Project title: Development and testing of an *in vitro* screen for Phytophthora root rot resistance

Summary
Lack of resistance to *P. cinnamomi*, the causative agent of Phytophthora root rot (PRR), may pose a major barrier to introducing the products of TACF's breeding program to the southern portion of chestnut's original range. Building on our existing collaboration with TACF to produce elite chestnut varieties via somatic embryogenesis, we propose to accelerate the development of varieties that are also resistant to PRR by developing and testing a rapid *in vitro* screen for PRR resistance using somatic seedlings. The screen will be developed using known susceptible (American), known resistant (Chinese), BC3F3 and transgenic chestnut plantlets.

Principal investigator: Scott Merkle
Performing institution: University of Georgia
Awardee institution: University of Georgia Research Foundation, Inc.
Duration of project: 2 years (first year to develop and test screen on known resistant and susceptible material; second year to screen material from putatively resistant parents)
Total amount requested: $20,000 ($10,000 per year)

Long-term goal: To develop and optimize a rapid in vitro screen for Phytophthora root rot resistance for chestnut plantlets
Short-term goal 1: Develop and test a +/- PRR resistance screen using susceptible American chestnut plantlets and resistant Chinse chestnut plantlets
Short-term goal 2: Test the ability of the in vitro screen to detect intermediate levels of PRR resistance in hybrid backcross chestnut material and identify promising clones for field testing
Short term goal 3: Test the ability of the in vitro screen to detect PRR resistance in transgenic chestnuts engineered with candidate PRR resistance genes from the FHI project
Short term goal 4: Validate results of in vitro screen with ex vitro tests in soil

Background
***P. cinnamomi* and Phytophthora root rot.** Until relatively recently, the effort to overcome chestnut blight has been the major focus of TACF's restoration program, while much less consideration has been given to the impact of Phytophthora root rot on American chestnut. In fact, the disease can have even more devastating effects than blight, since infection results in mortality of the entire tree, rather than just dieback of the stem, as is the case with chestnut blight. It is generally believed that *P. cinnamomi*, an oomycete, is native to southeastern Asia and, perhaps, South Africa (Crandall and Gravatt 1967, Zentmyer 1980, 1988) and that it was introduced accidently into the coastal region of the southeastern United States on or associated with ornamental plants imported from Asia in the late 1700s or early 1800s to landscape antebellum plantations (Crandall et al. 1945; Zentmyer 1980, 1988). It then spread inland along with movement of the human population (Crandall and Gravatt 1967, Crandall et al. 1945, Milburn and Gravatt 1932). During the late 1800s and early 1900s, PRR caused devastating losses to American chestnut and Allegheny chinkapin (*Castanea pumila*) in forests throughout the southeastern and mid-Atlantic states, with widespread death of trees occurring long before chestnut blight arrived in North America (Crandall et al. 1945). Rhoades et al. (2003) demonstrated high susceptibility of American chestnut to this pathogen when seedlings were planted into naturally infested soil, even in soils that were not heavily compacted or wet. Of serious importance to TACF's restoration program is the recent finding that the first field plantings of TACF hybrid backcross chestnut seedlings suffered up to 50% mortality from PRR before they could even be challenged by chestnut blight (Clark et al. 2014).

PRR resistance in chestnut. To date, no PRR-resistant American chestnut genotypes have been identified. Fortunately, Chinese chestnut is resistant to both chestnut blight and *P. cinnamomi* (Crandall et al. 1945). Efforts started in 2004 by TACF Carolinas Chapter member Dr. Joe James, in collaboration with Dr. Steve Jeffers (Clemson University) resulted in identification of hybrid backcross families with PRR resistance (James 2011, Jeffers et al., 2012). Early genetic analysis of PRR resistance from these screens provided evidence that PRR resistance may be controlled by a single locus (Jeffers et al. 2012). An initial genome-wide linkage map was constructed for a BC1F1 cross carrying PRR resistance from *C. mollissima* 'Nanking' by (Zhebentyayevo et al. 2016) detected a QTL that was mapped to linkage group E. However, another report by Zhebentyayevo et al. (2015) cited in TACF's 2015-2025 Science Plan offered evidence that loci in additional linkage groups may be involved in PRR resistance. The Science Plan also indicates that ongoing progeny testing of B3F2 trees from Meadowview for PRR resistance has confirmed that alleles of major effect control PRR resistance in the Graves lines of hybrid backcross chestnuts.

