Expression of Foreign Genes in Transgenic Yellow-Poplar Plants

H. Dayton Wilde*, Richard B. Meagher, and Scott A. Merkle

School of Forest Resources (H.D.W, S.A.M.) and Department of Genetics (R.B.M.), University of Georgia, Athens, Georgia 30602

ABSTRACT

Cells of yellow-poplar (Liriodendron tulipifera L.) were transformed by direct gene transfer and regenerated into plants by somatic embryogenesis. Plasmid DNA bearing marker genes encoding β-glucuronidase (GUS) and neomycin phosphotransferase (NPT II) were introduced by microprojectile bombardment into single cells and small cell clusters isolated from embryogenic suspension cultures. The number of full-length copies of the GUS gene in independently transformed callus lines ranged from approximately 3 to 30. An enzyme-linked immunosorbent assay for NPT II and a fluorometric assay for GUS showed that the expression of both enzymes varied by less than fourfold among callus lines. A histochemical assay for GUS activity revealed a heterogeneous pattern of staining with the substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid in some transformed cell cultures. However, callus clusters reacting positively (blue) or negatively (white) with 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid demonstrated both GUS activity and NPT II expression in quantitative assays. Somatic embryos induced from transformed cell cultures were found to be uniformly GUS positive by histochemical analysis. All transgenic plants sampled expressed the two marker genes in both root and shoot tissues. GUS activity was found to be higher in leaves than roots by fluorometric and histochemical assays. Conversely, roots expressed higher levels of NPT II than leaves.

Yellow-poplar (Liriodendron tulipifera L.) is a fast growing hardwood tree species native to the eastern United States that is used for wood and pulp products (22). Genetic improvement of yellow-poplar by traditional breeding strategies has been a slow process because, like all forest trees, it has a long generation time. Genetic engineering by recombinant DNA technology can accelerate the introduction of new traits into forest trees. Gene transfer systems would also be of use in the study of the molecular biology of developmental processes of woody plants, such as secondary xylem differentiation (14), lignification (30), and the juvenile-mature transition (9).

There has been limited success in the production of transgenic plants of woody perennials. Most frequently, plants have been regenerated by organogenesis from explants transformed by Agrobacterium tumefaciens-mediated gene transfer, as in the case of apple (Malus pumila [10]), grapevine (Vitis rupestris [25]), and hybrid poplars (Populus alba × grandidentata [5], Populus trichocarpa × deltoides and P. alba × tremula [4]). Tissues from some coniferous species have been stably transformed by A. tumefaciens (for example, see ref. 15), but regeneration of transformants has not been reported. Progress has recently been made in the regeneration of tree species through tissue culture, particularly in the area of somatic embryogenesis (reviewed in ref. 29). In one case, walnut (Juglans regia) somatic embryos, transformed by A. tumefaciens, were regenerated into plants after repetitive somatic embryogenesis (18).

Certain facets of tree biology and utilization play a role in transformation strategies for trees such as yellow poplar. For forest species, gene transfer to cells capable of somatic embryogenesis would be advantageous for reasons of economy and scale of production (29). In addition, transformation systems that minimize the occurrence of chimeras are more critical for trees than herbaceous plants, for which chimeras can be eliminated by sexual transmission of the transformed genotype (for example, see ref. 16). Protoplast transformation can, in principle, yield clonal, transformed cells, but regeneration of plants from protoplasts has been reported in only a few tree species (reviewed in ref. 21).

Direct gene transfer into intact cells by microprojectile bombardment (28) can circumvent limitations imposed by protoplast regeneration systems and the host range of Agrobacterium. McCown et al. (17) transformed cultured nodules of the poplar hybrid P. alba × grandidentata using microprojectile bombardment and regenerated transgenic plants by organogenesis. The introduction of plasmid DNA into cultured embryogenic cells of yellow-poplar by microprojectile bombardment demonstrated that a plasmid-borne gene encoding GUS2 could be expressed transiently in yellow-poplar cells (31). In this paper, we describe the stable transformation of yellow-poplar cells by microprojectile bombardment and the regeneration of transgenic plants by somatic embryogenesis. To determine whether chimeric tissue was produced by these methods, transformed cell cultures, somatic embryos, and plants were analyzed for the expression of GUS and NPT II.

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1 This research was supported by U.S. Department of Agriculture grant 87-FSTY-9-0249 to S.A.M and R.B.M.

2 Abbreviations: GUS, β-glucuronidase; NPT II, neomycin phosphotransferase; X-gluc, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid; CaMV 35S, cauliflower mosaic virus 35S gene; nos, nopaline synthase.
MATERIALS AND METHODS

Plant Material

An embryogenic yellow-poplar (Liriodendron tulipifera L.) line, 14 × 108–3, was chosen that showed high frequencies of somatic embryogenesis and conversion into plantlets. Tissue culture and plant regeneration procedures followed the protocol described in ref. 23, with the exceptions noted below. Suspension cultures were used for transformation 2 weeks after subculture into fresh yellow-poplar induction medium (23). Before microprojectile bombardment, the cell suspension was passed successively through stainless steel sieves of 860 and 140 µm pore size, and the concentration of the cells in the filtrate was determined with a hemocytometer. Approximately 10,000 cell clusters were collected onto a 4.25-cm filter paper disc (Eaton-Dikeman) using a Buchner funnel with mild vacuum. The filters were transferred into 6 cm Petri plates containing induction medium solidified with 0.8% Phytagar (GIBCO).

