

MICROPROJECTILE AND AGROBACTERIUM-MEDIATED TRANSFORMATION OF PIONEER ELM

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Abstract. We have transformed internode sections of the hybrid elm cultivar "Pioneer" to assess the possibilities of introducing significant genes into elms. Foreign DNA encoding beta-glucuronidase (GUS) was introduced into internode sections using microprojectile- or *Agrobacterium tumefaciens*-mediated transformation. Positive signals were detected by Southern analysis. Also, some enzymatic activity was detected in *Agrobacterium* transformed cultures using a fluorogenic assay.

Résumé. Nous avons transformés des sections internodales d'orme hybride de cultivar Pioneer afin d'évaluer la possibilité d'introduction de gènes significatifs chez les ormes. Le glucuronidase (GUS) encodé dans un ADN étranger était introduit dans les sections internodales par l'utilisation de microprojectiles ou par transformation de l'intermédiaire *Agrobacterium tumefaciens*. Les signaux positifs étaient détectés par l'analyse. De même, une certaine activité était détectée au sein des cultures d'*Agrobacterium* transformés par l'emploi d'un essai fluorogénique.

Dutch elm disease (DED) is a devastating vascular wilt disease caused by the fungus *Ophiostoma ulmi*, which has nearly annihilated the American elm in North America. Approaches toward protecting susceptible elms from DED include introducing disease resistance genes (to deter the fungal pathogen) or insect resistance genes (to ward off the beetle vector). These goals, which utilize biotechnology, require: 1) the identification of a single-gene trait associated with the desired phenotype, 2) access to the DNA responsible for this phenotype, and 3) techniques for the transformation and regeneration of the plant of interest.

Progress in these three areas, with respect to DED, has been slow. Because of the complexity of plant resistance and defense mechanisms, identifying the isolating single gene products has not yet been possible, although work is currently underway to identify restriction fragment length polymorphisms which could be used as molecular markers for resistance (R.K. Hajela and M.B. Sticklen, unpublished observations). In addition, some work has been done to establish regeneration procedures for the American elm (2, 8, 9, 17, and M.G. Bolyard, C. Srinivasan, and M.B. Sticklen, unpublished observations).

We have attempted to introduce foreign genes into a variety of elm for which regeneration protocols have been developed. The regeneration of shoots from the hybrid elm "Pioneer" (16) was reported following treatment of shoot tips, leaves and internodes (4) and protoplasts (15). Foreign DNA was inserted into "Pioneer" elm internode sections via techniques that have been used previously for major crop species, including microprojectile bombardment (13) or *Agrobacterium tumefaciens*-mediated transformation (5).

Materials and Methods

Plant Material. "Pioneer" elms were obtained as rooted cuttings from the Agricultural Research Service, Delaware, OH. Rapidly growing stem sections of "Pioneer" elm were excised from greenhouse grown plants and surface sterilized with 20% commercial bleach (Chlorox) and 1% Tween 20, for 20 minutes, then rinsed extensively with sterile distilled water. After two days of preculture on modified Murashige and Skoog medium (11) [Sigma #6899 supplemented with 100 mg/L casein enzymatic hydrolysate, 100 mg/L myo-inositol, 3% sucrose, and 200 mg/L thiamine HCl, hereafter referred to as MS] to test for contamination, internode sections were excised for transformation.

Transformation Procedures. Co-cultivation of internode sections with a 1:10 dilution of *Agrobacterium tumefaciens* (strain LBA4404) containing the binary vector pBI121 (Clontech, 7) was carried out overnight. Incubated internodes were washed twice in liquid MS medium containing 500 mg/L carbenicillin, then cultured in petri dishes containing the same medium supplemented with 10 μ M benzyl adenine (BA) and 6% phytagar (Gibco). Internodes obtained in the same fashion were bombarded with tungsten particles (1.2 μ m, Sylvania GTE M17) coated with pBI121 or pActF-1 (10) plasmid DNA via the "Biolistic" PDS-1000 microprojectile bombardment device (DuPont). Bombarded internodes were plated on MS medium containing 0.5 mg/L

pyridoxine, 0.5 mg/L nicotinic acid, 10 μ M BA, and 6% phytagar.

DNA Analysis. DNA from callus of putatively transformed cultures was extracted by the method of Dellaporta et al. (1). Total DNA was analyzed by Southern analysis (14) following transfer to Nytran (0.45 μ m, Schleicher and Schuell). Filters were hybridized with the appropriate probes which had been labeled with 32 P (800 Ci/mmol, New England Nuclear) using the random priming method (3). Following hybridization, filters were washed at high stringency (12) and exposed to X-ray film (X-omat AR-5, Kodak).

