Semi-annual Progress Report for July 1 to Dec. 31, 2011
Forest Health Initiative (FHI) at SUNY-ESF
American Chestnut Research & Restoration Program

by
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Highlights:

- Oxalate oxidase (OxO) is effective at reducing necrosis in the leaf assays. There appears to be an expression threshold, above which necrosis is significantly inhibited (more than the blight resistant Chinese chestnut control leaves). We have five events expressing above this threshold. These are promising events for either partial or full blight resistance, which will have to be confirmed when the trees are older. These results also demonstrate that gene expression studies will be critical for all transgenic events transformed with candidate genes to ensure expression is at effective levels.
- The very first cross of a transgenic American chestnut tree to a wild-type tree was completed. Catkins from our oldest transgenic event (Wirsig) were used to pollinate several wild-type American chestnut trees. Thirty-four nuts were harvested from the small pilot study. The nuts are undergoing cold stratification, but GFP reporter gene expression has been confirmed. We expect 50% of the nuts to contain the transgene. This proves that transgenic chestnut can go through an entire life cycle and can be bred with surviving wild-type chestnut trees.
- We showed that expression of the FT1 gene from poplar, sent to us by Steve Strauss, will induce catkin formation in transgenic American chestnut tissue culture shoots. We also determined that the heat shock promoter is inducible, but background expression is variable among the transgenic events. Three events had very tight control, with very low levels of background FT1 gene expression and high levels of induced expression. These transgenic events might be useful in future "rapid" breeding programs in greenhouses.
- In related research, we discovered a method to physiologically induce flowing in chestnut using high levels of light in a continuous long day regiment.
- From collaborations with the FHI genome sequencing group (John Carlson, PSU, and Meg Staton, Clemson), we developed a PCR based marker that appears to easily differentiate Chinese and American alleles of a laccase-like gene. The marker identifies a deletion in the promoters of the American alleles that might contribute to the differential expression of this gene.
- We are able to induce shoots from dormant chestnut stems so that they can be used as a source of material to start shoot cultures. This method will provide a quick way to clonally

propagate elite lines of chestnut and doesn't rely on embryos that are produced from a genetic cross of two trees. Therefore, one can propagate a true clone of the parent tree.

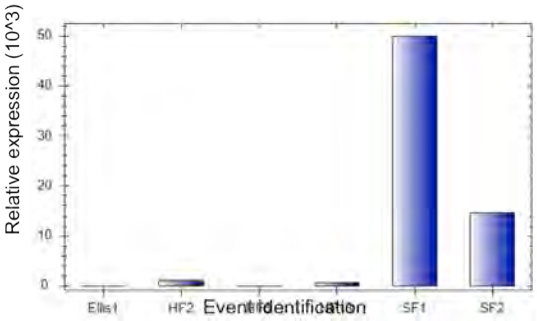
- We have now cloned a total of 24 candidate genes and sent them to UGA for vector construction. Of these, 21 have been put into vectors in Joe Nairn's lab and are being used for chestnut transformation at both SUNY-ESF and UGA. Three of these genes, which we had been unable to clone from *C. mollissima*, were cloned from the *C. sequinii* cDNA samples we brought back from China.
- We sent genomic DNA samples of wild-type *C. sequinii*, *C. henryi*, and *C. mollissima* collected from China to Dr. John Carlson (PSU) to help with the chestnut genome sequencing project.
- We currently have 21 gene constructs in our transformation pipeline at SUNY-ESF that have produced confirmed events. We are employing a rigorous testing protocol of early PCR screening, and later RT-qPCR for expression and qPCR for insert copy number to cull out the less promising events. This will allow us to retain the most promising events for each construct while reducing the total number of whole plants we have to deal with.
- We currently have 427 transgenic American chestnut trees, and about an equal number of non-transgenic controls, in the field spread among five locations in NY state. Currently we have 930 potted trees in our growth chambers and green house. Over half of these will be planted this summer to reach the high end of our deliverable goal of between 300 to 1000 trees in the field.