Application of somatic embryogenesis to chestnut restoration. Over the past 25 year, the Merkle lab at UGA has developed a somatic embryogenesis (SE)-based clonal propagation system for American chestnut (Merkle et al. 1991, Carraway and Merkle 1997, Robichaud et al. 2004, Andrade and Merkle 2005), and has recently demonstrated that the system can be used to clonally propagate BC3F3 genotypes with the same efficiency as pure American chestnut genotypes (Holtz et al. 2014). In fact, we have been collaborating with TACF for the past few years to initiate cultures from BC3F3 genotypes derived from controlled crosses between BC3F2 parents selected for blight resistance. The first set of these somatic seedlings is in production now. This year, we are planning to initiate cultures from parents selected for both blight and PRR resistance. Once generated, not only can these clonal trees be used for clonal testing, to provide clonal repeatability data for disease resistance and growth traits, but some of the clones demonstrating the best performance have the potential to be selected as "elite varieties" to provide planting stock to landowners who want to plant to very best chestnut material.

We have also used the SE system to produce transgenic chestnuts (Andrade et al. 2009, Kong et al. 2013). The SE-based gene transfer system has produced over 11,000 transgenic events representing 30 blight and PRR-resistance candidate genes. Hundreds of transgenic trees have already been planted in field tests. Specifically with regard to the *P. cinnamomi*-resistance candidate genes, we have produced chestnut somatic seedlings engineered with the RPH gene and the NPR3/4 gene (both from Chinese chestnut) and combinations of these genes. Some of these transgenic trees are currently being screened for PRR resistance by Dr. Joe James using his system.

Previous work and preliminary results on in vitro PRR screen development. During the past few years, we have been working with Dr. Steve Jeffers at Clemson University to develop an in vitro screen for *P. cinnamomi* resistance. Our first attempt at an in vitro screen was based on old reports from Spanish and French scientists that indicated that resistant Chinese chestnuts could be easily distinguished from susceptible European chestnuts using cambium-derived callus. European chestnut callus would blacken within 3 days of inoculation with *P. cinnamoni*, while Chinese chestnut callus would remain unchanged (Vieitez 1961, Grente and Sauret 1961). We reasoned that embryogenic chestnut callus may behave similarly to cambial callus, and conducted a series of experiments to test this concept. Unfortunately, we found that we were unable to distinguish embryogenic callus of resistant trees from that of susceptible trees using

this approach. Based on a much more recent report (Santos et al. 2014) that used excised chestnut shoots for PRR resistance screening, we designed another in vitro approach that employed micropropagated shoots in water agar (actually water-Phytagel) inoculated with a plug of *P. cinnamomi* (Figs. 1A, 1B). Shoots of susceptible American chestnut clones became darkened and leaves wilted within a week of inoculation (Fig. 1C). While we lacked Chinese chestnut shoots to use as controls, we found it intriguing that transgenic American chestnut shoots engineered with a *P. cinnamomi* resistance candidate genes (RPH and NPR3/4), remained green and unwilted for several days after the wildtype shoots darkened (Fig. 1C). However, even these shoots eventually died, but since none of the shoots had roots, we could not be sure what ultimately killed them. Most recently, we tested a method to test whole, rooted chestnut plantlets in vitro by substituting low melting agarose for Phytagel as the gelling agent, which allowed us to establish the root system in the gel before it sets up. A preliminary test showed that wildtype American chestnut plantlets in this system inoculated with *P. cinnamomi* rapidly darkened and wilted, while plantlets of the same genotype that were not inoculated grew well for two months, even though the gel was simply agarose and water, rather than tissue culture medium. We recently obtained shoot cultures of three Chinese chestnut varieties from Dr. Bill Powell's (SUNY-ESF) lab, and we are multiplying the shoots now to root and test as resistant controls.