Fluorogenic Assay. The fluorogenic assay was performed as described by Jefferson (6), using the substrate 4-methyl umbelliferyl beta-D-glucuronide (MUG). Briefly, approximately 50 mg of callus material was extracted in microcentrifuge tubes containing 500 μ l GUS extraction buffer. 50 μ l of extract was then added to 50 μ l of assay buffer containing 1 mM MUG. After 60 minutes of incubation at room temperature, samples were analysed by ultraviolet irradiation and photographed.

Results

Callus cultures were established following bombardment of "Pioneer" elm internodes by culturing the explants on modified MS medium supplemented with 10 μ M BA (4). Most callus cultures (Figure 1) did not regenerate shoots, although shoots were regenerated from cultures following a mock bombardment (Figure 2). After approximately six months of culture, the callus was pooled according to the genes used for transformation, and total DNA was extracted.

DNA from callus cultures bombarded with one of two plasmids containing a gene encoding beta-glucuronidase (GUS), pBI121 and pActF-1 (10), as well control cultures, was probed with a 32 P labeled Bam HI-Eco RI fragment from pBI121. As shown in Figure 3, a positive signal can be observed in each of the duplicate lanes containing pBI121 (lanes B and E), but no signal was observed in the control lanes (lanes A and D) or lanes containing pActF-1 (lanes C and F).

Total DNA was also isolated from callus cultures initiated from internode sections which had been co-cultivated with *Agrobacterium* containing PBI121. These cultures also failed to produce shoots, although some organization was detected

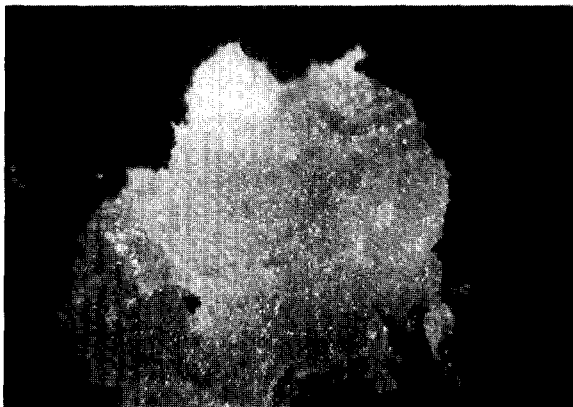


Figure 1. Callus culture from pioneer elm internode section cultured on MS medium supplemented with 10 μ M BA, photographed after approximately 3 months in culture following bombardment.

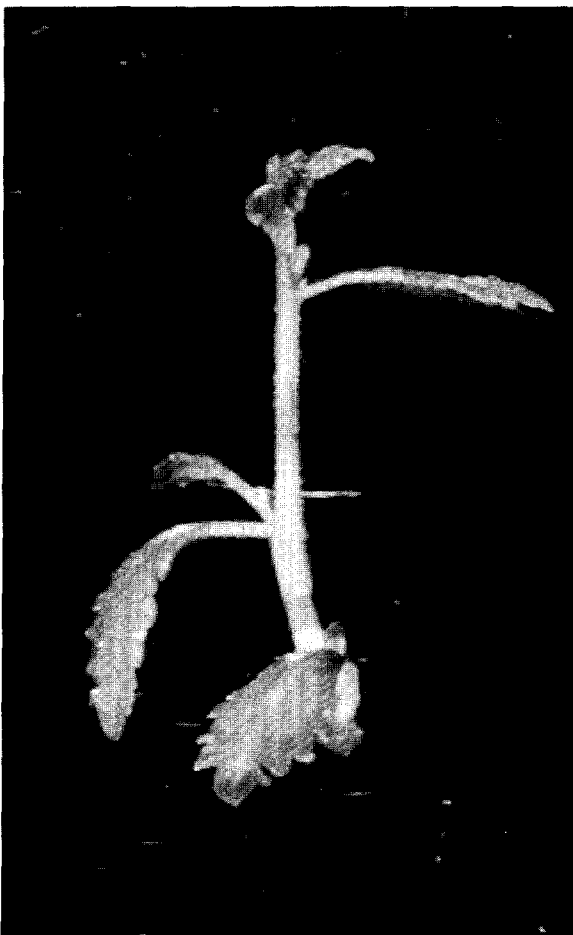


Figure 2. Shoot regenerated from an internode following a mock bombardment with tungsten particles.

