Forest Health Initiative Research at UGA

Clonal Testing/Gene Transfer Project

Milestones on the way to "the plantable tree"

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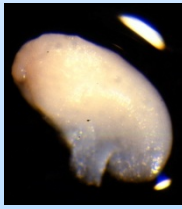
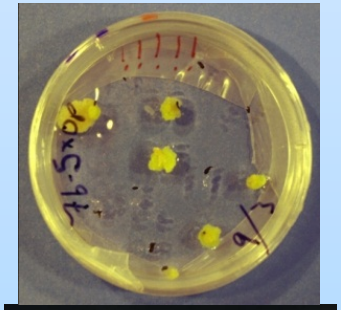
Objective 1: Initiate new embryogenic cultures from a broad diversity of chestnut germplasm

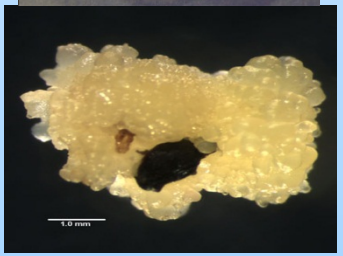
- Chestnuts from TACF, ACCF, NY-TACF, VDF and other suppliers cultured over 3 seasons of culture initiations (2009, 2010, 2011)
- Over **450** new embryogenic cultures from pure American chestnut, pure Chinese chestnut, OP B3F3 and other hybrid chestnuts captured to date
- A broad range of genotypes will help ensure “robust” plantable trees (broad genetic diversity)

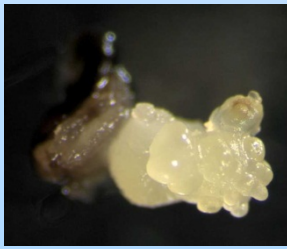
Gary Griffin collecting burs

August

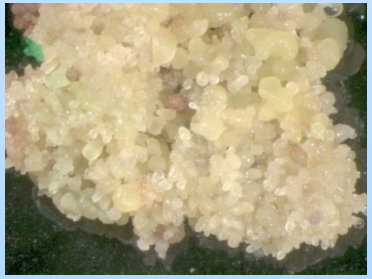








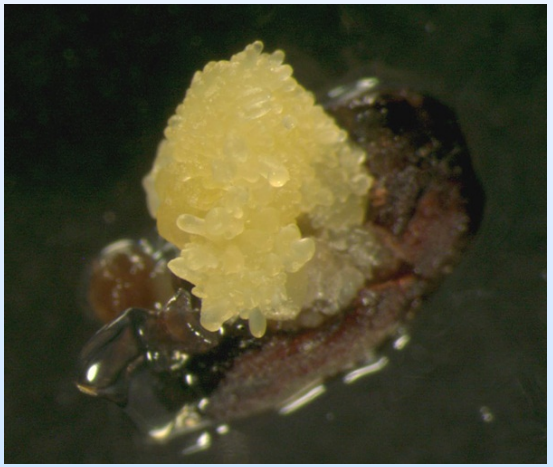
January



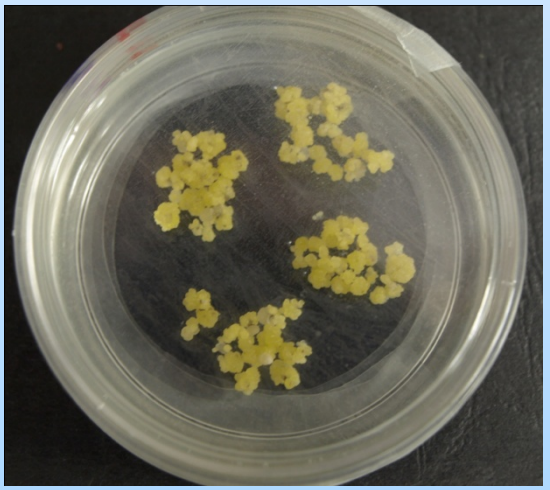
“Capture”

