SOMATIC EMBRYOGENESIS AND GENE TRANSFER IN AMERICAN CHESTNUT

D. T. CARRAWAY, H. D. WILDE, AND S. A. MERKLE[1]

Abstract. -- Leaves, stem sections, ovules and immature zygotic embryos of American chestnut were used as explants in experiments designed to induce formation of somatic embryos. Embryogenic cultures were induced from immature zygotic embryos under the influence of 2,4-D and BA and could be maintained on either semisolid medium or as suspensions in liquid medium. Suspension cultured cells were bombarded using gold particles coated with plasmid DNA encoding 13-glucuronidase (GUS), and neomycin phosphotransferase (NPT II). Stable integration of the introduced GUS gene in proliferating American chestnut cells was confirmed via fluorescence analysis, histochemical analysis, and Southern hybridization.

Key words: Castanea dentata, Cryphonectria parasitica, microprojectile bombardment, chestnut blight.

INTRODUCTION

American chestnut, Castanea dentata (Marshall) Borkhausen, was once a major component of climax forests in the eastern United States (Anagnostakis 1987). Until the early 1900s, as much as 25% of some eastern hardwood forests were composed of American chestnut (Burnham 1988). This species was the premier hardwood of the eastern United States by virtue of its abundance, decay resistant wood, rapid growth, size, ability for natural regeneration, and its myriad uses (Clapper and Gravatt 1943). During the early 1900s, American chestnut was nearly destroyed by chestnut blight which is caused by a parasitic fungus, Cryphonectria parasitica (Murrill) Barr, = Endothia parasitica (Murrill) P. J. and **H. W.** Anderson (Newhouse 1990). Chestnut blight is the most destructive forest disease known (Newhouse 1990). According to some estimates, the value represented by American chestnut, as it existed in the early 1900s would have been nearly $800 billion in 1991 dollars (Stoke 1940). In spite of attempts to control its advance, less than 50 years after its introduction, chestnut blight had reduced a species that once existed as a large, long-lived forest tree to a short-lived, understory shrub which persists only because of its ability to sprout profusely and repeatedly from living stumps and roots (Beattie and Diller 1954).

[1]J Daniel B. Warnell School of Forest Resources, University of Georgia, Athens GA 30602

American chestnut could potentially be restored as a component of eastern hardwood forests if a solution to the problem of mortality due to the chestnut blight fungus could be found. While some previous efforts to control chestnut blight have produced limited success, none have yet succeeded in restoring American chestnut to its former status. Control methods attempted to date include: clonal propagation and hybridization of native trees thought to possess low levels of resistance (Elkins et al. 1992); production of American chestnut-like hybrids between native trees and resistant Asian chestnuts (Burnham 1988); colchicine induced production of American chestnut polyploids (Dermen and Diller 1962); development of blight resistance in native trees by radiation mutagenesis (Dietz 1978); and use of hypovirulent fungal strains to reduce the degree of blight pathogenicity (Van Alfen et al. 1975).

Molecular biology techniques may have considerable impact on efforts to control chestnut blight. Choi and Nuss (1992) genetically engineered the chestnut blight fungus with a gene that may lead to a greater incidence of hypovirulence in chestnut blight fungus populations. However, hypovirulence alone is unlikely to bring about the restoration of American chestnut as a dominant forest tree species. Although hypovirulent fungal infections are normally nonlethal, American chestnut stems infected with hypovirulent strains are often badly damaged, and multiple infections, even with hypovirulent strains, can be lethal. Thus, there remains a need to develop American chestnut trees which are resistant to infection by the chestnut blight fungus. We believe that American chestnut trees which have been genetically engineered to be resistant to chestnut blight will, in combination with hypovirulent fungal strains, provide an integrated system of disease control that would be superior to either one alone.

Similar to the progress noted above with the chestnut blight fungus, recent advances in the fields of plant tissue culture and plant genetic engineering have provided powerful, new techniques that can be applied to development of an effective control for the chestnut blight fungus from the host side of the interaction. Recently, there has been increasing interest in somatic embryogenesis due to the tremendous potential of its application to plant improvement via high-volume, large-scale clonal propagation of elite genotypes. Successful transformation of embryogenic cells via microprojectile bombardment and subsequent regeneration of transgenic trees has been demonstrated in walnut, Juglans regia L., (McGranahan et al. 1988), yellow-poplar, Liriodendron tulipifera L., (Wilde et al. 1992), and white spruce, Picea glauca (Moench) Voss, (Ellis et al. 1993). Use of genetic engineering in conjunction with somatic embryogenesis could make possible the development of a practical, reliable gene transfer system for American chestnut. This gene transfer system may ultimately be used to incorporate fungal resistance genes into the American chestnut genome.

