PRELIMINARY EVIDENCE OF TRANSFORMATION AND FOREIGN GENE EXPRESSION IN SWEETGUM (Liquidambar styraciflua L.) AND LOBLOLLY PINE (Pinus taeda L.)

Abstract. Preliminary evidence has been obtained which documents the successful transformation of two commercially important forest tree species. A tumor was produced on an in vitro derived sweetgum 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 analyses, contained DNA originating from the A. tumefaciens binary vector. While no plants have been regenerated from the transformed cells, these findings encourage further work with regenerable sweetgum tissue culture systems. Transformation of loblolly pine was achieved via co-cultivation of an Agrobacterium vector with immature somatic embryos grown in suspension cultures. To date, transient expression of the GUS (B-glucuronidase) marker gene in intact loblolly pine cells has been demonstrated. The opportunity to transform embryogenic conifer cultures offers many advantages in the genetic transformation and subsequent propagation of economically important softwood species.

Keywords: Liquidambar styraciflua L., Pinus taeda L., Agrobacterium, transformation, CAT, GUS

INTRODUCTION

Assured supplies of fiber for the U.S. pulp and paper industry require reliable silvicultural systems for establishing and managing plantations from genetically improved material. Vegetative propagation and modern recombinant DNA techniques coupled with traditional genetic improvement programs may become part of such a system. Among the first applications of this new technology may be the transfer of foreign genes into genetically superior stock. After testing, the resulting transgenic individuals would then be incorporated into a traditional tree improvement/breeding program. While foreign traits under single-gene control have been successfully transferred into crop plants, progress with forest tree species has not been as rapid, with a notable exception being the transfer of a gene imparting glyphosate tolerance into a Populus hybrid (Filatti, et al., 1987). Clearly, more work is needed on the techniques for the genetic transformation of economically important tree species. An important step in this work is the transfer of marker genes into cells capable of regeneration/propagation in vitro.

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. 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 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 a foreign gene into this important southern hardwood forest species.

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Loblolly pine (Pinus taeda L.) is a softwood species of unquestionable importance for the production of fiber in the southeastern United States. The recent achievement of somatic embryogenesis in loblolly pine (Becwar et al., 189; Gupta and Durzan, 1987), as well as other conifers (Hakman et al., 1985), warrants the initiation of work on the genetic transformation of this species. Transient expression of a marker gene in electroporated loblolly pine protoplasts has been reported (Gupta et al., 1988), but problems related to the regeneration of plants from conifer protoplasts may hinder this approach. Successful infection of loblolly pine seedlings by Agrobacterium tumefaciens has been demonstrated by Sederoff, et al. (1986), suggesting that this biological vector can be used in the transfer of foreign DNA into pine cells. The use of intact cells in this procedure precludes problems related to the consequent regeneration of plants from protoplasts. Reported here are the results of initial attempts to transform cells in embryogenic loblolly pine suspension cultures.

MATERIALS AND METHODS

Transformation of Sweetgum

Small cuttings (8-10 inches long) 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.I%). 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/1 NAA and 1.0 mg/1 BA solidified with 0.8% agar (pH = 5.6). Following the procedure of Sutter and Barker (1986), shoot tips were transferred to fresh medium 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.

The Agrobacterium tumefaciens binary vector used (pGA515-47) was developed and described by An (1986). The oncogenic A, tumefaciens strain A281 carrying the binary vector, which contains neomycin phosphotransferase II (npt II) and chloramphenicol acetyltransferase (CAT) genes driven by nopaline-synthase (nos) promoters, was maintained as described (An, 1986). Shoots, 2-3 cm long, were excised from the cultures and inoculated on the stem. Inoculation was performed using an overnight culture of A. tumefaciens using a 22 gauge needle to make a small wound, into which 10 µl bacterial culture was applied. After 8 weeks a gall was observed on the stem of one of twelve shoots. Sixteen weeks after inoculation, the gall was excised and placed onto shoot proliferation medium (WPM medium with 0.05 mg/I NAA and 1.0 mg/I BA) containing carbenicillin (500 ug/mI) and cefotaxime (250 µg/ml) to kill any remaining Agrobacterium. A pale white callus proliferated from this gall was maintained in culture by monthly transfer to fresh medium (antibiotics not present).

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. Plant extract (20 µl) was incubated with 100 µl of reaction buffer, to which 0.1µCi [^{14}C]-chloramphenicol (54 mCi/mmol, Amersham Corp.) was added. After 30 min incubation at 37 °C, chloramphenicol and the resulting acetylated derivatives were separated on a silica gel TLC plate. The chloramphenicol and radioactive derivatives were then visualized by autoradiography.

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 R1 according to

manufacturer's instructions and electrophoresed in a 0.7% agarose gel. Undigested DNA from the transformed plant, as well as Eco R1 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 the npt II sequence (Church and Gilbert, 1984). Radioactive probes were prepared by nick translating the entire pGA515-47 plasmid or oligo-labeling a Bam H1 fragment (which contains only the npt II sequence) of pDO421. 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.