Embryogenic culture initiation from TACF B3F3 germplasm

- MOU between TACF and FHI and Germplasm Agreement between TACF and UGA reached in 2010 allowed culturing of TACF B3F3 material for the first time
- **10** OP B3F3 families cultured in 2010 and **11** more in 2011, representing both Graves (W) and Clapper (D) lines of blight resistance…and perhaps Graves *Phytophthora* resistance as well
- Embryogenic cultures "captured" for all **21** TACF B3F3 families cultured
- No significant difference in initiation rates between B3F3 and pure American
- First CP B3F3s being initiated <u>now</u> (August 2012)



B3F3 culture initiation



Captured B3F3 culture

Objective 2: Screen cultures to identify those with best somatic embryo/somatic seedling production capability

- All embryogenic cultures from 2009 and 2010 initiations have been screened (i.e. through somatic seedling performance in greenhouse)
- 2011 cultures are still in the process of being screened
- Several promising pure AC and hybrid lines identified for possible use as transformation targets and for clonal testing

RM x TH (ACCF) somatic seedlings

76-5 x OP (VDF) somatic seedlings

Thoroughfare Gap (VDF) somatic seedlings

Nagle (NY-TACF) somatic seedlings

Production of somatic seedlings for “clonal testing” of conventionally bred chestnut material by cooperators

- First ever TACF B3F3 somatic seedlings in greenhouse--more in hardening-off chamber and *in vitro*
- Somatic seedlings will begin flowing to cooperators (ACCF, TACF, VDF) to plant out this fall for clonal testing
  - i.e. these non-transgenic FHI clones are already “plantable” trees

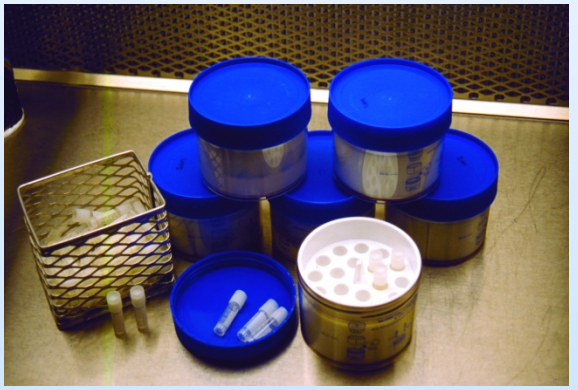
B3F3 somatic embryo

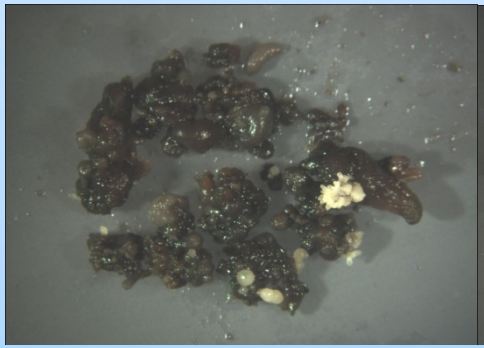
First W1-30-6 (Graves source) B3F3 somatic seedling

B3F3 somatic seedlings on August 6, 2012

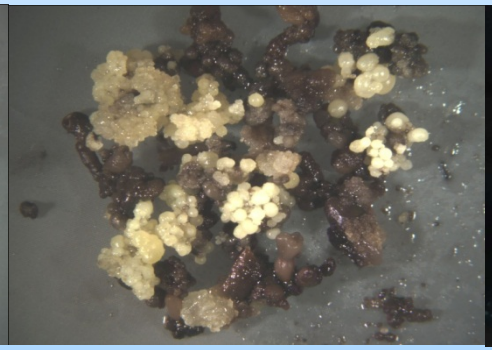
Objective 3: Cryostore copies of all embryogenic cultures

- All (**172**) 2009 and 2010 embryogenic culture lines have been cryostored (at least 3 copies of each)
- Cryostorage of 2011 lines in progress
- **>1100** transgenic events (40 per CG in "workhorse" line, at least 3 copies of each) have been cryostored for first 16 pFHI constructs
- Over **4000** vials total (out of space in cryo-freezer!)
- Cryostorage ensures that once the clone(s) that produce the "plantable tree" is identified, we can thaw out that clone and make millions more.

