

The Forest Health Initiative, American Chestnut (*Castanea dentata*) as a Model for Forest Tree Restoration: Biological Research Program

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Abstract

The Forest Health Initiative (FHI) was developed and implemented to test the hypothesis that a coordinated effort in biotechnology research could lead to resistant trees capable of restoring a species in a relevant time frame. As a test case, the American chestnut (*Castanea dentata*) was chosen for study as it is an iconic forest tree species in the eastern United States and southeastern Canada that has been nearly extirpated by chestnut blight which is caused by an introduced fungal pathogen (*Cryphonectria parasitica*). In addition, the species has attracted research investments over many decades, leading to some promising possibilities for effecting restoration. The FHI, now completing its third year, has integrated genomics-based candidate gene discovery with robust clonal propagation and gene transformation systems capable of producing hundreds of independent events for dozens of genes per year. A promising early leaf assay for blight reaction has been developed that will be instrumental in screening the large amount of material in production through these systems. Regulatory permits for testing some of the earliest transgene events have been obtained as small-scale field testing is beginning. High density genetic maps and various mapping populations are being analyzed to gain additional insights into the specific genes found in quantitative trait loci (QTLs) for resistances to blight and *Phytophthora* root rot (caused by *P. cinnamomi*, also known as ink disease). Nearly complete genome sequences of three of the blight resistance QTLs have been determined. These sequences are greatly assisting these analyses, as have comparisons with peach and other completed plant genomes, demonstrating the power of comparative genomics. These results show great promise for meeting the

challenge proposed by the FHI hypothesis for American chestnut. In addition, other forest tree species under threat should benefit from similarly designed initiatives.

INTRODUCTION

A forest health crisis is now widely apparent in the U.S. and various places globally (Rapport et al., 1998; Pautasso et al., 2010). Much of the crisis relates directly to introduced pests and pathogens, where native tree species have little to no evolved defenses (Leibhold et al., 1995; Lovett et al., 2006). Other aspects such as climate change and forest fragmentation are also at play in advancing the crisis (Woods et al., 2010). At the same time, unprecedented biotechnological advancements are available for application in many species (Merkle et al., 2007; Wheeler and Sederoff, 2009). These include genome mapping and sequencing, genome-wide gene expression analysis, candidate gene discovery, gene transformation and genetic engineering, and marker-assisted breeding. Clearly time is of the essence for developing and implementing systematic approaches to defining and solving forest health problems (Chornesky, 2005; Ellison, 2005; Hain, 2006). But what form would such a concerted effort take? One such approach, the Forest Health Initiative (FHI) initially developed in 2009, is currently being applied to American chestnut (*Castanea dentata*) and its two major diseases-chestnut blight (caused by *Cryphonectria parasitica*) and *Phytophthora* root rot (caused by *P. cinnamomi*). The FHI comprises a scientifically focused attempt to develop resistant trees under social (public acceptance, regulatory approval) engagement and a tight timeline. The social aspects can be gleaned from the www.foresthealthinitiative.org as can the overall science plan and various research results. A summary of the key scientific approaches and current results is provided here.

SCIENTIFIC APPROACH

Germplasm, Breeding and QTL Mapping

The core of any genetic improvement program is germplasm and the genetic variation contained within. Fortunately for American chestnut, several breeding programs are in place and their contribution to and collaboration with FHI is substantial and crucial. A wide array of materials from chestnut genetic improvement programs has been put into somatic embryogenesis (SE) culture and cryostorage (Table 1) as discussed below. In addition, certain later generation inter-species hybrid crosses and backcross (BC) families from The American Chestnut Foundation (TACF) (Hebard, 2005) have been made available for genetic analysis. These include an expanded version of an early F₂ blight resistance mapping population (Chinese chestnut (*C. mollissima*) ‘Mahogany’ resistance source) (Kubisiak et al., 1997; Sisco et al., 2005) and a set of BC₃ families derived from a single BC₁ selection (‘Graves’, resistance source unavailable). The former material is planted at TACF’s Meadowview Research Farms facility and is being phenotyped using standard artificial inoculation procedure with two strains of *C. parasitica*. The latter is planted at PA-TACF/Penn State’s research facility near State College, PA and is being phenotyped using the same methods and strains. Genotyping of these pedigrees has been conducted with an Illumina Infinium Bead-Array utilizing EST sequences as a genome-wide source of SNPs. Confirmation of the previously mapped QTLs for blight resistance (*Cbr1*, *Cbr2*, and *Cbr3*) (Kubisiak et al., 1997) was completed by genotyping the existing F₂ population with the new SNP markers and re-mapping the QTLs using all the marker data (Kubisiak et al., 2012).