Microprojectile Bombardment

Plasmid DNA (pBI121.1 [11]) was precipitated onto 1.1 µm tungsten particles using the CaCl2/spermidine method of Klein et al. (12), except that the final centrifugation was omitted. Two microliters of the tungsten suspension were loaded onto a macrocarrier and accelerated toward the target with a Biolistic particle gun (DuPont PDS-1000). Bombardment parameters were similar to those described by Wilde et al. (31). Each plate was bombarded twice and then incubated in a growth chamber at 25°C with a 16 h daylength.

Selection and Regeneration

After 2 d of incubation, filters were transferred onto solidified induction medium containing 100 µg/mL kanamycin and returned to the growth chamber. After 5 to 6 weeks, 40 kanamycin-resistant microcalli with a diameter of approximately 1 mm were transferred individually to fresh antibiotic-containing plates. Suspension cultures were initiated from calli of seven representative lines in which histochemically detectable GUS expression ranged from 0 to 100% of the cell culture. Cell suspensions were grown in liquid induction medium containing kanamycin at a level (50 µg/mL) sufficient to inhibit the growth of nontransformed yellow-poplar suspensions. Somatic embryogenesis was induced in cell clusters from suspension cultures by the procedure of Merkle et al. (23) except that the somatic embryos developed on media containing 50 µg/ml kanamycin. Mature somatic embryos were allowed to convert into plantlets GA7 vessels (Magenta Corp.) and then transferred to soil mix and placed in a humidifying chamber. From the five transformed lines (2H2, 2H4, 2D5, 2H7, 2H3) in which somatic embryogenesis was initiated, a total of 60 transgenic plants were regenerated for further analysis. The plantlets were gradually acclimated to ambient conditions and moved to a greenhouse.

DNA Isolation and Analysis

DNA was isolated from approximately 100 mg of callus tissue of seven independently transformed yellow-poplar lines by the cetyltrimethylammonium bromide nucleic acid extraction procedure of Rogers and Bendich (26). Isolated DNA was digested with EcoRI or a combination of EcoRI and HindIII. Restriction enzyme-digested DNA (10 µg/well) was loaded and electrophoresed through 0.8% agarose. Southern blot hybridizations were performed as described by Sambrook et al. (27) using a gel-purified, randomly primed probe for the GUS gene. The probe, an EcoRI/HindIII fragment from pBI121.1 encompassed the entire GUS gene.

NPT II ELISA

Callus tissue (50–100 mg) from eight transformed cell lines was ground in an Eppendorf tube with an equal volume of buffer containing 100 mm Tris (pH 7.5), 0.1% Tween, and 1.0% PVP (mol wt 40,000). Insoluble material was pelleted in a microcentrifuge, the supernatant removed, and the concentration of soluble protein determined using a protein assay kit (Bio-Rad Laboratories). Rabbit polyclonal antibody to NPT II and other components of the immunoassay were obtained from 5 Prime-3 Prime, Inc. (West Chester, PA), and the protocol of the manufacturer was followed, with minor modifications. Briefly, microtiter wells were coated with the NPT II antibody and then blocked with 1% BSA to prevent nonspecific adsorption. Callus cell extracts containing 200, 50, and 12.5 µg protein/mL were added to the wells. The dilution series was repeated twice for each callus extract. A second biotinylated antibody to NPT II and streptavidin-conjugated alkaline phosphatase were added sequentially to each well. Color development resulting from incubation of the bound alkaline phosphatase with p-nitrophenyl phosphate was quantified spectrophotometrically. The NPT II concentration of the callus extracts was determined from a standard curve produced from ELISA results of a serial dilution of purified NPT II. NPT II ELISAs of extracts of roots and leaves of transgenic plants were performed in a similar manner, except that only one protein concentration (100 µg/mL) was used.

GUS Assays

A fluorometric assay of GUS activity was performed using the substrate 4-methyl-umbelliferyl-β-D-glucuronide (11). Lysis buffer (50 µL) containing 20 µg of total protein was mixed with an equal volume of 2 mm 4-methyl-umbelliferyl-β-D-glucuronide. The reaction mixture was split, and one-half of the reaction was stopped immediately with 150 µL of 200 mm Na2CO3. The remainder was incubated at 37°C for 1 h and stopped. Each assay was performed in triplicate. The stopped reactions were diluted 1:100 in 200 mm Na2CO3 and the level of fluorescence was measured with a TK100 Mini-Fluorometer (Hoefer). GUS activity in the extracts was determined by comparison with the fluorescence of 4-methylumbellifereone standards. Background fluorescence, negligible in the initial time point of most samples except root extracts, was subtracted.
Histochemical detection of GUS expression with the substrate X-gluc generally followed the protocol of Klein et al. (13). Color development in transformed calli and somatic embryos was usually complete within 2 h. Heterogeneously staining callus cells were incubated overnight at 25°C in the presence of X-gluc, and blue and white cell clusters were separated under a dissecting scope. Cell clusters were then homogenized in ELISA extraction buffer, and the soluble fraction was assayed by the NPT II ELISA and the GUS fluorometric assay. Hand-cut sections of transgenic plants were incubated overnight at 25°C in the presence of X-gluc. Photographs were taken through a Wild M5 stereomicroscope using Kodak Ektachrome film (ASA 160).