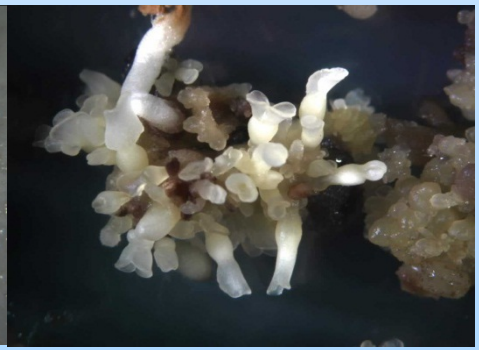




1 week



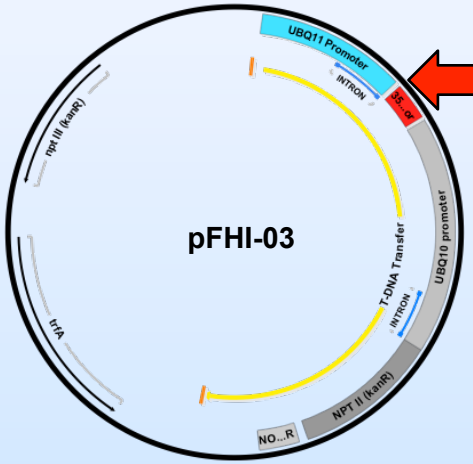
2 weeks



SE production

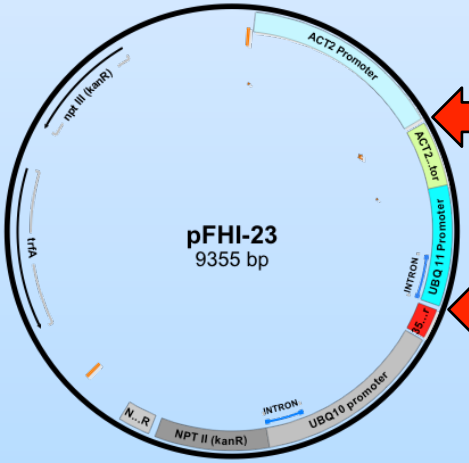
Objective 4: Construct vectors for cloning and expression of candidate resistance genes

***pFHI-03**:* A single gene vector to express genes in American chestnut. This vector is used to evaluate individual Candidate Genes.



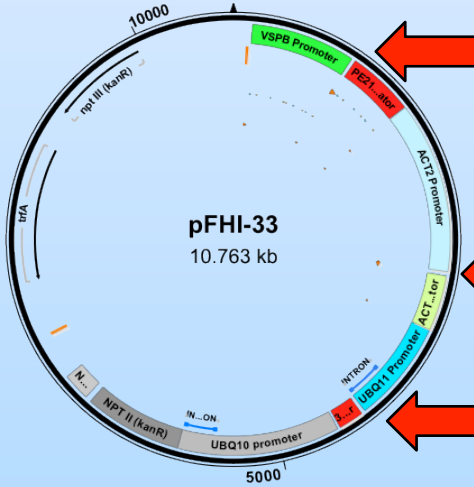
Candidate Gene

31 Candidate Genes (CG) have been cloned and transformed using this vector.



Candidate Gene 1

Candidate Gene 2



Candidate Gene 1

Candidate Gene 2

Candidate Gene 3

pFHI-23: A vector to express 2 genes in American chestnut. The vector is used to "stack" or "pyramid" candidate genes.

***pFHI-33**:* A vector to express 3 genes in American chestnut. The vector can be used to express genes from the 3 resistance QTLs.

Objective 5: Production of American chestnut transformed with candidate disease resistance genes