Genome Sequencing, QTL Sequencing and Gene Discovery

Developing genomic resources, namely a draft genome sequence, to support gene discovery and serve as a platform for molecular breeding and genetic engineering is an important and far-reaching goal of FHI. To optimize disease resistance gene discovery, a Chinese chestnut (resistant species) genotype ‘Vanuxem’ was used for genome sequencing because it is readily available to breeders, it is being used as a resistance

source parent in crosses for genetic mapping (Kubisiak et al., 2012) and in the TACF breeding program, and it was used as the source DNA for EST sequencing (Barakat et al., 2009) and for generating the BAC libraries that were used in constructing the physical map (Fang et al., 2012). Previous studies of the *Fagaceae* estimated the genome size of Chinese chestnut to be around 800 Mb (Kremer et al., 2007) suggesting 20 Gb of next generation sequence data (or 25X coverage) would be required for a draft genome assembly. Roche-454 pyrosequencing was used to obtain shotgun and paired-end sequence data followed by Illumina short read sequencing for closing gaps in the assembly and for verifying and correcting gene sequences. Assemblies were scheduled iteratively at increasing sequence depths of 454 data from 1 to 20X to assess sequence and assembly qualities. In parallel to whole genome sequencing, a strategy was developed and implemented to produce a reference-quality sequence across the three QTLs for blight resistance. Briefly, overgo probes representing genetic markers from the QTL regions were hybridized to the BAC libraries to identify their corresponding physical map contigs. Four physical map contigs were identified for *Cbr1*, and one contig was identified for each of *Cbr2* and *Cbr3*. From these physical map contigs a minimum tiling path of BAC clones that fully spanned the contig was selected for sequencing. The DNA from these BACs was extracted and pooled by contig. Contig pools were shotgun sequenced, producing 4.3 billion bases. Candidate gene discovery proceeded by searching the assembled QTL sequences for genes involved in pathways that may confer blight resistance such as stress response and signaling, lignin biosynthesis, and apoptosis.

Clonal Propagation, Transformation and Early Screening

1. University of Georgia, Warnell School of Forestry and Natural Resources. To obtain the embryogenic American chestnut cultures needed to generate trees for clonal testing and to provide target material for transformation, cultures were initiated from a diverse collection of chestnut germplasm following the protocol of Andrade and Merkle (2005). The new cultures were screened for their abilities to produce somatic embryos and somatic seedlings and a subset of the most productive cultures was selected to use as target material for *Agrobacterium*-mediated transformation (Andrade et al., 2009) with candidate genes for blight or root rot resistance, using new, modular vectors constructed specifically for the FHI project (C.J. Nairn, unpublished). Airlift bioreactors were tested as an alternative to growing embryogenic suspension cultures in shaken flasks (Kong, in preparation), for accelerating the production of embryogenic material for transformation. Following co-cultivation, transformation events were identified using liquid selection and individual putative events were grown up in liquid culture, PCR-screened to confirm presence of the transgene, and used to produce transgenic somatic embryos by the size-fractionation/plating technique as described previously (Andrade and Merkle, 2005). Somatic embryos were harvested, given a 12-week pre-germination cold treatment and germinated in vitro. Following germination, somatic seedlings were transferred to potting mix, hardened off in a humidifying chamber and transferred to the greenhouse or lath house for continued growth and development.