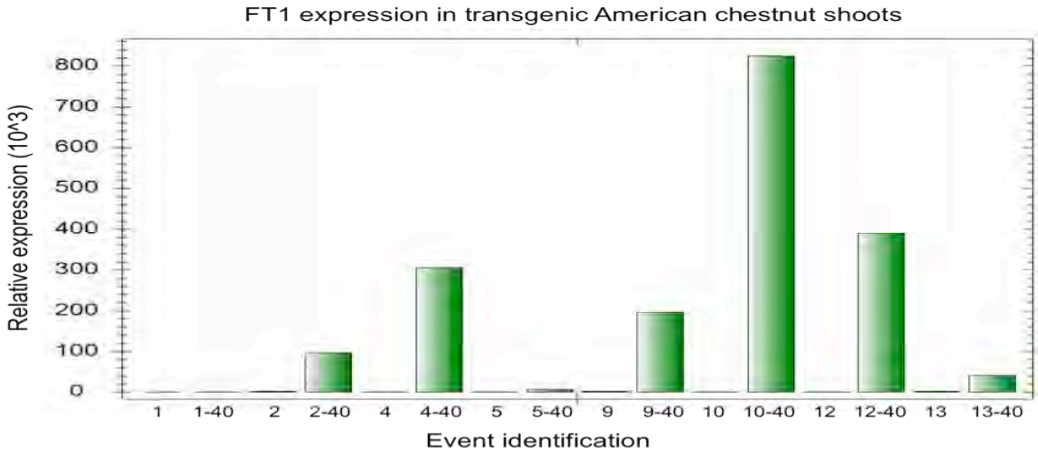
Samples of progress since our last report (objectives in reverse order):

Objective 4. ***Early flowering gene tests***

Transformation events for both the pOrStFor-35S::PtFT1 and the pOrStFor-HSP::PtFT1 vectors have reached the shoot stage. Only two events were obtained from the constitutively expressed, 35S vector and these immediately started forming catkins once shoots developed. From the gene expression studies it appears that high expression of the FT1 in early development is detrimental. The heat shock controlled vector produced nine events and had much lower expression at room temperature than the 35S vector events. There is, however considerable variability among these events, as expected, due to gene insert position effects. Some of these events form catkins from the background, uninduced expression of the FT1 gene (see figure and graphs below). Other events are more tightly regulated and these are being multiplied, rooted, and acclimatized for future greenhouse studies.
(A manuscript is in preparation to be submitted to a peer-reviewed journal.)

FT1 expression, non-induced, in transgenic American chestnut embryos. In HF events, the FT1 gene is under control of a heat shock promoter. In SF events, the FT1 gene is under control of a 35S constitutive promoter.





RNA was extracted from shoots at ambient temperature (22C) and from shoots induced at 40C for 4 hrs. Orange represents non-induced expression, green represents induced expression,

Physiologically inducing flowering

In addition to using the FT1 gene to induce flowering, we discover that high intensity light between 335 micro-Einsteins (micromoles of photons per meter squared per second) at pot height, to 890 µE at the highest catkins (87 cm) using High Pressure Sodium and Metal Halide lamps for continuous intensity, sixteen hour days, will induce catkin formation (in all chestnuts tested) and female flowers (in Chinese chestnut tested) in as little as six months. Not all plants tested were induced, but a significant proportion were induced. This treatment would allow crosses to be made throughout the year and much sooner than for field grown plants.