28 Chestnut CGs, 4 Heterologous CGs and 3 Reporter Genes to Date

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

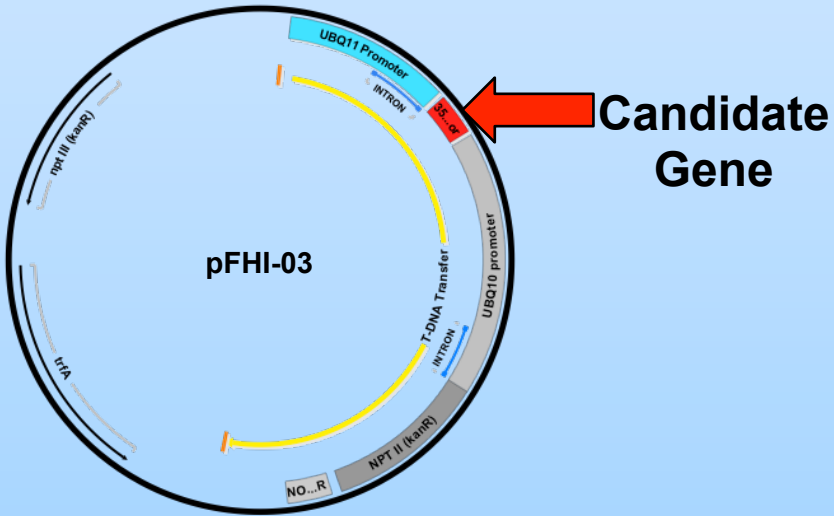
Vector Construct (*pFHI-*)	Heterologous Candidate Gene
GAFP	Gastrodia Anti-Fungal Protein
NPR1	Non-expressor of Pathogen Response
CAMP	Capsicum Anti-Microbial Peptide
VST	Vitis Stilbene synthase

Reporter Genes

GUSi	GUSintron
GUSiYFP	GUS intron-Yellow Fluorescent Protein fusion
GFP	Green Fluorescent Protein

In pFHI-23 (2 gene vector)

RGAF	RPH1 + GAFP



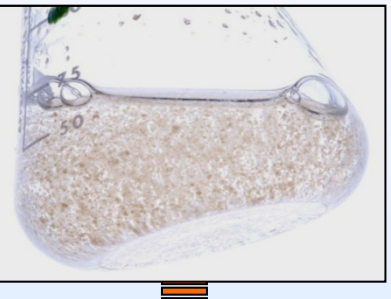
Progress through the “pipeline” as of June 1, 2011

Construct	Genotypes transformed	Geneticin resistant events	Events on plates	Events in flasks	Events in SE production	Somatic embryos harvested
pFHI-GUSi	3	>65	65	42	35	142
pFHI-GUSiYFP	4	360	360	16	7	NA
pFHI-NPR1	4	132	132	59	47	1,514
pFHI-Thaum	5	> 1,360	1,360	212	125	1,406
pFHI-ACPHOS	4	>708	708			
pFHI-UDPGTI	3					
pFHI-cmPRP	4	>1,307	1,307			
pFHI-cmLac	2	>500	367			
pFHI-B-Gluc	3	>109	109			
pFHI-CBS1	4	>600	312			
pFHI-ETF1	4	>600	366			
pFHI-GAFP1	>3	> 42	42			
pFHI-Cyst1	>2	NA				
pFHI-LTP1	>2	NA				
pFHI-GFP	NA	NA				
Totals			**5128**	**329**	**214**	**3062**

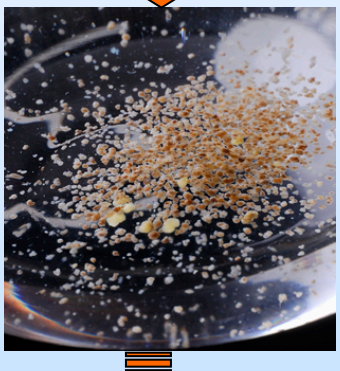
Progress through the “pipeline” as of August 6, 2012