No genes that code for products known to inhibit the growth of chestnut blight fungus *in vivo* have yet been isolated and cloned. Our goal is to develop a reliable system for generating transgenic American chestnut trees, so that when such genes are cloned, there will be a protocol readily available to engineer American chestnut trees with them. Thus, our approach involves achievement of two objectives: (1) To develop a system for *in*

vitro propagation of America chestnut via somatic embryogenesis, and (2) To define a protocol for incorporation and expression of foreign DNA in embryogenic American chestnut cells. Methods developed to achieve each separate goal will then be combined in order to produce transgenic trees from the transformed cell cultures via somatic embryogenesis.

MATERIALS AND METHODS

Explant Material

American chestnut ovules and zygotic embryos from developing burs were used as explants to initiate cultures. Explant material was collected from as many locations and genotypes as possible throughout the original American chestnut range. Since fertile American chestnut trees are not common, locations and available genotypes were dictated by the occurrence of fertile trees. During the two years (1991 and 1992) that cultures were initiated, twenty-five trees from locations in New York, Wisconsin, Connecticut, Pennsylvania, North Carolina and Georgia were sampled. Fruit from 5 different maturity stages was sampled in an attempt to include material within the developmental "window" required for the development of embryogenic cultures.

Culture initiation

Out-of-state collections of American chestnut material were sealed in plastic bags and shipped via overnight mail. In-state material was sealed in plastic bags and transported in an ice chest. All plant material was refrigerated upon arrival. Nuts were surface sterilized according to a procedure previously described for American chestnut (Merkle et al. 1991). Ovules were wounded before being placed onto culture medium. Cotyledons were removed from the embryonic axis of immature zygotic embryos and were cultured separately. Mature zygotic embryos were cultured in the same fashion except that cotyledons were cut into four quadrants before being placed onto culture medium:

Culture Media and Growth Regulators

Explants were cultured on two types of semisolid basal medium, woody plant medium [WPM] (Lloyd and McCown 1980) and Driver and Kuniyuki medium [DKM] (McGranahan et al. 1988). Media were solidified with 0.8% (wt/v) phytagar. During the two years that cultures have been initiated, four auxins and two cytokinins have been tested. Auxins tested were 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), napthaleneacetic acid (NAA), and 4-amino-3,5,6-trichloropicolinic acid (picloram). Cytokinins tested were benzyladenine (BA) and thidiazuron. Plant growth regulator treatments were as follows: (A) no growth regulators; (B) auxin only; (C) cytokinin only; (D) auxin + cytokinin (E) 1, 2, or 3 week pulse on B followed by transfer to A or C; (F) 1, 2, or 3 week pulse on C followed by transfer to A or B; (G) 1, 2, or 3 week pulse on D followed by transfer to A.

Microprojectile Bombardment

Suspension cultures were initiated by inoculating cells from cultures on semisolid medium into 125 ml Erlenmeyer flasks containing liquid WPM supplemented with 3 mg/1 2,4-D and 0.25 mg/1 BA (W3.25). Ten day old suspension cultures were fractionated through a stainless steel mesh with 380 pm grid size. The fraction that passed through the mesh was plated on 90 mm sterile filter paper disks over semisolid W3.25. After ten days growth on filter paper disks, the cells were bombarded with plasmid DNA (pBI121.1 [Jefferson et al. 1987]) which carries the *uidA* reporter gene encoding 13-glucuronidase (GUS), and the *nptII* selectable marker gene encoding neomycin phosphotransferase. Plasmid DNA was precipitated onto 1 µ.m diameter gold particles using the $CaCl_2$ method of Klein *et al.* (1989). Ten microliters of an alcohol suspension of the DNA-coated gold particles were dried onto a macrocarrier and accelerated into target cells using a BioRad PDS-1000/HE particle gun.