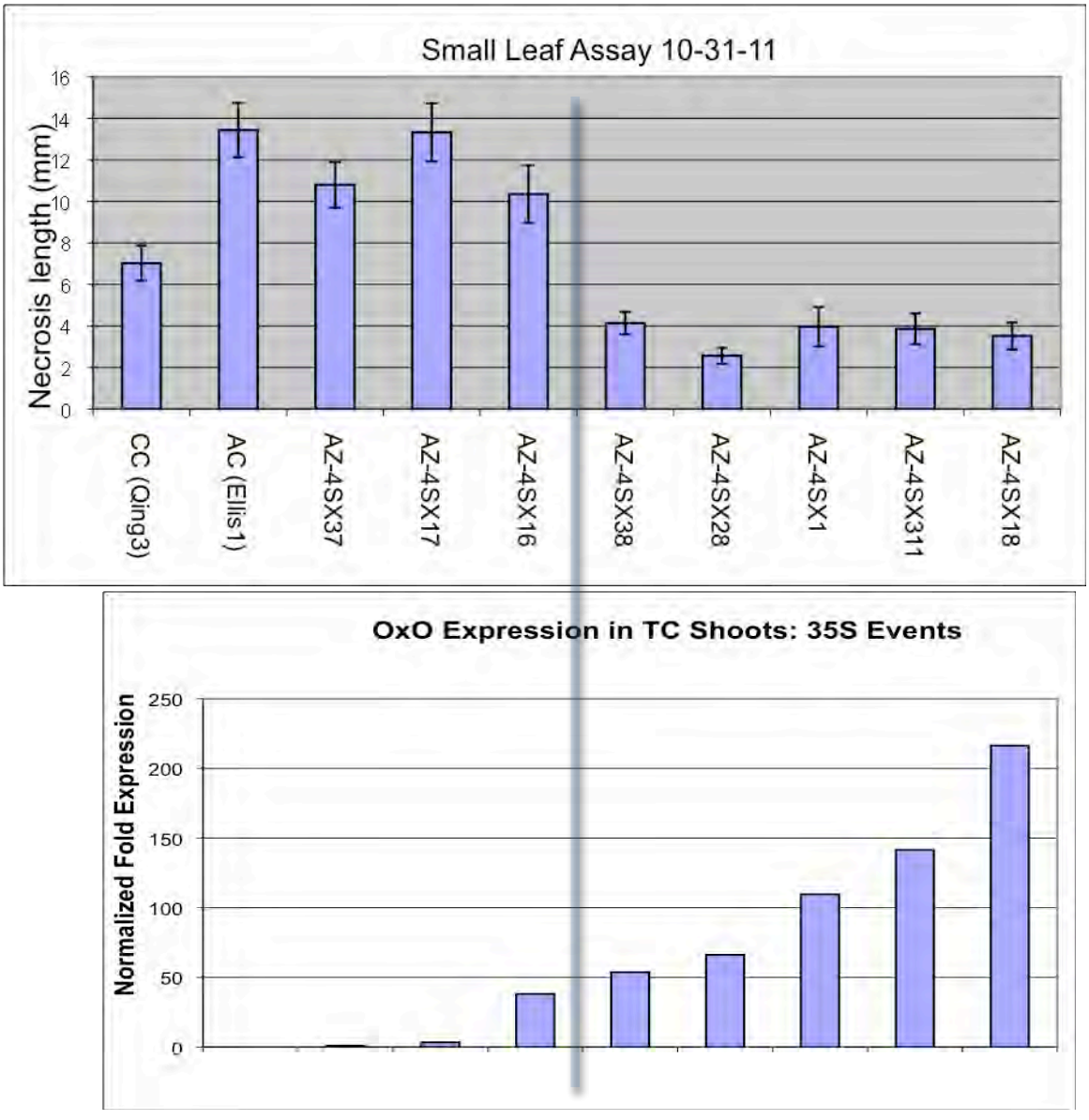
(A manuscript has been submitted for publication in the Journal of The American Chestnut Foundation.)

First cross between a transgenic American chestnut tree and a wild-type American chestnut tree in field grown trees.

During the 2011 summer, our first transgenic event (Wirsig) was producing pollen while some of our non-transgenic control trees produced female flowers. We completed a small pilot study where we collected transgenic pollen from a Wirsig tree and used it to pollinate several the non-transgenic trees, Both male and female flowers, and the resulting burs were contained through the whole season with bags to prevent any escapes. From these crosses, 34 filled nuts were produced and are currently being stratified. One nut, so far, has produced the first root and we confirmed that the GFP marker gene is being expressed. This is proof of the concept that transgenic American chestnut trees can produce viable nuts and the transgenes can be expressed in offspring. We will be following these further as they germinate and produce seedlings. This was a small study, so we are planning to perform a larger cross this summer, hopefully with some of our more recent transgenic events.