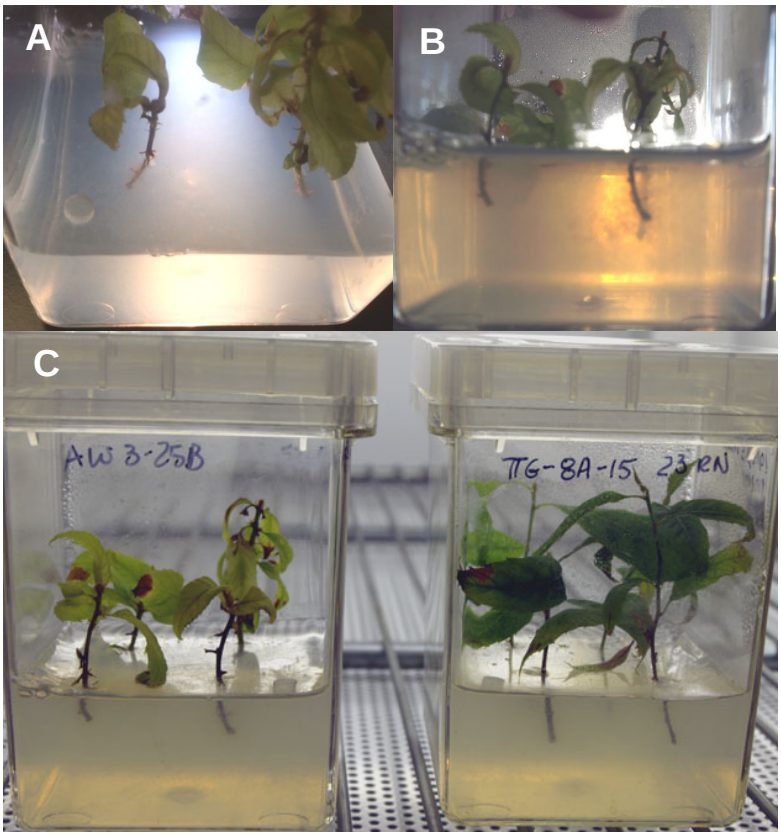

Fig. 1. Test of in vitro screen for Phytophthora resistance using chestnut shoots **A.** Shoots inserted into water-agar inoculated with a plug (left corner) of cultured *P. cinnamomi*. **B.** Back-lit view showing *P. cinnamomi* mycelia spreading through water-agar around cut end of shoot. **C.** Shoots of wildtype American chestnut (left) and transgenic chestnut expressing two candidate genes, RPH1 and NPR3/4 (right) one week following their insertion into the boxes with *P. cinnamomi*. Note that wildtype shoots are already blackened and leaves are wilting while transgenic shoots do not yet seem to be affected.

Rationale and Significance

As evidenced by the content of TACF's 2015-2025 Science Plan, producing planting stock with resistance to PRR, as well as blight resistance, has assumed a high priority in TACF's breeding program. Under Objective 3 in the Science Plan, the authors propose to combine resistance to *C. parasitica* and *P. cinnamomi* resistance by conducting controlled crosses between the most blight resistant B3F2s and the most PRR resistant B3F2s, followed by 1-2 generations of recurrent selection, to enhance resistance to both pathogens. The Merkle Lab is already collaborating with TACF to propagate the products of some of these controlled pollinations via somatic embryogenesis, with the long-term goal of generating elite chestnut varieties to offer landowners. We believe that by combining SE-based propagation of elite varieties with a reliable in vitro screen for PRR resistance, we can help to validate chestnut material thought to carry PRR resistance genes (via clonal testing) and at the same accelerate the production of the elite varieties with both blight resistance and PRR resistance for planting throughout the eastern U.S. Before the screen can be applied to hybrid backcross genotypes, however, we need to make sure the screen can at least clearly distinguish resistant Chinese chestnut plantlets from susceptible American chestnut plantlets. Then, we can proceed to test different clonal plantlets we regenerate from PRR resistant parents to test for clonal repeatability.

In addition to identifying and multiplying genotypes from TACF's breeding program, the availability of a reliable in vitro screen could ultimately help us identify the Chinese chestnut gene(s) involved in PRR resistance. In collaboration with other chestnut researchers, including TACF collaborators, we have twice submitted proposals to the USDA-AFRI competitive grants program in Plant Health and Production and Plant Products, with the goal of conducting functional analysis of gene candidates conferring resistance to *P. cinnamomi* using transgenic chestnuts. The proposals received mostly positive reviews, but a major criticism by the panel was the lack of an in vitro screen for *P. cinnamomi* resistance. If we can demonstrate the availability of a reliable in vitro screen for resistance, this may give us the tool we need to successfully compete for major funding from this program, which will ultimately aid efforts in the identification of the Chinese chestnut gene(s) involved in resistance.