(data not shown), which was later covered with callus. DNA was extracted from ten pools of callus material for Southern analysis (14). Six of the ten samples showed a signal when probed with a portion of pBI121 (data not shown). In addition, part of the callus material was used in a fluorogenic assay which is routinely used to detect beta-glucuronidase activity in transgenic plants (6). One of the extracts, which gave a positive signal by Southern analysis, showed fluorescence (Figure 4, tube F).

Discussion

We have demonstrated the transformation of Pioneer elm internodes using the microprojectile and *Agrobacterium* methods. Southern analysis revealed bands from samples transformed with pBI121, while no signal has been detected from material treated with pActF-1, each following transformation by microprojectile bombardment. In addition, plants were regenerated from internodes following a mock bombardment, indicating that the physical stress to the internodes caused by bombardment with tungsten particles was not sufficient to prohibit regeneration. However, it is

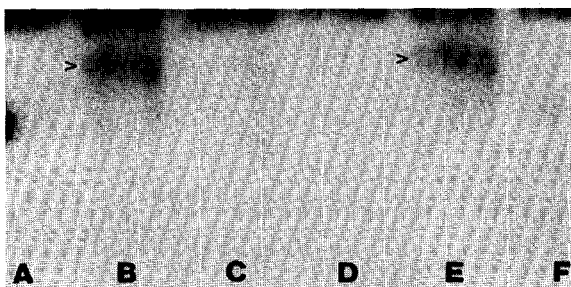


Figure 3. Southern analysis of total DNA isolated from control (Lanes A and D), pBI121 (lanes B and E), or pActF-1 (lanes C and F), following hybridization with a ^{32}P labelled fragment from the GUS gene in pBI121.

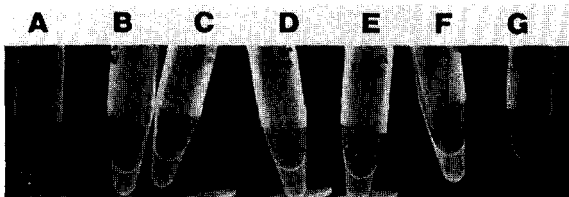


Figure 4. Microcentrifuge tubes containing pioneer elm callus extracts treated with buffer containing the substrate MUG. The tubes are arranged on a UV light box at a wavelength of 302 nm. Letters denote tubes containing extracts from different callus cultures.

not clear why the transformed callus cultures did not produce shoots.

Also, fluorescence activity and positive signals following Southern analysis have been detected from samples co-cultivated with *Agrobacterium*. However, none of the callus cultures produced shoots, indicating that perhaps co-cultivation with *Agrobacterium*, culture on media containing carbenicillin, or the lower levels of vitamins in the media could have interfered with regeneration.

These experiments provide the first evidence of elm transformation. It is to be hoped that these results will be the beginning of more investigations into the applications of the tools of molecular biology for the improvement of elms, particularly in the area of increasing resistance to Dutch elm disease, including the fungal pathogen and the beetle carriers, in order to maintain the American elm as part of the North American landscape.

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ABSTRACTS

POWELL, C.C. 1990. **Fungicides: an overview.** Am. Nurseryman 171(6):61-70.

Hundreds of infectious diseases can threaten the health of today's landscape plants. However, many will yield to fungicidal sprays. Most of the effective fungicides available today will work—if they are combined with good plant care practices and an environment manipulated to curb pathogen-favoring conditions. Fungicides work best as a preventive measure. Their effectiveness depends on treating your crop before a pathogen strikes and you see actual damage. Preventive fungicide treatments can help control the diseases leading to damping off and root or crown rot. Many products prevent diseases caused by water molds. The fungicides now available to combat powdery mildews are extremely effective. Fungicides can adequately control rust if they are applied before the disease strikes. Mancozeb remains the best general fungicide available for the many miscellaneous leaf spots that occur in the nursery and landscape. Preventive spray programs properly managed are the secret to successful disease management.

SANDERS, PATRICIA L. 1989. **Resistance to fungicides.** Grounds Maintenance 24(9): 74, 76, 104.

Fungicides can be divided into two groups. Contact fungicides coat plant surfaces, providing a barrier against disease-causing fungi. Systemic fungicides also act as surface barriers, but have the added advantage of being absorbed by plants. The major problem with systemic fungicides is the development of fungal resistance. To understand fungicide resistance, we must understand the concept of population. Because systemic fungicides usually poison fungi at a single point in their growth and development, it is likely that some individuals in a population will be unaffected by the fungicide. When the same fungicide is used repeatedly and exclusively, these resistant individuals will increase in number until most of the population is resistant, and disease control fails.