Objective 3. ***Early blight-resistance assay development***

Constitutive, CaMV 35S promoter driven, oxalate oxidase expression (bottom graph) in transgenic American chestnut events, was correlated to leaf assay necrosis (top graph) of the same events. It was found that a threshold level of OxO expression (vertical line) was needed to reduce the necrosis levels to below the Chinese chestnut (CC) control. The events that didn't meet that level of expression had similar necrosis length as the wild-type, clonal line of American chestnut (AC). This indicates that the OxO gene has some inhibitory effect on the growth of *Cryphonectria parasitica* at high expression levels. These five events are very promising and will be tested for enhanced blight resistance when they reach an older age. (A manuscript is in preparation to be submitted to a peer-reviewed journal.)

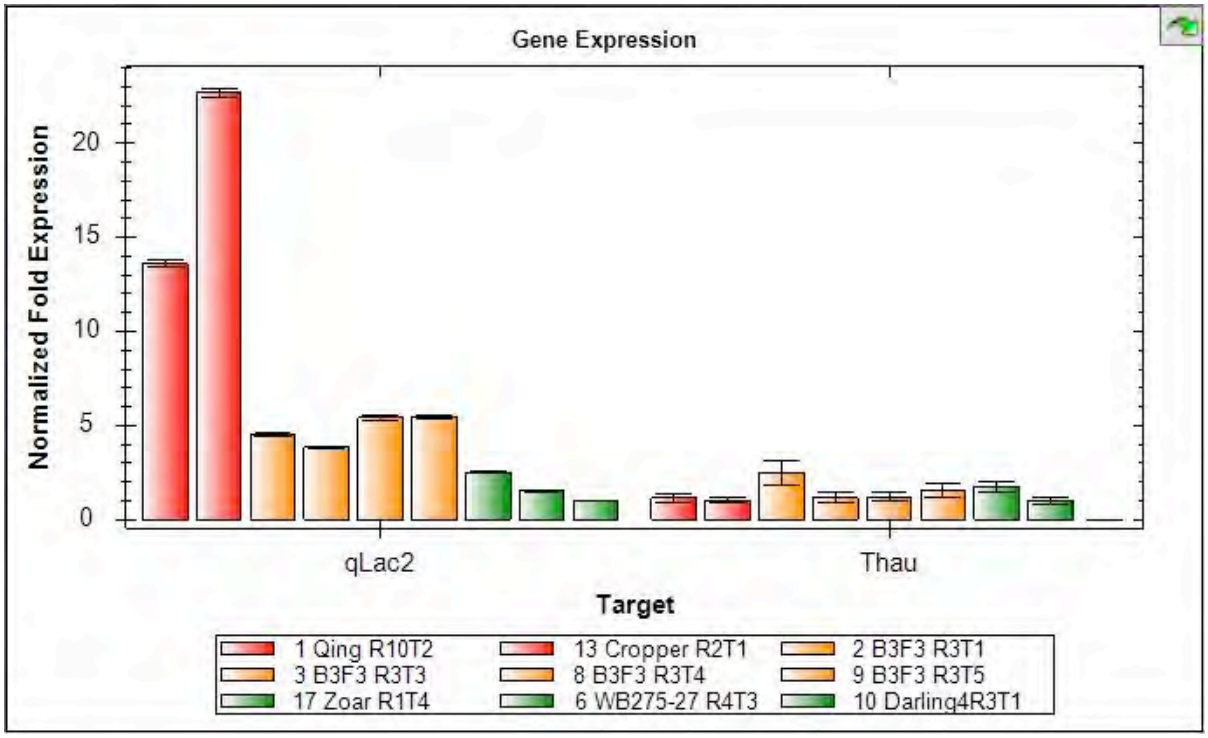


Objective 2. ***Candidate Genes, second-generation gene constructs and transformations***