2. State University of New York, Environmental Sciences and Forestry. Transformation of American chestnut somatic embryos was accomplished using previously developed procedures (Polin et al., 2006; Maynard et al., 2008). Briefly, somatic embryos were co-transformed with *Agrobacterium*, desiccated, and selected on a semisolid medium. PCR was used to confirm transgene insertion. Confirmed transgenic events were regenerated into shoots, multiplied, rooted, potted, acclimatized, and planted in the field. Quantitative PCR and RT-qPCR were used to determine insert copy number and relative transgene expression, respectively. Early blight resistance screening assays were tested and developed by inoculating mid-veins of leaves with the SG2-3 (moderately virulent) strain of *C. parasitica* and measuring lesion length. Chinese chestnut and American chestnut seedlings were used as resistant and susceptible controls, respectively, for comparison with transgenic trees (A. Newhouse and W.A. Powell, unpublished). Field based assays were performed on stems ~2.5 cm in diameter using the Ep155 (highly

virulent) and SG2-3 strains of *C. parasitica* and again using Chinese chestnut and American chestnut as controls compared to the transgenic trees (A. Newhouse and W.A. Powell, unpublished).

RESULTS AND DISCUSSION

Germplasm, Breeding and QTL Mapping

Valuable germplasm from several breeding programs has been captured using SE of immature seeds and cryopreservation. Details are discussed below. Breeding efforts have provided valuable material for disease resistance gene mapping. In the F₂ mapping population, 129 new progeny trees were obtained and phenotyped for blight resistance in Virginia. In the BC₃ mapping, 724 progeny were phenotyped representing 11 families. Each family consists of a separate BC₂ tree selected for blight resistance crossed to one or more unrelated American chestnuts. An example of the genotypic distributions for one BC₃ family is provided in Figure 1. The genotypic data are derived from the bead-array where over 1000 SNP markers were segregating in this cross among the 128 progeny. Proportion of Chinese chestnut genome was estimated using the proportion of heterozygous loci (Fig. 1). This gives an idea of the resolution possible in evaluating interspecies populations for the proportion of genomes remaining for the two parental species. Using these data to pinpoint the blight resistance QTLs and estimate genome proportions should prove to be a valuable marker-assisted selection tool. Genotyping methods are needed to optimize these applications at costs affordable for routine use. Adding SNP marker data to the existing QTL mapping data set confirmed the location of the QTLs *Cbr1*, *Cbr2* and *Cbr3* on linkage groups LG_B, LG_F, and LG_G, respectively. In addition, these markers allowed the placement of these QTL regions within the larger consensus map developed for Chinese chestnut (Kubisiak et al., 2012) and the integration of these regions into the Chinese chestnut physical map (Fang et al., 2012). This later feature facilitated QTL sequencing as discussed below.

Genome Sequencing, QTL Sequencing and Gene Discovery

Genomic DNA sequencing yielded 14.2 Gb (~18X depth) of 454 shotgun sequence (355 bp average read length), 3.6 Gb (4.5X) of 454 mate-paired sequences and 916 Mb of Sanger sequence of 43,143 BAC-ends that were selected from a minimum tiling path across the physical map (Fang et al., 2012). From this total 25X depth of sequence, assembly with Newbler v2.5.3 software resulted in 1.14 M contigs containing 925 Mb of DNA sequence and 51,766 scaffolds covering 587 Mb (0.73X) of the genome, with N50 length of 52 kb. Finally, over 37 Gb of Illumina GA paired-end sequence (75 bp average read length) was included, which unexpectedly did not further improve the assembly. This sub-optimal result suggested that DNA contamination and/or high levels of heterozygosity might be interfering with assembly. To address this concern, a second round of sequencing of a new preparation of SSR-marker verified 'Vanuxem' DNA was initiated which to date has yielded 26.5 Gb of filtered sequence data (consisting of ~20 M reads averaging 516 bp using Roche 454 FLX+ and ~65 Mb of 250-bp paired-end reads using Illumina MiSeq). The current *de novo* assembly, performed with the second round of genome sequence data using the Newbler v2.6 assembly program, yielded 82,590 scaffolds containing about 556 Mb of DNA, with a scaffold N50 size of 9.4 kb, from 355,714 assembled contigs containing 768 Mb of DNA.