Transformation of Loblolly Pine

Embryogenic calli of loblolly pine were initiated from immature embryo explants on MSG medium as described by Becwar et al. (1988; 1989). Suspension cultures were derived by transfer of calli to liquid DCR medium (Gupta and Durzan, 1985) containing 2,4-D (3 mg/I) and BA (0.5 mg/I). These cultures were grown on a gyratory shaker under a 16-hour photoperiod at 24 °C and maintained by transfer to fresh medium every 14 days.

LBA4404, a disarmed Agrobacterium tumefaciens, carrying the binary vector pBII21 was obtained from Clontech Laboratories, Palo Alto, CA. This plasmid vector contains the E. cob derived GUS (beta-glucuronidase) gene joined to a CaMV 35S promoter and a NOS poly-A site (Jefferson et al., 1986). Infection of suspension cultured loblolly pine cells was carried out by adding 100 µl of an overnight culture of Agrobacterium to 25 ml of fresh medium (DCR) containing I,300 µl (packed cell volume) of plant cells. The 125 ml shake flasks were then incubated at 28 °C on a gyratory shaker. After a 24 hr cocultivation period, plant cells were collected from the flasks, washed with fresh medium (DCR), and placed into 100 mM phosphate buffer (pH = 7.0) containing 100 mM sucrose as an osmoticum and 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) (Jefferson, 1987). Although some color development was observed after 1 hr, cells generally had to be incubated in this substrate solution 4 to 6 hr before blue coloration of transformed cells became obvious.

RESULTS AND DISCUSSION

Sweetgum transformation

Approximately 8 weeks after inoculation, a small growth was noted on one of twelve treated shoots. This growth formed a callus and exhibited characteristics of a crown gall phenotype. After eliminating the Agrobacterium with cefotaxime, the callus was maintained on WPM (NAA and BA at 0.05 and I.0 mg/I, respectively) containing kanamycin at 100 ug/ml. WPM containing NAA and BA at these levels did not typically support normal callus growth of sweetgum. The callus thus exhibited two characteristics of transformed tissue; the ability to grow in the absence or at reduced levels of plant growth regulators and the ability to grow in the presence of kanamycin, a trait imparted by the npt II gene present in the T-DNA. Untransformed control sweetgum callus proved unable to grow in the presence of kanamycin.

Chloramphenicol acetyltransferase activity assays were carried out to demonstrate the presence and expression of the CAT gene in the transformed tissue. Acetylated derivatives of chloramphenicol were formed with extracts of transformed tissue but not untransformed control tissue (data not shown). Although present at measurable levels, the CAT activity in the transformed tissue was very low. Higher levels of CAT expression might have been obtained using the 35S cauliflower mosaic virus promoter, which has been reported to be 10-15 fold stronger than the NOS promoter (Morelli et al., 1985). Insertion of the CAT gene into an inactive region 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. Extracts of non-transformed control tissue were found to reduce the apparent activity of authentic CAT isolated from E coli (data not shown), suggesting the presence of such an inhibitor.

The most convincing evidence demonstrating transformation of the tissues was 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 R1 digest of the T-DNA of pGA515-47. 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 (undigested plant) DNA. No bands characteristic of those obtained when the probe hybridizes to undigested pGA515-47 plasmid DNA were observed. Thus, hybridization signals in the lanes containing DNA isolated from transformed tissue result from the presence of T-DNA in the sweetgum genomic DNA.

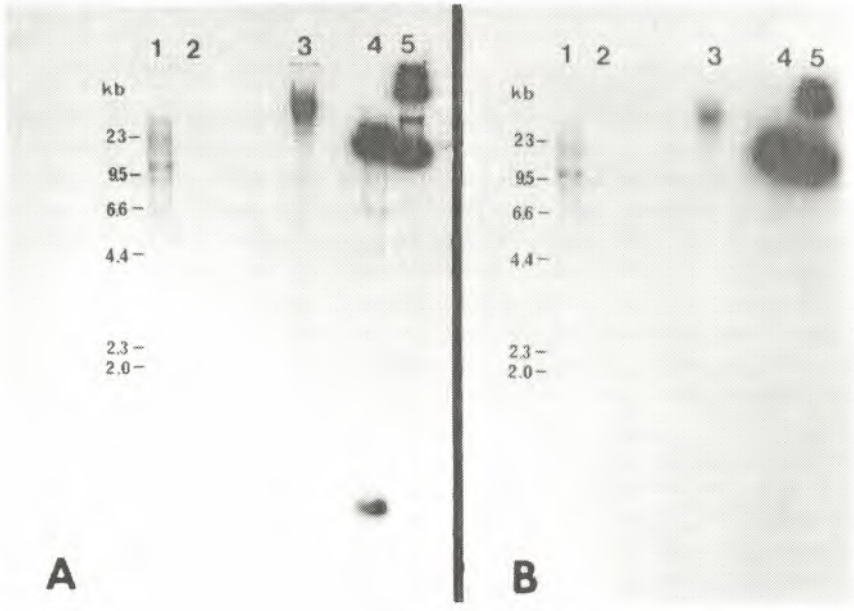

Figure I. Southern blot analyses of Eco R1 digested DNA isolated from transformed (lane I) and untransformed (lane 2) sweetgum calli. Lane 3 contained uncut, high molecular weight DNA isolated from transformed callus. Controls included uncut (lane 5) and Eco R1 digested (lane 4) pGA515-47 plasmid. Membranes were probed with nick translated p515-47 (A) and an oligo-labelled npt 1I gene fragment (B).