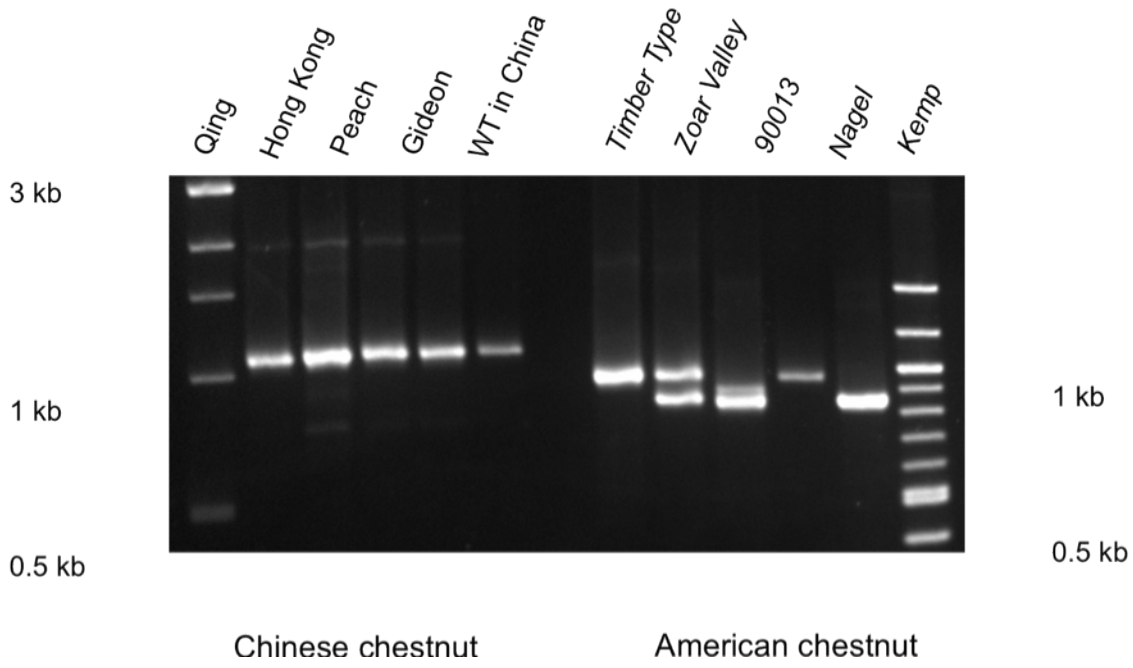
Laccase-like gene:

One of the candidate genes from Chinese chestnut that looks very interesting encodes a unique laccase-like protein. The protein sequences are identical and the DNA coding sequences are 99% similar between American and Chinese chestnut, but the expression levels are very different, with much higher expression in the Chinese chestnut. In the RT-qPCR graph below, the gene expression is highest in two Chinese chestnut (red), intermediate in four B3F3 backcross trees (orange), and lowest in the three American chestnut tree stems (green). With the help of the FHI genomics project, we were able to compare the promoter sequences and found that even though the DNA coding regions are very similar, the promoters are significantly different, which may account for the differences in expression levels. One of the differences is a deletion in the American sequences as compared to the Chinese sequences. This has allow us to develop a simple PCR-based marker (see picture of gel below). The marker appears very consistent for identifying the Chinese chestnut allele of the laccase-like gene. Even a DNA sample isolated from a tree in China gave the same band as Chinese varieties grown in the U.S. The American chestnut trees appear to have two alleles that are different than the Chinese allele. We will continue to exam this gene to determine if it is one of the blight resistance genes on linkage group G.

The promoter of the Chinese laccase-like gene allele also appears to be a strong, constitutive promoter, as compared to the expression of a Thamatin-like protein gene (top graph). Therefore it might be useful in constructing a new vector for cisgenic transformations. (A manuscript is in preparation to be submitted to a peer-reviewed journal.)



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



Cloning of cisgenic candidate genes:
Significant progress has been made in the cloning of the cisgenic candidate genes that we send to UGA for vector construction. To date we have cloned a total of 24 genes, 21 from *C. mollissima* and 3 from *C. seguinii*. To date we have received 21, second generation vectors to be used in the chestnut transformations. These are added to the 8 first generation vectors previously constructed at SUNY-ESF, the 2 first generation vectors constructed at UGA, and the 2 flowering gene vectors from OSU.
(publications on the cloning of the Chinese CG genes and transformation should be done in collaboration with UGA collaborators)

Chinese chestnut candidate gene (CG) cloning and vector construction list:

