SCALING-UP TRANSGENIC AMERICAN CHESTNUT SOMATIC SEEDLING PRODUCTION FOR THE FOREST HEALTH INITIATIVE

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American chestnut (*Castanea dentata*) was once one of the most important forest trees of eastern North America, both ecologically and economically. With a range stretching from Maine to Alabama, it reached its greatest size and density on the ridges and benches of the Southern Appalachians, where it is estimated that one in four trees was an American chestnut. Huge volumes of American chestnut were harvested annually in the region, and the durable wood was used for construction of houses, barns, fences and furniture. The nutritious nuts were consumed by wildlife and people. The central role of chestnut in Appalachian life changed dramatically following the accidental introduction of the chestnut blight fungus (*Cryphonectria parasitica*) on Asian chestnut planting stock late in the 19th Century. The fungus is a necrotroph, entering any wound in the bark and producing a mycelia fan under the bark that kills the cambium and young xylem and phloem with oxalic acid and then consumes the dead tissue. Eventually, the stem is girdled, killing the tree. First documented in New York City, the blight spread southward at the rate of 200 miles every ten years, killing almost every American chestnut tree in its path. Since its appearance, multiple approaches to combat chestnut blight and restore the American chestnut to the forest have been attempted. These approaches have included application of fungicides, searches for naturally resistant American chestnut trees, mutation breeding via gamma irradiation of nuts, hypovirulence and breeding with resistant Asian chestnut species. While the initial attempt by the USDA to use this last approach ended in apparent failure, a more recent hybrid backcross breeding program initiated by The American Chestnut Foundation in the 1980s has made substantial progress toward producing hybrid trees with near-Chinese chestnut levels of resistance.

Another recent approach to production of blight-resistant trees involves application of *in vitro* propagation and genetic engineering. Laboratories at the University of Georgia (UGA) and The State University of New York-Environmental Science and Forestry (SUNY-ESF) have been conducting research on genetic engineering of chestnut for over 20 years. While these labs made substantial progress developing protocols for producing transgenic chestnut trees, production of trees with genes with potential anti-fungal activity lagged until recently, when significant new support for the research was provided by the Forest Health Initiative (FHI). FHI chose American chestnut as its first target for research in its mission to demonstrate the application of biotechnological tools to address forest health threats in the U.S. The application of in vitro clonal propagation and transgenics is part of a "braided" approach to bring the tools of biotechnology to bear on the chestnut blight problem, which also includes efforts in the areas of germplasm, breeding, genomics and gene discovery. In addition to biological sciences research, the FHI also includes teams focusing on social and environmental issues and on regulatory and legal affairs associated with biotechnology and forest health. As part of this effort, we are collaborating with scientists from multiple universities (SUNY-ESF, Penn State, Clemson), The

American Chestnut Foundation (TACF) and the USDA Forest Service to employ somatic embryogenesis (SE) for several project objectives. SE will be used to propagate blight-resistant hybrid backcross-derived material from TACF's breeding program for clonal testing. SE will also provide target material for testing candidate genes (CGs) from Chinese chestnut and heterologous sources that may provide resistance to the blight fungus and/or *Phytophthora*, which is a particular problem in the southern part of the range. With regard to transgenics, screening of hundreds of embryogenic cultures has already been conducted to identify a handful of "workhorse" culture lines that will be the main targets of transformation with all CGs. However, in order to be selected as a "workhorse" line, a culture line not only has to be "captured" and grow well in suspension culture, but has to successfully pass a number of other bottlenecks, including unambiguous sensitivity to selection agents and ability to produce high-quality somatic embryos and somatic seedlings following transformation.

New embryogenic cultures were initiated from several American chestnut full-sib and half-sib families from different parts of the range hybrid backcross material in 2009 and 2010. In 2009, over 9000 seeds were cultured, resulting in 64 new embryogenic cultures lines or an overall capture rate of 0.7%, while in 2010, over 8500 seeds were cultured, producing 107 new embryogenic cultures for an overall capture rate of 1.23%. A new germplasm agreement with TACF enabled culture, for the first time, of TACF B3F3 hybrid backcross material, resulting in capture of embryogenic cultures from 10 B3F3 families representing two lines of blight resistance. Copies of all embryogenic chestnut cultures were cryostored following our published protocol (Holliday and Merkle 2000). Once established, 2009 embryogenic cultures were screened for potential to produce somatic embryos and somatic seedlings and displayed a range of productivities. Some lines could produce over 100 well-formed somatic embryos per 0.5 g of starting material, following our published protocol for somatic embryo production from suspension cultures (Andrade and Merkle 2005), and some culture lines demonstrated conversion frequencies over 50 percent. Lines initiated in 2010 are currently being screened for somatic embryo and somatic seedling production potential. Culture lines capable of producing a minimum of 10 somatic seedlings are needed to test the concept of clonal testing of genotypes for blight resistance. In addition, information from these culture screens was used to choose three of the most productive lines to uses as "workhorse lines" for transformation with CGs.

Transformation of workhorse lines with new, modular CG and reporter gene constructs began in 2010, using the transformation protocol detailed in Andrade and Merkle (2009). At the start of the project, we projected that we could move only 2-3 CGs through the transformation/regeneration "pipeline" per year, based on the shaken flask-based embryogenic culture system we were using at that time. However, our recent adoption of airlift bioreactors for growing embryogenic suspension cultures, rather than shaken flasks, greatly accelerated production of embryogenic material for both somatic embryo production and *Agrobacterium*-mediated genetic transformation. Currently, sufficient new target material for transformation experiments can be produced every two weeks. This change allowed us to transform 12 CG constructs and 3 reporter gene constructs into embryogenic American chestnut cells to in 8-9 months. Transformation frequencies for some target lines have been very high, producing almost 700 putative transformation events per 50 mg of inoculated tissue of one target line. Over 5000 transgenic events in 3-4 backgrounds (target lines) have been captured. Somatic seedlings

carrying the first FHI CG, an anti-fungal peptide gene from the *Gastrodia* orchid, will be transferred to the greenhouse soon and somatic embryos with four more CGs are in production. Our goal is to generate 20 plant-producing transgenic events per CG, each capable of producing at least 10 transgenic trees to be screened in the field for blight and/or *Phytophthora* resistance. The first field planting of transgenic chestnuts produced at UGA was installed in May 2011. Over 100 transgenic chestnuts carrying the ESF39 synthetic antimicrobial peptide gene (Powell et al. 1995) were planted and some of these trees should be sufficiently large by summer 2011 to inoculate with the blight fungus for resistance screening.

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