DELIVERY AND CHARACTERIZATION OF CANDIDATE GENES FOR DISEASE RESISTANCE IN AMERICAN CHESTNUT: VECTOR CONSTRUCTION AND SCREENING

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American chestnut (*Castanea dentata*) was an abundant tree and a keystone species of eastern US forest ecosystems. "It has been said that an enterprising squirrel could once travel from Maine to Georgia on the interlocking branches of chestnut trees" (Anon). A blight caused by the fungal pathogen *Cryphonectria parasitica* was introduced to the eastern US around the turn of the 20th century. The pathogen spread rapidly and, although root sprouts persist, the American chestnut was essentially lost from the eastern forests by the mid-1900s. The near extirpation of American chestnut has been considered one of the worst ecological disasters in North American history.

Restoration of American chestnut has been selected as the Forest Health Initiative's first project to explore the potential of biotechnology for addressing threats to forest health (Nelson et al. 2009). The multi-institutional collaboration is focused on development and application of biotechnologies for production of disease resistant American chestnut germplasm. Chinese chestnut (*Castanea mollissima*) is resistant to chestnut blight and candidate genes for resistance to pathogens such as *Cryphonectria* and *Phytophthora* have been identified in Chinese chestnut through genomic and bioinformatic approaches. A number of genes conferring anti-fungal activity have been reported in other plant species as well.

Current approaches to obtain candidate resistance genes fall into three broad categories. The first has been comparative transcriptomics of *Cryphonectria*-inoculated American and Chinese chestnut. High-throughput sequence analyses of genes expressed in the two chestnut species have identified specific gene transcripts that are differentially regulated in Chinese chestnut relative to American chestnut. Copies of identified genes were isolated from Chinese chestnut cDNA libraries. The second approach, which also targets genes from Chinese chestnut, is to map quantitative trait loci (QTL) conferring resistance and integrate these data with high-throughput genomic sequencing to identify candidate resistance genes located within the QTL intervals. The third approach is to leverage advances from fungal pathogen resistance studies in other plant species to identify heterologous candidate resistance genes for transformation and testing in American chestnut.

To evaluate the relatively large number of candidate genes that have been identified for *Cryphonectria* and *Phytophthora* resistance, an efficient system for cloning, transformation, expression, and molecular characterization was developed. Variation in delivery and expression of individual genes is minimized through standardization of genetic elements and inter-element sequences so that phenotypic characteristics conferred by individual candidate genes can be

evaluated. A modular vector design facilitates gene insertion, multi-gene construct assembly, and the potential for integrating genome-derived promoter-gene regions.

A binary vector, *pFHI-03*, was constructed for use as a standard, constitutive expression vector for *Agrobacterium*-mediated transformation of chestnut embryogenic cultures (Figure 1). The vector includes the *npt II* gene driven by the Ubiquitin-10 promoter from *Arabidopsis* for antibiotic selection of transformed chestnut cultures. The Ubiquitin-11 promoter from *Arabidopsis* is included to drive expression of the candidate gene of interest. A multiple cloning site is integrated at the 3' end of the promoter and is followed by the CaMV 35S terminator sequence. The *Pac I* recognition sequence within the multiple cloning site provides an invariant AT-rich sequence fusing the 3' end of the promoter directly to the methionine initiator codon sequence of the inserted candidate gene. Unique restriction sites border the elements of the vector to facilitate substitution of promoters, terminators, and selectable markers. These unique restriction sites also facilitate modification of the vector for multi-gene delivery and expression.