	Chinese CG, putative ID, contig	**cDNA cloned/sent (SUNY-ESF)**	**Binary vector constructed (UGA)**	**SSH diff. expressed (SUNY-ESF)**	**454 seq diff expressed (Penn State)**	**Linkage group (US For. Serv. & Clemson)**
1	**β-1,3 glucanase,** CCall-contig8901_v2	Cloned & sent	**pFHI-B13Gluc** completed	✔ (CC1.2-DO3)		?
2	**CBS domain protein** CCall-contig2586_v2	Cloned & sent	**pFHI-CBS1** completed	✔ (CC2.2-E07)		?
3	**UDP glucosyltransferase** CCall-contig11269_v2	Cloned & sent	**pFHI-UDP** completed	✔ (CC2.2-C08)	✔	LG-B & LG-G Peach G6 & G8
4	**Thaumatin-like protein** CCall-contig8443_v2	Cloned & sent	**pFHI-Thau** completed			LG-G (center)

5	**DAHP synthase (DHS1) (3-Deoxy-d-*arabino*-Heptulosonate 7-Phosphate Synthase)** CCall-contig9278_v2	Cloned & sent	**pFHI-DAHP** completed	✔ (CC1.2-D06)	✔	LG-G
6	**Acid phosphatase** CCall-contig8996_v2	Cloned & sent	**pFHI-AcPhos** completed	✔ (CC1.2-A11)		LG-G
7	**Laccase / diphenol oxidase** CC454-contig42836_v2	Cloned & sent	**pFHI-CmLac1** completed	✔ (CC1.2-E04)	✔	(old LG-1) LG-B? Dana – **LG-G** Peach G8
8	**Proline-rich protein** CCall-contig18406_v2	Cloned & sent	**pFHI-PRP1** completed	✔ (CC3.2-F07)		LG-G
9	**Ethylene-response transcription factor** CCall-contig19527_v2	Cloned & sent	**pFHI-ETF1** received 5/12/11	✔ (CC2.2-DO6)		LG-F
10	**Cysteine proteinase inhibitor** CCall_contig4992_v2	Cloned & sent	**pFHI-Cyst1** received 5/12/11			LG-E
11	**Lipid transfer protein SSH** CCall_contig_2055_v2	Cloned & sent	**pFHI-LTP1** received 5/12/11	✔ (CC1.2-H07)		?
12	**RPH1 (*Phytophthora* resistance)** CC454_contig44620_v2	Cloned & sent	**pFHI-RPH1** received 7/7/11			LG-E (end40-44cM); PeachG3
13	**SKDH (**Shikimate dehydrogenase) CCall_contig19078_v2	Cloned & sent	**pFHI-SKDH1** received 7/7/11	✔		?
14	**Myo-inositol-1 phosphate synthase** CCall_contig11468_v2	Cloned & sent 5/12/11	**pFHI-MIP1** received 7/7/11	✔ (CC1.2 – B07)		LG-B
15	**Triacylglycerol lipase** CCall_contig47573_v2	Cloned & sent 5/12/11	**pFHI-TAGL1** received 7/7/11		✔	LG-E
16	**ACC oxidase (1-Aminocyclopropane-1-Carboxylic Acid)** CCall_contig26701_v2	Cloned & sent 5/25/11	**pFHI-AcOx1** received 7/7/11		✔	LG-E
17	**CAD** (Cinnamy-alcohol dehydrogenase) CCall_contig4088_v2	Cloned & sent 7/28/11	**pFHI-CAD** received 11/4/11	✔ (CC1.2-C04)		LG-B
18	**Peroxidase** CC454_contig1568_v2	Cloned & sent 7/28/11	**pFHI-PrOx** received 11/4/11	✔ (CC1.2-F11)		LG-B
19	**CCoAOMT** (caffeoyl-CoA-O-methyltransferase) CCall_contig7500_v2	Cloned & sent 8/10/11	**pFHI-CcAOMT** received 11/4/11	✔ (CC1.2-G10)		?
20	**GST U7** (glutathione S transferase) CCall_contig4267_v2	Cloned & sent 9/1/11	**pFHI-GST7** received 11/4/11			LG-E