The large number of contigs and scaffolds of small size in the genome assemblies has several possible explanations. First, the total amount of assembled sequence is less than the estimated genome size of 800 Mb, indicating that a significant portion of the genome is either not assembling or not sequencing, leading to fragmentation. Second, based on the empirical evidence from the genome assemblies, the Newbler program estimates the final genome assembly size to be ~1,364 Mb (vs. 800 Mb from flow cytometry) suggesting that chestnut DNA is more heterozygous than expected from a single diploid genotype or that multiple genotypes are being sequenced. Third, the current

assembly lacks mate-pair sequences of long insert sizes, reducing the assembler's ability to extend the genome coverage by pulling contigs into scaffolds. The second issue is being addressed by stringent quality control of new input DNA. The first and third issues should be resolved as new mate-pair libraries (3, 8, and 20 kb inserts) are sequenced.

Even though the draft genome is not complete, it is proving quite useful for gene discovery work. For example 96% of the 48,355 transcript unigene sequences (Barakat et al., 2012), were detected in the contigs of the current assembly. In addition, the G+C content of the assembled contigs is 35.4% and repetitive content (based on RepeatMasker, www.repeatmasker.org) is 8.4% both as would be expected for an angiosperm tree genome. In addition, the largest chestnut scaffold analyzed (~100 Mb), showed strong gene order micro-synteny with the *Populus trichocarpa* and *Prunus persica* genomes and, across all contigs, predicted chestnut gene models show strongest sequence similarities with genes from the *P. persica*, *Oryza sativa*, *Vitis vinifera* and *P. trichocarpa* genomes (23, 11, 7 and 6%, respectively). Sequencing the blight resistance QTLs yielded the following sequence assembly and gene content.

Cbr1, LG_B QTL = 6.8 Mb, 214 contigs, N50 of 75 kb, 432 genes

Cbr2, LG_F QTL = 4.1 Mb, 128 contigs, N50 of 72 kb, 219 genes

Cbr3, LG_G QTL = 3.0 Mb, 53 contigs, N50 of 158 kb, 131 genes

Of the almost 800 genes discovered, approximately 100 could be considered candidates for disease resistance. Combining the functional annotation of these genes and the previous gene expression studies of canker versus healthy tissue, a much smaller subset of candidate genes has been selected for functional testing in transgenic American chestnut.

Clonal Propagation, Transformation, and Early Screening

1. University of Georgia, Warnell School of Forestry and Natural Resources. During 2009-2011, over 450 new embryogenic cultures were initiated from several American chestnut full-sib and half-sib families and, for the first time, from BC₃-F₃ families (source TACF), as well as some other hybrid chestnuts. Table 1 shows the diversity of chestnut material provided by cooperators that was captured as proliferating embryogenic cultures during the first two years of the FHI. Capture rates for BC₃-F₃ material were not significantly different from those for American chestnut, indicating that our SE protocol can be used for propagation of elite hybrid backcross material. Copies of all cultures were cryostored for germplasm conservation using a protocol previously described (Holliday and Merkle, 2000). Somatic seedlings have been produced from multiple large surviving American chestnut (LSA) and BC₃-F₃ trees (Fig. 4) and are ready for clonal testing in field environments. The combination of breeding, SE and cryopreservation has the potential to greatly aid American chestnut restoration. This will also help satisfy future demand for elite chestnut planting stock, since it allows clones to be held in storage for years while propagules from them are field tested. Clones showing the best performance in the field can then be recovered from cryostorage and propagated to produce planting stock.

