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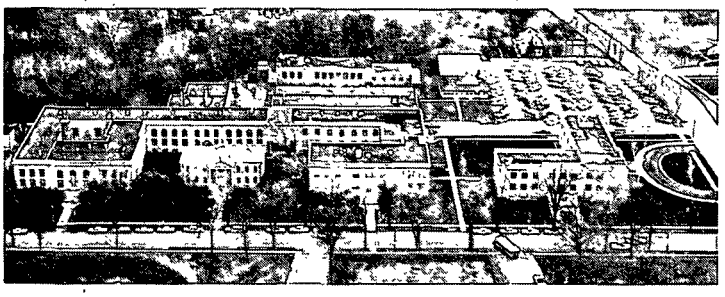

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES
NUMBER 270

TRANSFORMATION AND FOREIGN GENE EXPRESSION IN MICROPROPAGATED SWEETGUM (*LIQUIDAMBAR STYRACIFLUA* L.)

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JANUARY, 1988

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This manuscript is based on results obtained in IPC Project 3223 and has been submitted for consideration for publication in Plant Cell Reports

TRANSFORMATION AND FOREIGN GENE EXPRESSION IN MICROPROPAGATED SWEETGUM (*LIQUIDAMBAR STYRACIFLUA* L.)

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ABSTRACT

Evidence has been obtained which documents the successful transformation of sweetgum, a commercially important forest tree species. A tumor was produced on an in-vitro derived shoot inoculated with a binary Agrobacterium vector. Tumor-derived callus displayed growth in the presence of kanamycin, exhibited measurable levels of CAT activity and, as shown by Southern blot analysis, contains genes originating from the A. tumefaciens binary vector. While no plants have been regenerated from the transformed cells, these findings encourage further work with more regenerable sweetgum tissue culture systems.

INTRODUCTION

Assured supplies of hardwood fiber for the U.S. pulp and paper industry require a reliable silvicultural system for establishing and managing hardwood plantations from genetically improved material. Vegetative propagation and modern recombinant DNA techniques may become part of such a system. In the Southeastern United States, sweetgum (Liquidambar styraciflua L.) is a candidate for plantation forestry, as it exhibits fast growth on a wide variety of sites and produces a pulp that blends well with pulp of southern pines. A further demonstration of the utility of this species is its consideration for use in biomass production (Raney et al., 1987) as well as in the horticultural industry.

Sweetgum has been propagated in vitro from shoot cultures established from seedling (Sommer et al., 1985) and mature tree explants (Sutter and Barker, 1985). In addition to serving as a system for the clonal propagation, shoot cultures also provide a convenient source of sterile plant material to explore gene transfer with Agrobacterium tumefaciens. Using micropropagated shoots, we describe the first successful introduction of foreign genes into a commercially important southern hardwood forest species.

MATERIALS AND METHODS

Establishment of Shoot Cultures

Small cuttings (8-10 inches) containing dormant lateral buds were harvested in March. Cuttings were obtained from trees selected in 3-year-old progeny tests (G. Hansen, Union Camp Corporation, Franklin, VA). Buds were picked from the cuttings and soaked for 30 minutes in a solution containing a small amount of surfactant (Tween 20, 0.1%). Buds were sterilized with 10% commercial household bleach (Hilex, 0.525%) for 15 minutes. After three rinses with sterile water, the apical meristems (plus 3-4 primordial leaves) were excised and sterilized with 1% Hilex for five minutes. After three additional rinses with sterile water, shoot tips were placed on WPM medium (Lloyd and McCown, 1980) containing 0.05 mg/L NAA and 1.0 mg/L BA solidified with 0.8% agar (pH = 5.6). Following the procedure of Sutter and Barker (1986), shoot tips were transferred to fresh medium (by simply moving the bud to a different part of the petri dish) every 3-4 days. Within 3-4 months, each shoot tip had proliferated a number of shoots and, by the end of six months, stable, rapidly growing shoot cultures were established.