Selection of transgenic cells

Prior to microprojectile bombardment, a kanamycin sensitivity curve was developed for 3 different lines of embryogenic American chestnut cells. Cell lines were subcultured to W3.25 supplemented with kanamycin sulfate in concentrations ranging from 20 to 200 µg/ml in 20 pg/ml increments. After 2 weeks on the selection medium, all cells that had been subcultured to medium containing more than 80 pg/ml kanamycin sulfate had been killed. Bombarded cells were allowed to stabilize for 12 days on antibiotic free medium before being transferred to selection medium with 100 µg/ml kanamycin. After 8 weeks on selection medium, kanamycin-resistant cell clusters were transferred to fresh selection medium and maintained by monthly subcultures to fresh selection medium.

GUS Assays

Expression of the *uidA* gene in kanamycin resistant cells was verified with a histochemical assay using 5-bromo-4-chloro-3-indoyl glucuronic acid (X-gluc). Approximately 10 mg of kanamycin resistant cells per well were placed in 96-well tissue culture plates with 20 p.1 of 2mM X-gluc solution per well. A fluorescence assay was also performed using 4-methyl umbelliferyl glucuronic acid (MUG). A protein extract was prepared by homogenizing 100 mg of cells in 1.5 ml Eppendorf tubes containing 200 µl of GUS extraction buffer (Jefferson et al. 1987). The homogenate was centrifuged at 15 g for 1 minute and the supernatant was removed and split into two equal parts. An equal volume of GUS extraction buffer containing 2 mM MUG was added to the protein extracts. The reaction in one tube was stopped immediately with 100 µl of 200 mM Na_2CO_3. The other half of the protein extract/MUG reaction was incubated at 37° C for one hour and stopped. Fluorescence, as visualized with long wave UV light, was compared between the two reactions.

DNA isolation and analysis

A cetyltrimethylammonium bromide nucleic acid extraction procedure modified from Doyle and Doyle (1990) was used to isolate DNA from approximately 150 mg of callus tissue from seven different GUS positive cultures and one nontransformed control. Isolated DNA was digested with the restriction enzymes EcoRI and HindIII. Digested DNA from the seven putative transformants (10 µg/well), and the EcoRI/HindIII excised GUS fragment from pBI121.1 were separated by electrophoresis through a 1% agarose gel. Southern blot hybridizations (Southern 1975) were performed on the resulting gel. The probe was a randomly labeled EcoRI/HindIII excised GUS sequence from pBI121.1. Probe labeling, hybridization, and detection was performed as per the protocol in the Boehringer Mannheim Genius III digoxigenin DNA labeling and detection kit.

RESULTS AND DISCUSSION

Somatic Embryogenesis

Embryogenic response varied according to tree source, type and maturity stage of explant tissue, and growth regulator treatment. Explants from 16 of the 25 trees sampled during 1991 and 1992 produced somatic embryos. However, only 5 trees produced cell lines that exhibited repetitive somatic embryo production over several months. Repetitive embryogenic cultures were initiated from developing ovules and zygotic embryos less than 4 mm in length (Figure 1). The cell lines that consistently produced somatic embryos had been continuously exposed to a combination of 3 mg/1 $2,4^{-D}$ and .25 mg/1 BA. There was no noticeable difference in culture performance between the two basal media.