21	**Glucanase** (glucan endo-1,3-glucosidase) CC454_contig4907_v2	Cloned & sent 8/24/11	**pFHI-Gluc2** received 11/4/11			Like LG-B
22	**NPR3/4 (*Phytophthora* resistance)** CC454_contig43310_v2 *Castanea seguinii* gene cloned	Cloned & sent 1/12/12				LG-E (end40-44cM); PeachG3
23	**Lipid transfer protein (LTP) /proteinase inhibitor** CCall_contig_39658_v2 *Castanea seguinii* gene cloned	Cloned & sent 1/12/12				LG-G
24	**Subtilisin-like protease (Cucumisin precursor) LG?** CC454_contig46020_v2 *Castanea seguinii* gene cloned	Cloned & sent 1/12/12		✔		LG-B, LG-E, LG-G Peach G3, G6

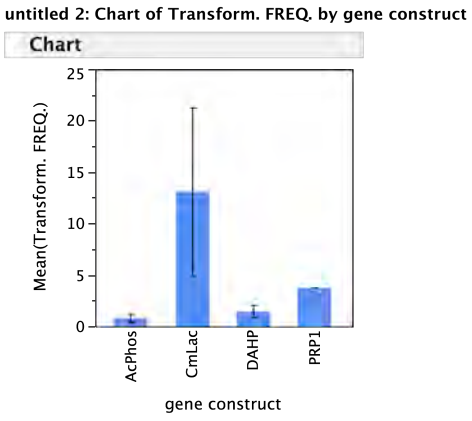
Other non-chestnut genes from SUNY-ESF constructed before FHI started

1	Oxalate Oxidase, defective VspB promoter		pVspB-OxO
2	Oxalate Oxidase, corrected VspB promoter		pTACF3
3	Oxalate Oxidase & ESF39 AMP		pTACF7
4	Oxalate oxidase, CaMV 35S promoter		p35S-OxO
5	CC laccase gene, sequence optimized		pESF-KBL
6	Oxalate Oxidase (same as TACF3 but modified terminator)		pESF-KBO
7	CC laccase gene, sequence optimized, & Oxalate Oxidase		pESF-KBLO
8	CC laccase gene, sequence optimized, Oxalate Oxidase, & ESF39 AMP		pESF-KBLOE
	Note: pHFT1 and pSFT1 (early flowering genes) only for greenhouse		

Other non-chestnut genes from UGA

1	Antimicrobial peptide (pepper?)		pFHI-CaAMP
2	stilbene synthase gene (grape?) – phytoalexin resveratrol synthesis		pFHI-VST1

Objective 1. ***Transformations with constructs on hand and characterization of transgenic events***



Good progress is being made with the American chestnut transformation and molecular characterization of the events. The table below shows that we have 21 gene constructs in our transformation pipeline showing confirmed events. The others are still being used for transformation. We have seen that some candidate genes produce more events than others (for a preliminary example, see graph to the left showing data from 4 vector constructs) and some have been

recalcitrant using our transformation methods. This makes sense because all the second generation candidate genes are constitutively expressed and therefore might be detrimental at some stage of development, as we have seen with the FT1 flowering gene. We have developed a culling process by PCR testing at the embryo stage, and RT-qPCR expression assays and qPCR insert copy number assays at the shoot stage. This helps remove the less promising events before they reach the leaf assay stage and field planting, thereby making the pipeline more efficient. We currently have 427 trees in the field and expect to more than double that number this summer.

Transgenic events in the pipeline:

Table 2. Transformations & Transgenic Events (Events, columns 2-4, are cell lines with unique insertions of the transgenes from which many trees, columns 5&6, can be made. Events are culled after PCR testing during embryo stage and RT-qPCR during shoot multiplication stage. Therefore these numbers change as more information is gained and less desirable events are removed.)