Vectors were successfully constructed for 27 chestnut and 4 heterologous candidate genes, shared with the ESF group, and are at various stages in the UGA and ESF transformation pipelines. The chestnut candidate genes were selected based on several factors including differential gene expression (Barakat et al., 2009, 2012; Baier, 2009), location within the resistance QTL intervals (Kubisiak et al., 2012), and sequence annotation indicating disease or stress resistance. Production of transgenic material using these vectors was greatly accelerated at UGA by the application of airlift bioreactors. Comparisons of chestnut embryogenic suspension culture growth in bioreactors versus shaken flasks showed that the bioreactors produced about 1.5-fold more cell clumps than shaken flasks within 6 weeks following inoculation. Thus, we were able to generate sufficient target material to conduct transformations every 2 weeks and meet our goal of

completing transformations with over 30 genes during the three years of the FHI. Over 1300 transgenic events in multiple American chestnut backgrounds have been confirmed using PCR and somatic embryos have been produced for over 900 of these events. Thousands of transgenic somatic embryos have been harvested and over 800 transgenic somatic seedlings, representing multiple transformation events for the first 9 candidate genes, are currently growing in the greenhouse in preparation for screening for blight.

2. State University of New York, Environmental Sciences and Forestry. To date, 21 Chinese chestnut and 6 Seguin chestnut (*C. seguinii*) candidate genes were cloned from cDNA libraries and sent to UGA for vector construction (as discussed above) and are in various stages of the transformation pipeline. Additionally, 12 constructs containing heterologous candidate genes are in this pipeline. As the transgenic events move through, they are tested for transgene presence and copy number and relative transgene expression levels, compared to their orthologs endogenous to the cell line, where appropriate. This allows culling of escapes, and less promising and redundant transgenic events. Once selected events are regenerated into whole plants with six or more leaves, the early screening (leaf assay) is used to predict blight resistance relative to resistant and susceptible controls (Newhouse and Powell, unpublished results). At present these leaf assays predict that seven of the older transgenic chestnut events, each containing a wheat oxalate oxidase gene, are likely to have enhanced blight resistance (Fig. 2) (Darling 4 example), confirming earlier results where transgenic poplars expressing this oxalate oxidase transgene were shown to have higher pathogen resistance (Liang et al., 2001).

In the summer 2012, an early transgenic American chestnut event, Darling 4, had reached a size (>2.5 cm stem diameter) in field tests to begin traditional blight resistance assays. To confirm results of the Darling 4 leaf assays (Fig. 2), which predicted higher than intermediate levels of resistance, eight trees each of Darling 4, American chestnut (susceptible control) and Chinese chestnut (resistant control) were tested in the field using traditional stem assays. Half of the tested trees of each type were inoculated with *C. parasitica* strain SG2-3 and half with strain Ep155. After 15 weeks, the canker areas of the Darling 4 trees were similar to the resistant control trees (Fig. 3) (Ep155 example, SG2-3 results were similar). Assessment of these test trees will continue for a full year to determine if the Darling 4 cankers heal over time, as seen in Chinese chestnut, or if they only slow the progression of canker growth and disease development. These field-based stem assay data appear to confirm the results from the leaf assays and also indicate that the heterologous oxalate oxidase gene enhances blight resistance, at least to a level intermediate to American and Chinese chestnuts. Newer Darling events with higher levels of oxalate oxidase expression compared to Darling 4 are showing smaller necrotic lesions in the leaf assays compared to resistant controls, indicating blight resistance to the level of Chinese chestnut. At the same time, in concert with the UGA group, candidate genes from Chinese and Seguin chestnuts are being processed through the respective pipelines to provide a range of disease resistance-enhancing genes for application in American chestnut.