Experimental Plan

Short-term goal 1: *Develop and test a +/- PRR resistance screen using susceptible American chestnut plantlets and resistant Chinese chestnut plantlets*. American chestnut somatic seedlings will be produced from at least two pure American embryogenic culture lines using procedures described in Andrade and Merkle (2005). Briefly, existing embryogenic culture lines will be inoculated into liquid induction-maintenance medium (IMM), grown in suspension culture and allowed to proliferate for approximately 6 weeks before size-fractionating on stainless steel sieves and plating on 30 µm pore size nylon mesh overlaid on semisolid embryo development medium (EDM). Somatic embryos will be harvested, given a 12-week pre-germination treatment at 8 C and transferred to semisolid germination medium (GM) in GA7 vessels (Magenta) to produce plantlets. Chinese chestnut plantlets will be produced by harvesting shoots from existing proliferating shoot cultures of at least two cultivars and rooting them following the procedure modified from that described by Oakes et al. (2013). Briefly, excised shoots, 2-3 cm long, will be dipped in 2 mg/ml K-IBA dissolved in a mixture of DMSO and water (50/50 v/v), then inserted into semisolid GM in GA7 vessels and incubated in the dark for 9 days, followed by growth in a lighted incubator until roots appear. Once American chestnut and Chinese chestnut plantlets have developed root systems, they will be carefully removed from the germination medium under aseptic conditions and transferred, singly, to empty, sterile GA7 vessels that will immediately be filled with 100 ml of molten 1.5% water-agarose (SeaPlaque, Lonza), held at 35º

C in a water bath to keep it molten. Each plantlet will be held in place with forceps to keep it upright until the gel sets up. A day later, each GA7 will be inoculated with a 3 mm diameter plug of a *P. cinnamomi* isolate known to be pathogenic on American chestnut, supplied by Dr. Steve Jeffers (Clemson University) and maintained in culture in the lab. Inoculated GA7 vessels with chestnut plantlets will be maintained in a lighted incubator at 22º C with 16 hour day-lengths and plantlets will be observed for PRR symptoms, including darkening of roots and stems and wilting. Data to be collected will include the number of days from *P. cinnamomi* inoculation to appearance of first symptoms and days from inoculation until plantlet death. After 6 weeks, all plantlets will be scored for severity of symptoms using the following scale: 0 = no symptoms, 1 = moderate root or stem lesions/darkening, 2 = extensive root or stem lesions/darkening, 3 = plantlet death. Symptoms will be photo-documented.

Short-term goal 2: *Test the ability of the in vitro screen to detect intermediate levels of PRR resistance in hybrid backcross chestnut material and identify promising clones for field testing.* Production of BC3F3 somatic seedlings from cultures derived from putatively-resistant BC3F2 parents, initiated in 2015 and 2016, will be accomplished following the same procedure as that described above for American chestnut somatic seedlings. Once the ability of the screen to discriminate between susceptible American chestnut plantlets and resistant Chinese chestnut plantlets is established, we will add BC3F3 somatic seedlings representing clones from multiple putatively PRR-resistant parents to the screens. BC3F3 plantlets will be tested in the same water-agarose format described above, along with susceptible American chestnut controls and resistant Chinese chestnut controls. Since BC3F3 plantlets are expected to display resistance levels between those of the two pure species, we will attempt to quantify the intermediate levels of resistance using the same symptom severity scoring system described above, as well as the numbers of days between inoculation and first appearance of symptoms and between inoculation and plantlet death.

Short term goal 3: *Test the ability of the in vitro screen to detect PRR resistance in transgenic chestnuts engineered with candidate PRR resistance genes from the FHI project.* American chestnut somatic seedlings engineered with different PRR resistance candidate genes, singly and in combination, are already in production in the lab. New plantlets with the PRR resistance candidate genes RPH1 and NPR3/4, cloned from Chinese chestnut, and plantlets engineered with both genes, will be tested using the same format as described above, and compared to the resistant and susceptible controls. As with the BC3F3s, we will attempt to quantify the intermediate levels of resistance using the same symptom severity scoring system described above, as well as the numbers of days between inoculation and first appearance of symptoms and between inoculation and plantlet death. The only difference with the testing of the transgenics will be that the American chestnut wildtype controls will be the same background genotypes as the transgenic chestnuts.

Short term goal 4: *Validate results of in vitro screen with ex vitro tests in soil*. It is certainly possible that apparent PRR resistance demonstrated by chestnut plantlets under in vitro conditions may not accurately reflect resistance in soil, since the environments are completely different. Therefore, the performance of genotypes scored as showing resistance will need to be confirmed in potting mix or soil in the greenhouse or in……Additional plantlets representing BC3F3 clones and transgenic lines that show evidence of enhanced PRR resistance compared to wildtype American chestnut plantlets in the in vitro screens will be produced for validation of PRR resistance in potting mix. Currently, we do not have the capacity or expertise to perform this testing in a greenhouse at UGA, but by 2017, we hope to collaborate with Steve Jeffers

(Clemson University) to set up a pot-based screen in one of our greenhouses, since he routinely conducts these screens in his greenhouse. Alternatively, we are also open to supplying these plantlets to others who routinely conduct these kind of screens, including John Frampton at NC State, Hill Craddock at UT Chattanooga, or Joe James.