Plant Transformation

The A. tumefaciens employed is a binary vector system developed and described by An (1986). Oncogenic A. tumefaciens strain A281 carrying the binary vector designated pGA515-47, which contains neomycin phosphotransferase (npt) and chloramphenicol transferase (CAT) genes driven by nos promoters, was maintained as described (An, 1986). Twelve shoots, 2-3 cm in length, were excised from the shoot cultures and inoculated on the stem portion. An additional twelve shoots were inoculated on the second or third leaves down from the

shoot apex. Inoculation was performed with an overnight culture of A. tumefaciens using a 22 gauge needle to make a small wound, into which ca. 10 µL bacterial culture was applied. After 8 weeks a gall was observed on the stem of one of the twelve shoots. Sixteen weeks after inoculation, the gall was dissected free of the shoot and placed onto shoot proliferation medium (WPM medium with 0.05 mg/L NAA and 1.0 mg/L BA) containing carbenicillin (500 ug/mL) and cefotaxime (250 mg/mL) to kill any remaining Agrobacterium. A pale white callus proliferated from this gall and has been maintained in culture by monthly transfer to fresh medium (antibiotics not present).

CAT Analysis

Chloramphenicol acetyl transferase (CAT) activity was determined by the method of Gorman et al. (1982) employing the modifications of An (1986). Additionally, the plant extract was incubated at 65°C for 10 min before use in the assay. Twenty microliters of plant extract was incubated with 100 µL of reaction buffer, to which 0.1 µCi [^{14}C]-chloramphenicol (54 mCi/mmol, Amersham Corp.) was added. After 30 min incubation at 37°C, chloramphenicol and the resulting acetyltylated derivatives were separated on a silica gel TLC plate. The chloramphenicol and radioactive derivatives were then visualized by autoradiography.

DNA Isolation and Southern Blot Analysis

DNA was isolated from transformed and untransformed control callus using the method described by Dellaporta et al. (1983). Approximately 3 µg of DNA was digested with Eco Rl according to manufacturer's instructions and electrophoresed in a 0.7% agarose gel. Undigested DNA from the transformed plant, as well as Eco Rl digested and undigested plasmid DNA (pGA515-47) were

also included on the gel. Following electrophoresis, the DNA was transferred to Zeta-Probe nylon blotting membranes (Bio-Rad Laboratories) using an alkaline blotting technique (Read and Mann, 1985). The membranes were then hybridized with radioactive probes consisting of either the pGA515-47 plasmid or just the npt sequence (Church and Gilbert, 1984). Radioactive probes were prepared by nick translating the entire pGA515-47 plasmid or oligo-labeling a Bam Hl fragment of pDO421 (which contains only the npt II sequence). Labeling reactions were carried out according to the manufacturer's instructions. After hybridization, the membranes were washed at 65°C (Maniatis et al., 1982) and exposed to Kodak XAR-5 film.

RESULTS AND DISCUSSION

Approximately 8 weeks after inoculation, a small growth was noted on one of twelve treated shoots. Later, this growth enlarged and exhibited characteristics of a crown gall phenotype. After freeing the cultures of Agrobacterium, the callus was transferred to and is now maintained on WPM (NAA and BA at 0.05 and 1.0 mg/L, respectively) containing kanamycin at 100 µg/mL. It should be noted that WPM containing NAA and BA at these levels is not typically conducive to normal callus growth of sweetgum. The callus thus exhibits two characteristics of transformed tissue; those being the ability to grow in the absence or at reduced levels of plant growth regulators and the ability to grow well in the presence of kanamycin, a trait imparted by the nptII gene present in the T-DNA. Untransformed control sweetgum callus proved unable to grow in the presence of kanamycin.

CAT Activity

Chloramphenicol acetyl transferase activity assays were carried out to demonstrate the presence and expression of the CAT gene in the transformed

tissue. As shown in Figure 1, acetylated derivatives of chloramphenicol were formed with extracts of transformed tissue but not untransformed control tissue. Although present, the CAT activity in the transformed tissue appeared to be very low. Higher levels of CAT expression might have been obtained using the 35s cauliflower mosaic virus promotor, which has been reported to be 10-15 fold stronger than the nos promotor (Morelli et al., 1985). Insertion of the CAT gene into a relatively inactive area of the genome or the presence of an inhibitor of CAT activity in extracts of the transformed tissue might also have been responsible for the low CAT activity observed. While not obvious in Figure 2, it appeared that extracts of nontransformed control tissue reduced the apparent activity of authentic CAT isolated from E. coli (data not shown), suggesting the presence of such an inhibitor.