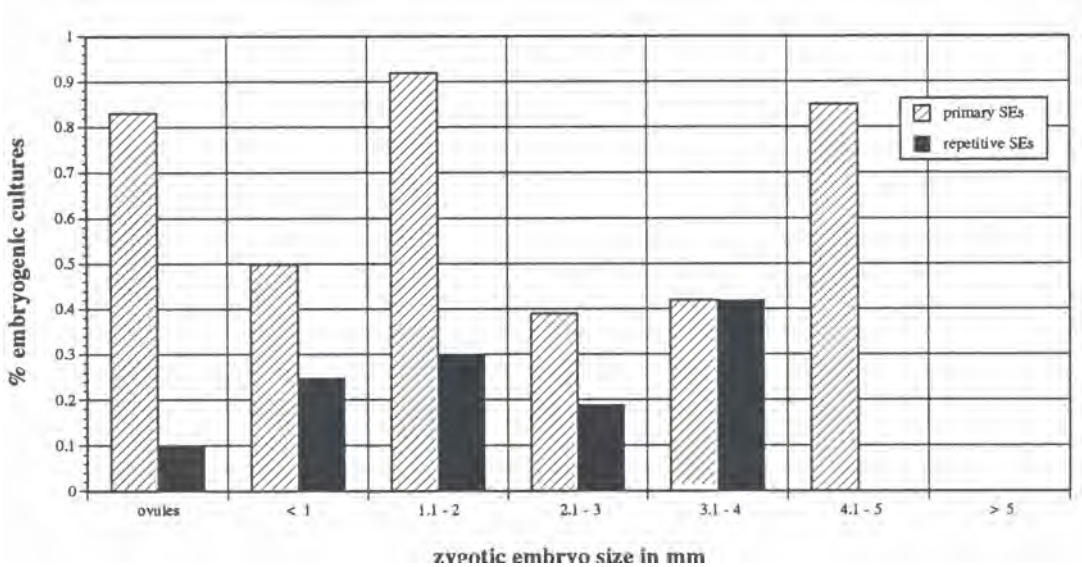

Figure 1. Relationship of zygotic embryo maturity stage (measured by length in mm) and embryogenic frequency after 3 months on W3.25 medium.

Callus from two cotyledonary explants produced adventitious shoots. Non-expanded leaves and primary growth of stem sections from these adventitious shoots were explanted to the same medium used in production of embryogenic cultures from immature zygotic embryos. Nonembryogenic callus resulted from the vegetative explants.

Embryogenic cultures are being maintained on semisolid by monthly transfer to W3.25. Somatic embryos can be produced from these cell lines after subculture to WPM without growth regulators, but with the addition of 1% wt/v activated charcoal. American chestnut somatic embryos are now receiving treatments in order to promote maturation and germination. These treatments include addition of a non-plasmolyzing osmoticum, altering the carbon source, cold stratification, desiccation, and exposure to abscisic acid.

Gene Transfer

Microprojectile bombardment produced 16 putatively transformed cell lines. Approximately 8 weeks after bombardment, kanamycin-resistant colonies of cells were visible against a background of senescent cells. Each resistant colony was transferred to its own plate of fresh selection medium to continue growth. Approximately 10 weeks later, each putatively transformed line was tested for expression of the GUS reporter gene, using both the fluorescence assay and the histochemical assay. Of the 16 kanamycin-resistant lines, 11 were GUS positive and 5 had no GUS activity detectable by the X-gluc or MUG assays. Following 6 months of maintenance on selection medium, Southern blot analysis was performed on seven GUS positive cell lines. The GUS gene was detected in all seven lines (Fig. 2). To date, no mature somatic embryos have been produced from these transformed cell lines. However, all of the transformed lines were derived from a single embryogenic American chestnut suspension culture which had only infrequently produced well-formed somatic embryos prior to the gene transfer experiments. Other, more highly embryogenic lines are available for bombardment, and we intend to apply what we have learned to date to obtain transgenic somatic embryos from those lines.

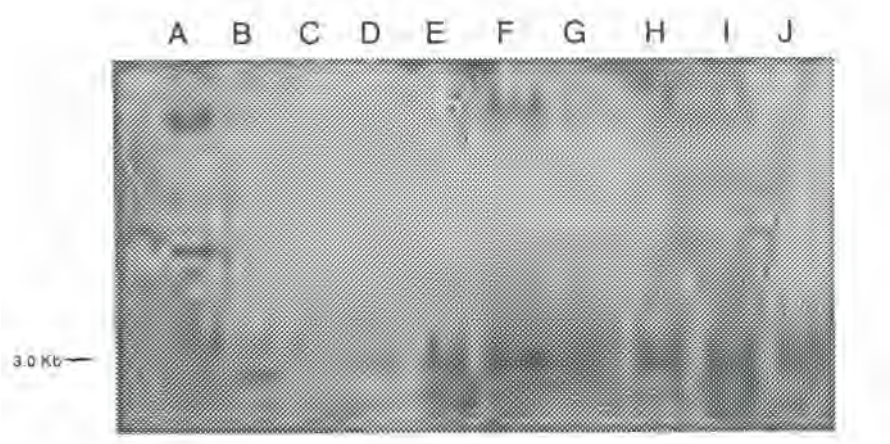

Figure 2. Southern blot analysis of the GUS gene in transformed American chestnut cultures. Lane A, DNA molecular weight markers. Lane B, pBI121.1 digested with EcoRI and HindIII. Lane C, nontransformed cell line. Lanes D-J, transformed cell lines.

CONCLUSION

We have demonstrated that embryogenic cultures can be induced from immature zygotic embryos of American chestnut. In addition, we have shown that microprojectile bombardment is an effective method for genetic engineering of American chestnut cells. We believe that the integration of somatic embryogenesis and microprojectile-mediated gene transfer will allow development of a system for producing transgenic American chestnut trees.

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