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 H 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 were 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 npt II gene is not only present, but is being expressed.

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 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 analyses, and expression of the transferred genes was suggested 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. That this sweetgum transformation scheme must be further optimized is affirmed by the low frequency of gall formation on inoculated plants. This work may lead, however, to studies of gene expression and transfer of economically important traits in this commercially important hardwood species. In this regard, it is noteworthy that leaf disc regeneration systems that have found extensive application in Agrobacterium-mediated transformations (Horsch, et al., 1985) have been developed for sweetgum (Brand and Lineberger, 1988).

Loblolly pine transformation

After incubation of washed, cocultivated loblolly pine cells in a solution containing the colorless, synthetic substrate X-Gluc, a significant proportion of the cells appeared blue upon examination by brightfield microscopy. While the distribution of the blue color within the cell compartments (i.e. vacuole or cytoplasm exclusively) was difficult to judge, the color was clearly located intracellularly. This was especially evident in those cells that had plasmolyzed, with the dye clearly being limited to the area inside the plasma membrane. Cocultivation periods of 24 and 48 hr were tested, with no apparent improvement in transformation success at 48 hr. Embryogenic conifer calli and suspension cultured cells typically contain two cell types; large, elongated, highly vacuolated "suspensor" cells and smaller (pro)embryonic cells exhibiting dense cytoplasm. In these transformation studies, both cell types exhibited blue coloration, evidence of transient expression of the GUS gene.

Removal of Agrobacterium from the cocultivated plant cells remains a problem. While the bacteria can easily be killed in cocultivated carrot suspensions by the addition of antibiotics to the culture medium (Scott and Draper, 1987; Feirer, 1988), the Agrobacterium persisted in the loblolly pine cultures (similar problems have been encountered with Norway spruce cultures in our laboratory). Consequently, bacterial contamination of the cultures was a problem, complicating attempts to obtain stably transformed plant cell lines. Therefore this report demonstrates only the transient expression of the foreign gene.

Transformation of loblolly pine protoplasts and cells in intact seedlings has previously been reported (Dandekar et al., 1987; Gupta et al., 1988; Sederoff et al., 1986). The work reported in this study differs, however, in the use of Agrobacterium as a vector to deliver foreign DNA to embryogenic, intact cells. Use of intact cells rather than protoplasts should facilitate easier regeneration of transformed plants. Especially notable is the use and apparent transformation of competent cells in embryogenic cultures. Although regeneration of intact plants from embryogenic loblolly pine cultures remains difficult, when the tissue culture techniques are optimized to successfully produce whole plants this transformation technique will be immediately useful. The cocultivation of embryogenic cells has been successfully used in the production of both transgenic carrot cells and intact plants (Scott and Draper, 1987; Feirer, 1988). This technique offers the advantages of high transformation efficiencies, 60 to 70 % (Scott and Draper, 1987) and the ability to use regenerable, easily manipulated cultured cells.

The E. coli derived GUS gene has served as a reliable model gene for use in plant transformation studies with a number of plant species, including coniferous tree species (Jefferson, 1987; Wilson et al., 1989; Zhang and Wu, 1988). Bekkaoui et al. (1988) have recently reported that this marker gene may be unsuitable for use in white spruce cells due to high levels of endogenous compounds which interfere with the fluorometric measurement of GUS activity. These authors, using the marker gene in protoplast electroporation studies, suggested that the interfering compound(s) might possibly be released upon the electroporation process and related lysis of protoplasts. Wilson et al. (1989), reported that background "GUS-like" activity in the fluorometric assay was not a major problem in PEG-mediated transformation of white spruce protoplasts, however. Our results were uncomplicated by high background levels of GUS activity (cells in control cultures remained colorless in presence of x-Gluc), and this may be related to the use of intact cells or the use of the histochemical rather than the fluorometric detection of enzyme activity.

CONCLUSION

While reports of transformation of agronomic crops are plentiful in the literature, work with forest trees has lagged. This report provides evidence for the transformation of two commercially important forest tree species. The use of intact cells, rather than protoplasts, should simplify the regeneration of transformed plants from the shoot cultures of sweetgum and embryogenic loblolly pine cultures. The results of this study also reinforce the earlier observations (Bekkaoui et al., 1988; Wilson et al., 1989) that foreign genes and promoters are functional in conifers, including loblolly pine.

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