(Figure 1 and 2 here)

Southern Blot Analysis

The most convincing evidence demonstrating transformation of the tissues is provided by Southern blot analysis. When probed with the entire pGA515-47 plasmid, the probe hybridized to several bands in the lane containing DNA isolated from the transformed tissue (Figure 2a). The lengths of these fragments agree with those expected from an Eco Rl digest of the T-DNA of pGA515-47. The probe actually hybridized to more fragments than expected, indicating that the transformed callus may not be of clonal origin. Several transformation events could have occurred, in which full length T-DNA as well as fragments of the T-DNA may have been inserted into different cells. The probe did not bind to any fragment of the DNA isolated from untransformed control callus, providing evidence that the probe did not hybridize to a sequence normally present in the sweetgum genome. To prove that the probe was hybridizing

to T-DNA that was incorporated into the sweetgum genomic DNA, and not to plasmid DNA that might have been present in Agrobacterium contaminating in the callus tissue, uncut DNA isolated from transformed tissue was probed (Figure 2a, lane 3). The probe hybridized to high molecular weight (plant) DNA and no bands characteristic of those obtained when the probe hybridizes to undigested pGA515-47 plasmid DNA were observed. Thus, hybridization signals in the DNA isolated from transformed tissue result from the presence of T-DNA in the sweetgum genomic DNA.

A more specific probe was also used to confirm transformation. Using only the npt II sequence as a probe in Southern blot analysis, the presence of the npt II gene was demonstrated in the DNA of the transformed tissue (Figure 2b). Several bands are missing in this blot when compared with the results obtained using the entire Ti-plasmid as a probe, presumably due to hybridization of pGA515-47 to fragments containing the CAT gene or T-DNA border sequences. As shown on the previous blot, the probe hybridized with high molecular weight plant DNA in the lane containing the uncut DNA from transformed tissue, and did not give a hybridization pattern expected if Agrobacterium was still present in the transformed tissues. This blot again confirms the presence of foreign genes, specifically npt II sequences, in the genome of the transformed sweetgum callus. The apparent ability of the transformed tissue to grow in the presence of kanamycin suggests that the nptII gene is not only present, but is being expressed.

CONCLUSION

While reports of transformation of agronomic crops are plentiful in the literature, work with forest trees has lagged. Conifer shoots and seedlings

have been successfully infected with A. tumefaciens and transformed calli have been obtained from these tissues (Dandekar et al., 1987; Sederoff et al., 1986). Work with angiosperm forest tree species has progressed significantly farther. Transformation as well as regeneration of transformed Populus hybrids has been reported (Pythoud et al., 1987; Fillatti et al., 1987; Parsons et al., 1986).

The transfer of foreign genes into sweetgum, a hardwood species commercially important in the southern United States, and the subsequent expression of those genes in the transformed tissue are demonstrated in this report. The presence of the foreign DNA was confirmed by Southern blot analysis, and expression of the transferred genes was demonstrated by tumor formation on the inoculated explant, the ability of the transformed tissue to grow in the presence of kanamycin and measurable levels of CAT activity in extracts of the tissue. This work may lead to studies of gene expression and transfer of economically important traits in this commercially important hardwood tree species.

ACKNOWLEDGMENTS

The authors wish to thank Dr. David Ow (Plant Gene Expression Center, Albany, CA) for generously supplying the plasmid pDO421, which contains an npt gene. Helpful discussions with Dr. R. Amasino (University of Wisconsin) are also appreciated.