Construct	Genotypes transformed	Geneticin resistant events	Events on plates	Events in flasks	Events in SE production	Somatic embryos harvested	Plants in-vitro	Plants in soil
pFHI-GUSi	3	>65	65	42	42	914		14
pFHI-GUSiYFP	4	360	360	16	16	679		14
pFHI-NPR1	6	>682	570	87	87	4,029	13	18
pFHI-THAUM	6	> 1,360	1,360	201	172	8,855		107
pFHI-ACPHOS	6	>1062	308	56	14	46	2	
pFHI-UDPGT	7	>1030	335	36	5	249		
pFHI-PRP	4	>1,307	1,307	59	53	3,221	9	258
pFHI-LAC	4	>500	367	74	65	2,112	17	154
pFHI-BGLUC	3	>109	109	40	31	2,388	67	87
pFHI-DAPH	4	>800	556	39	34	1,380	6	15
pFHI-CBS	4	>600	312	81	71	2,200	70	35
pFHI-ETF	4	>600	366	79	79	2,898	192	164
pFHI-GAFP	4	> 372	255	61	44	1,281	4	9
pFHI-CYST	5	>497	334	61	50	2,591	47	
pFHI-LTP	4	>201	171	66	61	3,266	24	
pFHI-GFP	4	>100	94	15	2			
pFHI-RPH	3	>230	138	35	35	2,481		
pFHI-ACOX	3	>394	175	31	28			
pFHI-MIP	3	>420	205	35	25	921		
pFHI-VST	4	>1159	287	15	15			
pFHI-SKDH	3	>495	242	20	18	106		
pFHI-CAD	3	>520	194	28				
pFHI-PROX	3	>615	282	33	10			
pFHI-CCAOMT	5	>1541	458	5				
pFHI-GST7	4	>555	355	20				
pFHI-CAMP	4	>1101	301	5	5			
pFHI-GLUC2	4	>615	362					
pFHI-TAGL	3	>202	191	30	10	812		
pFHI-SBTL	3	>300	300					
pFHI-NPR34	3	>318	318					
pFHI-LTP2	3	>357	357					
8/6/12 Totals			**11034**	**1270**	**972**	**40,429**	**451**	**875**
6/1/11 Totals			**5128**	**329**	**214**	**3062**	**0**	**0**

Overall Status of Transgenic Chestnut Pipeline as of August 6, 2012



Agrobacterium (AGL1) infection of chestnut PEMS



Selection of growing colonies with Geneticin

Air-lift bioreactors make cells for transformation every 2 weeks

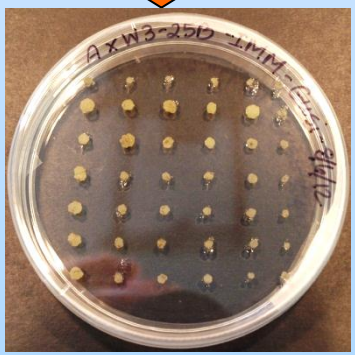
>800 transgenic somatic seedlings in pots

>400 transgenic plants in vitro

>40,000 somatic embryos harvested

>900 events through SE production

>1200 events grown in flasks



>11,000 events grown on plates

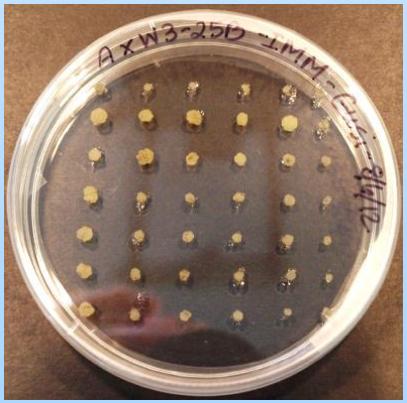
Molecular characterization of expression and delivery vectors in transformed AC lines

- Screened over **1,300** transgenic events for CG integration and confirmed over **1,240** transgenic lines each containing one of **31** CGs or **3** reporter genes.
- Standardized protocol to screen for CG in embryogenic cultures at 6-8 weeks post-transformation
- Confirmed transcription and translation of the GUS-intron and GFP reporter genes in AC embryogenic cultures by GUS assay and imaging in live tissues
- Confirmed gene expression of ESF39A in roots and stems of 12 transgenic AC plants prior to screening plants for *Phytophthora* resistance