Vector[1] (Variety name)	**Candidate genes (CGs) (note: laccase is from Chinese chestnut)**	**Events as SE (total events)**	**Events multiplying as shoots in TC**	**Events in pots**	**Events currently in field plots**	**Total trees currently in field**	**Estimated new trees for possible 2012 planting**
Totals:		**140**	**80**	**33**	**15**	**427**	**930**
By vector:							
pESF-KBO pTACF3 (Darling) pVspB-OxO (Wirsig)	Vascular (V) - Oxalate oxidase (OxO) = V-OxO	9	9	9	9	204	121
pTACF7 (Hinchee)	V-OxO + V-ESF39 AMP	2	2	2	2	158	259
p35S-OxO (Travis)	Constitutive (C) – C-OxO	17	17	11	1	1	102
pESF-KBLOE (Radel)	C-laccase (optimized) + V-OxO + V-ESF39 AMP	7	7	6		9	281
pESF-KBL (Mansfield)	C-laccase (optimized)	6	1	1	1	1	82
pESF-KBLO	C-laccase (optimized) + V-OxO	3					
pWVK147	Empty vector control – only marker genes	2	2	2			28
pGFP	Transgenic control with GFP vector	2	2	2	2	54	57
pTACF6	V-ESF39 AMP	1	1				
pFHI-DAPH	C- DAPH synthase (DHS1)	7	4				
pFHI-AcPhos	C- Acid Phosphatase	4	2				
pFHI-CmLac	C- CC laccase / diphenol	40	16				

	oxidase						
pFHI-PRP1	C- Proline-rich protein (PRP1)	11	9				
pFHI-LTP1	C- Lipid transfer protein identified by SSH	8					
pFHI-ETF1	C- Ethylene response transcription factor	5					
pFHI-AcOx1	C- ACC oxidase (1-Aminocyclopropane-1-Carboxylic Acid)	3					
pFHI-MIP1	C- Myo-inositol-1 phosphate synthase	2					
pFHI-RPH1 (selection)[1]	C- RPH1 (*Phytophthora* resistance)						
pFHI-CcAOMT (selection)	C- caffeoyl-CoA-O-methyltransferase						
pFHI-VST1 (selection)	C- stilbene synthase gene (grape?) – phytoalexin resveratrol synthesis						
pFHI-CaAMP (selection)	C- Antimicrobial peptide (pepper?)						
pFHI-TAGL1 (selection)	C- Triacylglycerol lipase						
pFHI-SKDH1 (selection)[1]	C- Shikimate dehydrogenase						
pFHI-CAD (selection)	C- Cinnamy-alcohol dehydrogenase						
pFHI-GST7 (selection)	C- GST U7 (glutathione S transferase)						
pFHI-Gluc2 (selection)	C- Glucanase (glucan endo-1,3-glucosidase)						
pFHI-PrOx (selection)	C- Peroxidase						
pFHI-Cyst1 (selection)[1]	C- Cysteine proteinase inhibitor						
pOrStFor-35S::PtFT1[2]	C – FT1 (early flowering gene)	2	2 (but start flowering and stop growing)				
pOrStFor-HSP::PtFT1[2]	Heat shock inducible – FT1	9	8				

1. pFHI-CBS1, pFHI-Csyt1, pFHI-RPH1, and pFHI-SKDH1 have not produced any events after several transformation attempts. They are currently be used again, but the constitutive expression might be detrimental to development in our transformation/regeneration system.
2. The FT1 events are only for greenhouse use.

Special event!

We will be planting 5 of our first generation transgenic American chestnut events in the New York Botanical Garden on April 18, 2012. This is significant for two reasons. First, this is very near to where the chestnut blight was first discovered. Second, these will be the first transgenic trees in the NYBG and therefore will be a great public education tool. The SUNY-ESF alumni office is organizing this planting event. Below is a draft of the cover and schedule information. Invitations with more information, including costs, should go out soon.

The State University of New York College of Environmental Science and Forestry requests the pleasure of your company as we celebrate

The Return of an American Icon

Wednesday, April 18, 2012

New York Botanical Garden
2900 Southern Boulevard
Bronx, NY 10458-5126

Schedule of Events

3 p.m. Lecture on the Restoration of the American Chestnut
Ross Lecture Hall

4:30 p.m. Planting of Transgenic American Chestnuts
Planting site on the Garden grounds

6 p.m. Reception and Dinner
The Stone Mill

Please see the enclosed card for information about ticket purchases and sponsorship opportunities.

Please RSVP by April 4, 2012.

Seating is limited; early responses are encouraged.