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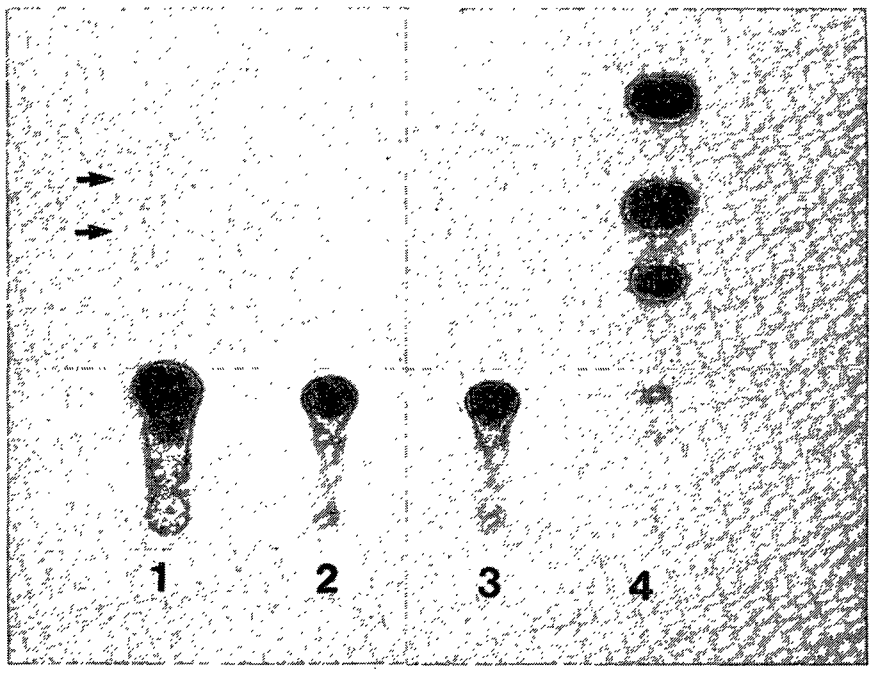

Figure 1. Results of CAT activity assays of transformed (1) and untransformed (2) sweetgum calli. Control treatments included chloramphenicol incubated without a plant extract (3) and incubated with authentic CAT obtained from E. coli (4). Arrows mark positions of weak radioactive spots representing acetylated chloramphenicol derivatives present in incubated extracts of transformed callus.

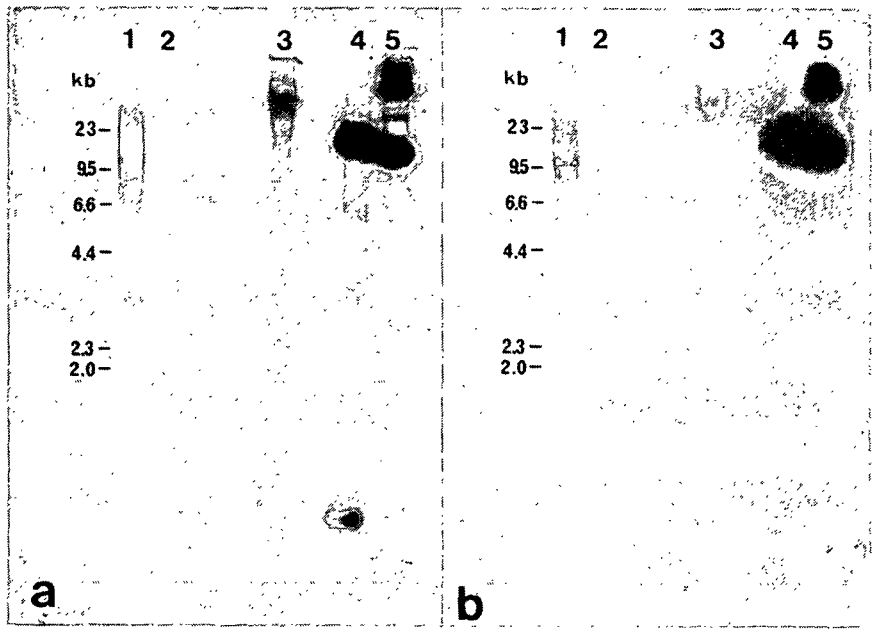

Figure 2. Southern blot analysis of Eco Rl digested DNA isolated from transformed (lane 1) and untransformed (lane 2) sweetgum calli. Lane 3 contained uncut, high molecular weight DNA isolated from transformed callus. Controls included uncut (lane 5) and Eco Rl digested (lane 4) pGA515-47 plasmid. Membranes were probed with nick translated p515-47 (A) and an oligo-labelled nptII gene fragment (B).