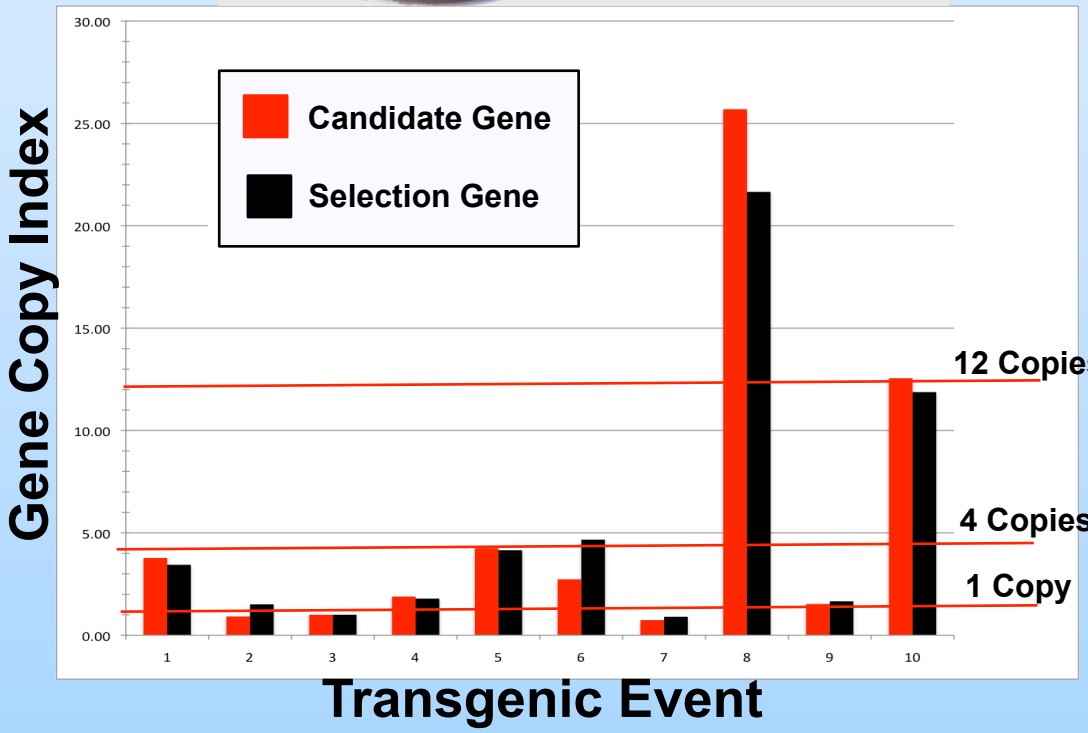
Screening for Candidate Gene insertion and copy number

1st screen at 6-8 weeks post-transformation

Lane	Well#	Sample ID#	*Gene*	Result	Lane	Well#	Sample ID#	*Control*	Result
1	A1	**1144**	UDPGT1	+	2	B1	**1144**	CdCyst	+
3	A2	**1145**	UDPGT1	+	4	B2	**1145**	CdCyst	+
5	A3	**1146**	UDPGT1	+	6	B3	**1146**	CdCyst	+
7	A4	**1147**	UDPGT1	+	8	B4	**1147**	CdCyst	+
9	A5	**1148**	UDPGT1	+	10	B5	**1148**	CdCyst	+
11	A6	**1149**	UDPGT1	+	12	B6	**1149**	CdCyst	+
13	A7	**1150**	UDPGT1	+	14	B7	**1150**	CdCyst	+
15	A8	**1151**	UDPGT1	+	16	B8	**1151**	CdCyst	+
17	A9	**1152**	UDPGT1	+	18	B9	**1152**	CdCyst	+
19	A10	**1153**	UDPGT1	+	20	B10	**1153**	CdCyst	+
21	A11	**NT**	UDPGT1	-	22	B11	**NT**	CdCyst	+
23	A12	**H_2O**	UDPGT1	-	24	B12	**H_2O**	CdCyst	-



Screened for gene insertion and copy number when fractionated for embryo production