CONCLUSIONS

Results to date for the FHI support the hypothesis that biotechnology can be successfully brought to bear on a difficult forest health problem in a short period of time. The partnership and funding structure utilized (but not discussed here) should be further developed and implemented to support focused research where important forest species are threatened by introduced pathogens or pests. Each species will require its own analyses of the current biological and cultural situations before work plans can be developed, but we suggest there are some common ingredients for a successful biological sciences program:

- 1) develop and integrate genomic libraries, maps and sequences for comparison to a model or reference species for candidate gene selection;
- 2) develop tissue culture and transformation systems for the species of interest, even if only for functional analysis of candidate genes;

- 3) develop early, standardized assays for resistance, measured as directly as possible for the response of interest; and
- 4) collaborate with current breeding programs and arboretums to make sure the most important and potentially useful materials are included at the earliest and most appropriate opportunity.

For American chestnut, we see a much improved future for developing resistant materials capable of initiating and enhancing populations for re-introduction efforts aimed at ecosystem restoration (Jacobs et al., 2012). This includes resistance for both blight and root rot diseases and using both molecular breeding and genetic engineering methods for concentrating resistance in a large number of parents representing a diverse set of adapted populations. Coordinating and integrating such a range of biotechnologies clearly requires a concerted effort to educate the public on both the underlying science and the ecologic and economic costs and benefits of the various alternatives while gaining their confidence in working together to enhance the region's natural and cultural resources.

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Tables

Table 1. American and hybrid chestnut embryogenic culture initiation and cryopreservation summary for 2009 and 2010.

Supplier	Mother tree	Pollinator	# of nuts	# of seeds cultured	No. of embryogenic cultures	Capture frequency (%)
ACCF ¹	Ragged Mountain (RM)	TH	57	855	25	2.9
ACCF	Thompson (TH)	RM	43	645	13	2.0
CAES ²	3-48	OP ⁸	50	750	1	0.1
TACF ³	D6-26	OP	32	212	8	3.8
TACF	W3-32-97	OP	30	457	1	0.2
TACF	W1-31-63	OP	46	591	8	1.4
TACF	W5-24-74	OP	31	353	2	0.6
TACF	W3-32-68	OP	30	374	2	0.5
TACF	W3-32-123	OP	30	461	1	0.2
TACF	W2-37-52	OP	27	354	2	0.6
TACF	W1-31-60	OP	33	487	2	0.4
TACF	W1-30-6	OP	33	404	9	2.2
VDF	76-5	OP	33	495	26	5.2
VDF ⁴	Thoroughfare Gap	OP	21	314	19	6.1
Darling ⁵	Nagle	OP	35	553	11	2.0
Darling	P-38 DeGolyer	OP	35	517	7	1.4
Darling	15-C Moss Lake	OP	32	316	1	0.3
Darling	A-2 Zoar	OP	12	106	4	3.8
Darling	A-8 Zoar	OP	12	170	1	0.6
Darling	A-11 Zoar	OP	12	163	1	0.6
Micsky	Haun Orch. 1-6	OP	23	158	1	0.6
Gurney ⁶	VT-CC028	OP	24	297	3	1.0
Gurney	VT-CC040	OP	10	125	6	4.8
Donowick ⁷	Donowick #9001	OP	46	618	18	2.9
Total (or Mean)			737	9775	172	1.8

¹American Chestnut Cooperators Foundation (Gary and Lucille Griffin).

²Connecticut Agricultural Experiment Station (Sandy Anagnostakis).

³The American Chestnut Foundation B₂F₂ mother trees (F.V.H.).

⁴Virginia Department of Forestry hybrid mother tree (Jerre Creighton and Wayne Bowman).

⁵Herb Darling (TACF-NY).

⁶Gary Micsky (TACF-PA).

⁶Kendra Gurney (TACF-VT).

⁷James Donowick (TACF-NY).

⁸OP = open-pollinated.