Experimental design and statistical analysis. Replication within all in vitro screen experiments will be achieved by using at least 4 plantlets per clone to evaluate resistance/susceptibility, using the scoring system detailed above, as well as days to appearance of symptoms and death. Experiments involving BC3F3 clones and transgenics will employ American chestnut plantlets as susceptible controls and Chinese chestnut plantlets as resistant controls. Entire experiments will be conducted (replicated) twice. Scoring data will be plotted using Excel and analyzed by analysis of variance and using PROC ANOVA of SAS (SAS Institute Inc. 2011). Means separation will be performed by Tukey's test using PROC MEANS of SAS (SAS Institute Inc. 2011).

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Project schedule

Milestones (tasks):

1. Conduct +/- in vitro resistance screens on American chestnut and Chinese chestnut plantlets
2. Test in vitro resistance screen on hybrid backcross chestnut material
3. Test in vitro screen on transgenic chestnuts engineered with candidate PRR resistance genes
4. Conduct validation resistance screens in soil

Timeline (Month 1 = December 2016)

Month 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Task 1 xxxxxxxxxx
Task 2 xxxxxxxxxxxxxxxx xxxxxxxxxxxxxxxxxxx
Task 3 xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
Task 4 xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Measurement and reporting of results

Measurements, experimental design and statistical analyses are described above in the Experimental Plan. Results from our experiment will be reported at annual meetings of TACF, meetings of the Georgia Chapter-TACF and/or Southeast Regional TACF meetings and tree improvement meetings such as SFTIC. Results will be included in manuscript to be submitted to the Journal of The American Chestnut Foundation or a plant biotechnology journal such as Trees Structure and Function. An annual report will be submitted to TACF by December 1 of each year the project is active.

Budget justification and funding timing

Funds are requested for 2 months of salary and benefits (estimated at 68%) per year for a Research Technician III. Funds are also requested for tissue culture supplies (chemicals, plasticware, glassware) and greenhouse supplies (pots, potting mix, insect control). Year 1 funds will be needed by December 1, 2016 and Year 2 funds will be needed by December 1, 2017.

Budget (Year 1 only)

	Year 1
Personnel	
Research Technician III (2 months)	5210
Fringe benefits (68%)	3543
Operating expense	
Tissue culture & greenhouse supplies	1247
Total direct costs	10,000
Indirect costs (F&A)	0
Total costs	10,000

CURRICULUM VITAE

Scott A. Merkle

Education

Doctor of Philosophy, Virginia Polytechnic Institute and State University, 1982. Major: Forest Genetics and Tree Improvement.
Master of Science, Virginia Polytechnic Institute and State University, 1978. Major: Forest Biology.
Bachelor of Science, College of William and Mary, 1976. Major: Biology.

Academic and professional positions held

February 2014 - present, Associate Dean for Research and Professor, School of Forestry and Natural Resources, University of Georgia
July, 1997 – January 2014. Professor, School of Forestry and Natural Resources, University of Georgia
May, 2000 – December, 2002. Graduate Coordinator, School of Forest Resources, University of Georgia
July, 1992 – June, 1997. Associate Professor, School of Forest Resources, University of Georgia
July, 1987 - June, 1992. Assistant Professor, School of Forest Resources, University of Georgia
January, 1987 - July, 1987. Temporary Assistant Professor, School of Forest Resources, University of Georgia
April, 1984 - January, 1987. Postdoctoral Associate, School of Forest Resources, University of Georgia
January, 1983 - April, 1984. Postdoctoral Associate, Department of Forest Science, Oregon State University
September, 1976 - August, 1982. Research Associate and Research Assistant, Forestry Department, Virginia Polytechnic Institute and State University

Publications (last 5 years)

Merkle, S.A., and M. Cunningham. 2016. Application of hybrid breeding and somatic embryogenesis to develop sweetgum varieties for the bioenergy and pulp and paper industries. In: Proceedings of the 33rd Southern Forest Tree Improvement Conference, June 8-11, 2015, Hot Springs, AR, pp. 55-57.

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Grants (last 5 years)

American Chestnut Foundation, Mass propagation of elite American chestnut varietals for timber and nut production, $20,000 2015-2016

US Endowment Forestry & Commun, Forest Health Initiative: Biological Research Component Phase II, $88,332 2015-2016

USDA Forest Service, Forest Health Initiative-Phase II Year 2 Discrete Project, $45,000 2015

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