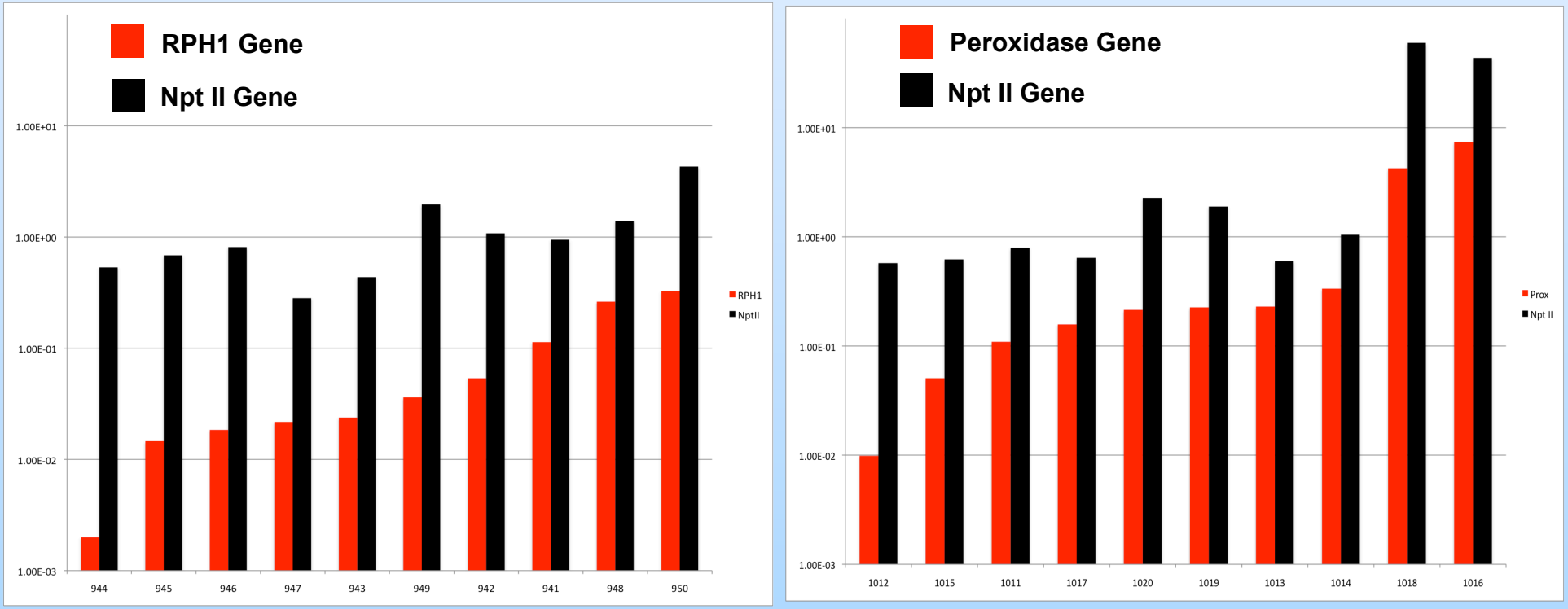


Candidate Gene Expression in Independent Transgenic American Chestnut Events

Candidate gene expression among independent lines varies 10 – 100+ fold

Will expression in embryogenic cultures correlate with that in regenerated plants ?

Select and test transgenic lines with different expression levels



Objective 6: Establish field testing sites in South for field testing of transgenic lines

- Over 100 trees representing 10 different ESF39A events and 10 transgenic controls growing at Whitehall Nursery
- 60 ESF39A/OXO trees and controls now in lath house ready to be planted at Whitehall Nursery this fall
- Somatic seedlings with GAFP and other FHI CGs for *Phytophthora* resistance will be ready for screening at James Farm in 2013

May 2011

May 2012

Dr. Joe James

ESF39A/OXOs in lath house

Summary:
Milestones on the way to “the plantable tree”

- Over **450** new embryogenic cultures from American chestnut, Chinese chestnut, B3F3 and other hybrid chestnuts captured and cryostored
- **First ever** TACF B3F3 somatic seedlings generated
- **27** chestnut candidate genes and **4** heterologous candidate genes transformed into multiple chestnut genotypes
- Over **800** transgenic somatic seedlings in pots, with thousands more in the pipeline
  - The first trickle of transgenic trees should grow into a flood over the next few years
  - **Really just “hitting our stride” this year**

UGA Group Personnel and Collaborators

Scott Merkle and Joe Nairn

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SE culture initiation & screening

Allison Newman
Part-time Research Technician II
Transgenic SE and SS production; greenhouse; nursery; screening

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Undergraduate Student Researcher
SE culture initiation & bioreactor testing

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- Vector construction & sequencing
- Candidate Gene vector construction
- Transgenic/transgene screening

Acknowledgments

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