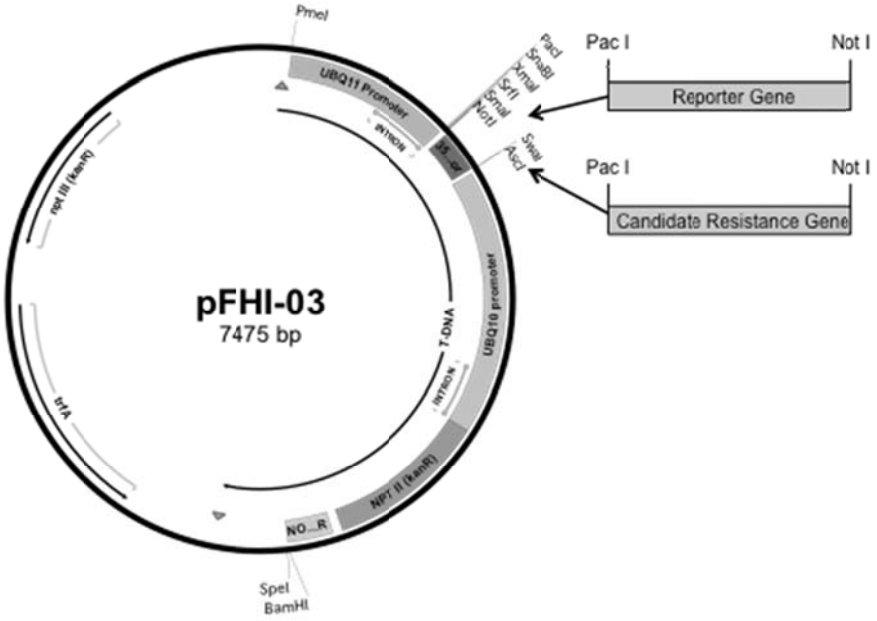

Figure 1. Map of the *pFH-03* vector. A multiple cloning site bordered by *Pac I* and *Not I* recognition sequences is located between the 3' end of Ubiquitin-11 constitutive promoter and the 5' end of the CaMV 35S terminator. A candidate gene or a reporter gene was inserted at the *Pac I* and *Not I* sites to produce individual vectors for transformation into American chestnut embryogenic cultures. A unique *Pme I* site is present to facilitate substitution of promoters and for insertion of multi-gene constructs. Unique restriction sites to permit substitution of alternate selectable markers border the *npt II* gene with the associated Ubiquitin-10 promoter and NOS terminator. Triangles indicate T-DNA borders and an arrow indicates the region of T-DNA transfer.

Reporter gene vectors were constructed containing an intron-*β*-glucuronidase (GUSi) gene (*pFHI-GUSi*), a green fluorescent protein gene (*pFHI-GFP*), and a GUS-YFP fusion (*pFHI-GUSiYFP*). Each of the reporter gene vectors was transformed into embryogenic chestnut cultures and assayed for expression. PCR-based analyses of transgene integration indicated stringent selection with less than 1% escapes and PCR-positive embryogenic tissues exhibited high levels of reporter gene expression as determined by GUS assays and *in vivo* fluorescent imaging of green and yellow fluorescent proteins.

To date, fourteen candidate genes from Chinese chestnut, identified by comparative transcriptomics of *Cryphonectria*-inoculated American and Chinese chestnut, and two heterologous genes have each been cloned into the *pFHI-03* vector and transformed into American chestnut embryogenic cultures. Early screening methods were developed to identify transformed lines for each of the candidate gene vectors. Total genomic DNA was isolated from 5-10 mg of embryogenic tissue and analyzed by PCR using vector-specific primers (Figure 2). The approach reduces the transformation to screening interval from several months to six weeks and minimizes the number of cultures that are maintained to achieve the target of 40 translines per vector.

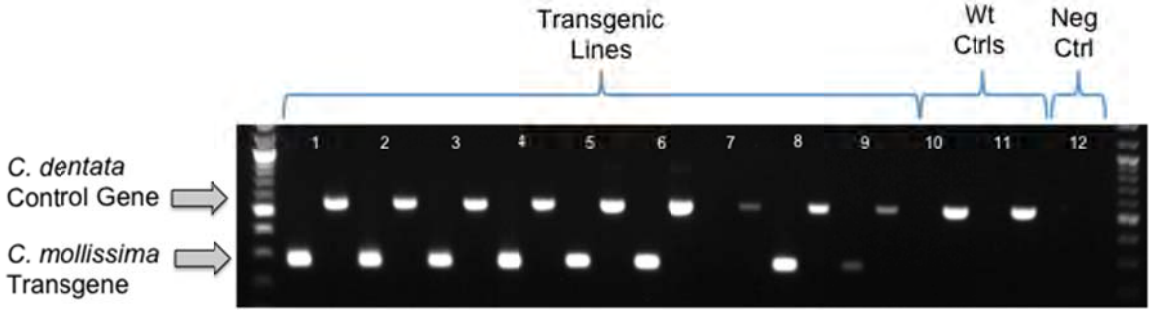

Figure 2. An example of results from PCR screens of transformed embryogenic chestnut tissues. Total genomic DNA was isolated from 5-10 mg tissue samples. DNA from each embryogenic line was assayed by PCR for the Chinese chestnut transgene (1-9). A second PCR reaction assays for an endogenous American chestnut gene was used as an internal control. Wild type American chestnut samples (10 and 11) were positive for the endogenous gene and negative for the transgene. No-template reactions were included as negative controls (12).

Following conversion to somatic seedlings and regeneration of plants, the materials are re-assayed to confirm transgene stability. Independent lines for each candidate gene and clonal materials derived from each line are evaluated to confirm transgene expression. Regenerated plantlets are transplanted into potting mix, then successively moved from growth chambers to the greenhouse and then into the nursery. Potted plants from the greenhouse containing candidate genes for *Phytophthora* resistance genes are subjected to *Phytophthora* screening. Plants with candidate genes for blight resistance will be grown in the nursery for two to three years until they reach a stem diameter suitable for blight resistance screening. Correlations between resistance levels, individual candidate genes, and gene expression levels will be examined.

Acknowledgements

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References Cited

Nelson, CD, Carlson, JE, Merkle SA, Powell, WA. 2009. Biotechnological tools for accelerating species restoration (Forest Health Initiative). International Fagaceae Conference, Research Triangle Park, North Carolina.