Figures

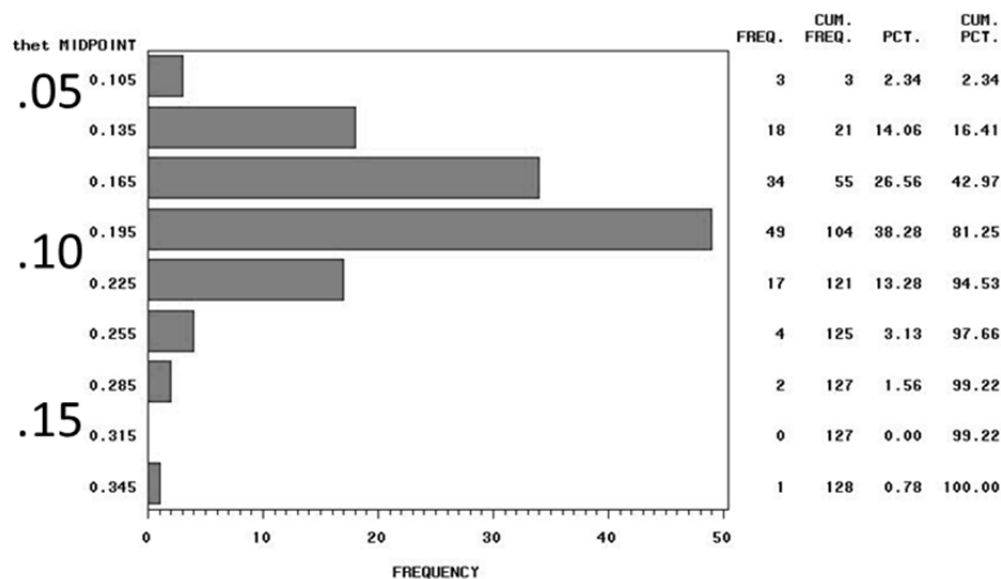


Fig. 1. Frequency distribution of proportion of heterozygous SNP loci within BC₃ family GL443 (n=128). An approximation of the Chinese chestnut genome remaining in these BC₃ trees is about 0.5 × the proportion of heterozygous loci (large font on Y-axis).

Transgenic Leaf Assays

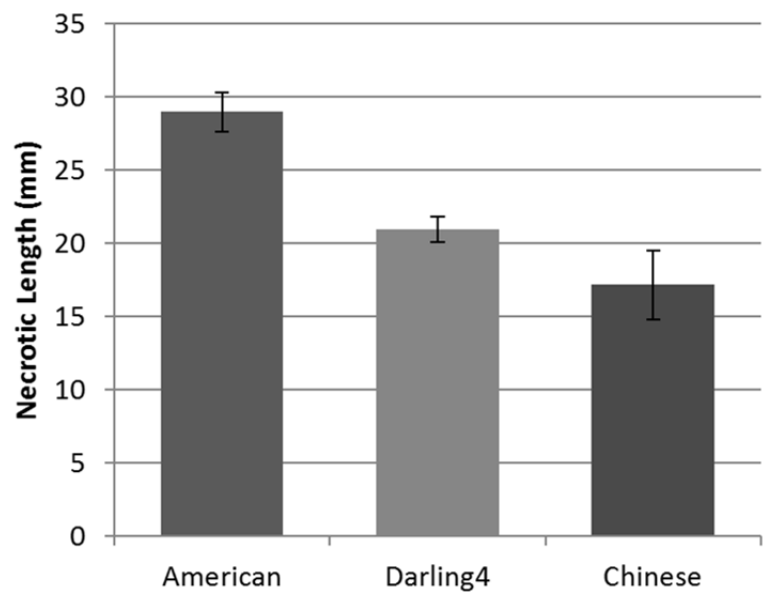


Fig. 2. Early blight screening leaf assays. All inoculations were with *C. parasitica* strain SG2-3, n=25 to 40 inoculations per leaf type, error bars = ±1 standard error of the mean. American = susceptible control, Darling 4 = transgenic expressing oxalate oxidase, Chinese = resistant control.

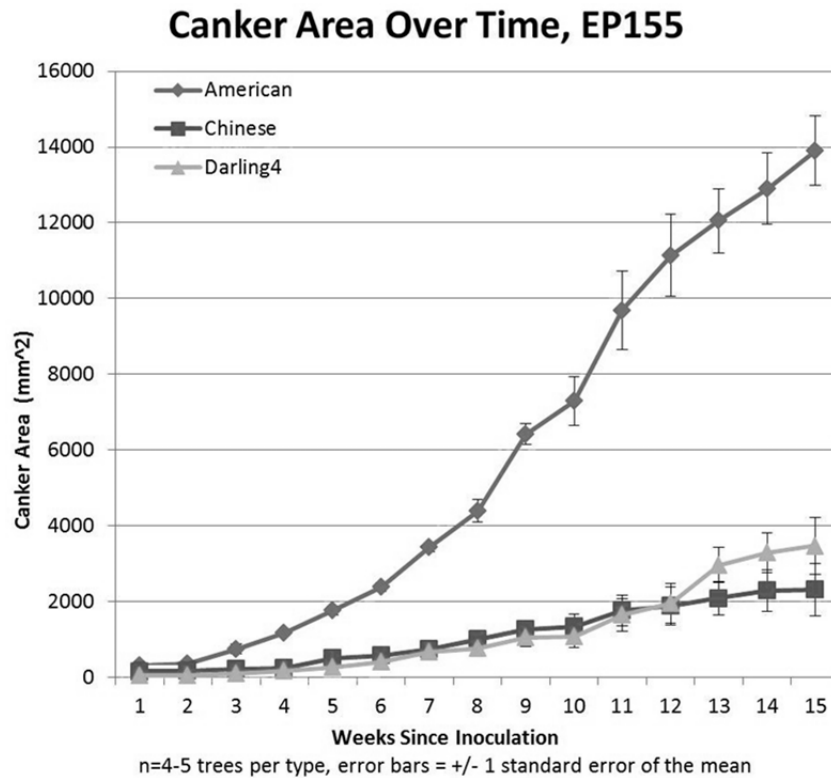


Fig. 3. Chestnut blight assays on stems 2.5 to 2.8 cm in diameter during the summer 2012. Four trees each of the transgenic event, Darling 4, and the resistant (Chinese) and susceptible (American) controls were inoculated with Ep155. The height and width of the cankers were measured weekly for 15 weeks and area was calculated as if an oval.

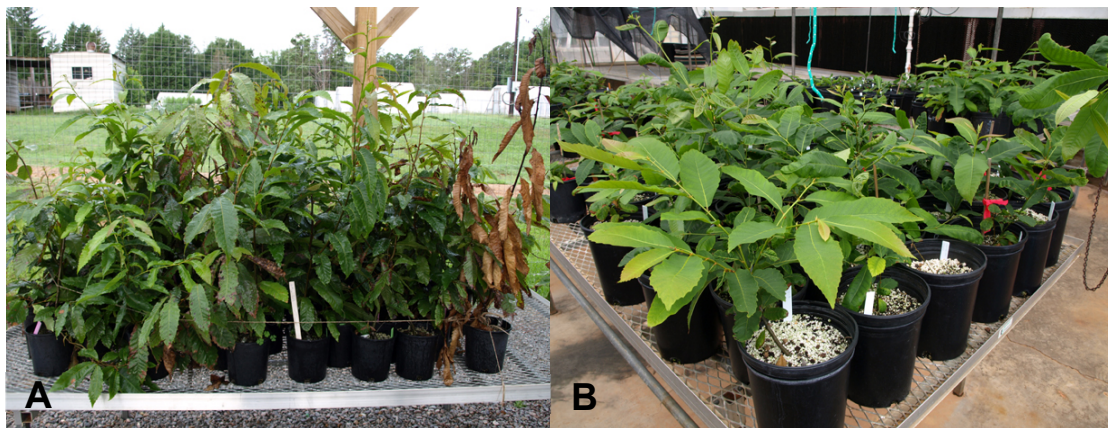


Fig. 4. Chestnut somatic seedlings from large surviving American chestnut and BC₃-F₃ sources. A) Somatic seedlings derived from embryogenic cultures of seeds from Ragged Mountain × Thompson crosses. B) Somatic seedlings derived from embryogenic cultures of BC₃-F₃ seeds.