RESULTS

Transformation and Regeneration

A transformation strategy for yellow-poplar was designed using the plasmid pBI121.1, which carries the marker genes encoding GUS and NPT II driven by the CaMV 35S promoter and the nos promoter, respectively. The plasmid was introduced by microprojectile bombardment into cells from an embryogenic suspension culture. Suspension cultures were sieved through stainless steel mesh before bombardment to yield a fraction of single cells and small cell clusters (<140 μm in diameter (Fig. 1A). Approximately 10,000 cell clusters were dispersed evenly on a filter paper disc and bombarded with plasmid-coated tungsten particles. Kanamycin-resistant microcalli appeared 5 to 6 weeks after transferring filters onto solid selection medium. Putatively transformed cells formed light-colored microcalli growing rapidly among dark, inactive nontransformed cells. There was an average of four transformants per filter which, assuming 10 cells per cluster, resulted in a transformation efficiency of 0.004%.

Somatic embryogenesis in transformed suspension lines was initiated with equal efficiency on phytohormone-free medium in the presence or absence of kanamycin (data not shown). No somatic embryos developed from a nontransformed yellow-poplar culture under antibiotic selection. Somatic embryos from transformed lines converted into plantlets that grew vigorously in soil (Fig. 1B). Transformed cell suspensions could still produce GUS-positive somatic embryos after 1 year of culture under antibiotic selection.

Southern Analysis of Kanamycin-Resistant Calli

Kanamycin-resistant lines exhibited a heterogeneous pattern of GUS activity in the histochemical assay using X-gluc. For example, the percentage of cells reacting positively for GUS was estimated to be >90% in lines 1A3 and 3B2, 80 to 90% in lines 2H4 and 2H7, 60 to 70% in line 2H2, 40 to 50% in line 1A1, and 0% in line 2H3. Genomic DNA was isolated from calli of these seven antibiotic-resistant lines and analyzed for the presence of integrated copies of the GUS gene.

Hybridization of the 3.0-kilobase HindIII-EcoRI fragment of pBI121.1 containing the entire GUS gene to genomic DNA demonstrated the presence of full-length copies of the GUS expression unit in six of these lines (1A3, 2H2, 2H4, 2H7, 3B2, 1A1; Fig. 2A). The estimated copy number of the GUS gene ranged from three (2H7) to 30 (1A3). Hybridization of the GUS probe to EcoRI-digested genomic DNA showed that the plasmid pBI121.1 had integrated into the yellow-poplar genome and, in many cases, formed head-to-tail concatemers. No hybridization was detected in genomic DNA from a nontransformed line (lane a, Fig. 2). In genomic DNA from line 2H3, the GUS probe did not hybridize to a DNA fragment the size of a full-length GUS gene, indicating a rearrangement of the transforming plasmid DNA (lane h, Fig. 2A). However, line 2H3 expressed NPT II (Table I) and Southern analysis detected an intact copy of the NPT II gene (data not shown). There was no apparent correlation between the amount of histochemically detectable GUS activity and the number of copies of the GUS gene (cf. lines 2H7 and 1A1).

Gene Expression in Transformed Callus Tissue

The level of expression of the genes encoding NPT II and GUS was quantified in calli from eight transformed lines, and the results are presented in Table I. An ELISA for NPT II detected the protein at levels at least 10-fold over background in all transformed lines. A linear response was obtained in extracts from transformed calli containing between 12.5 and 200 μg/ml of total protein (data not shown). The amount of NPT II in transformed lines ranged from 399 to 1455 pg NPT II/mg total protein.

GUS expression, as determined by a fluorometric assay, was evident in all transformed calli with the exception of 2H3, which lacks an intact copy of the GUS gene (Fig. 2A). The level of GUS activity between different transformed lines containing a full-length GUS gene varied by an approximate factor of 4 (Table I). There appeared to be no correlation between the level of GUS activity and NPT II expression in the lines sampled.

The histochemical assay for GUS often demonstrated a heterogeneous pattern of enzyme activity in cells maintained under kanamycin selection in liquid (Fig. 1C) or on solid medium. Nontransformed yellow-poplar cells did not stain positively (blue) with the X-gluc substrate (not shown). To determine whether kanamycin-resistant cells that failed to react with X-gluc were transformed, blue and white cell clusters were separated and assayed quantitatively for GUS and NPT II expression. Cells that had stained positively for GUS in the histochemical assay also reacted positively for GUS in the fluorometric assay and for NPT II by ELISA (Table I). White cell clusters, however, demonstrated levels of GUS and NPT II expression slightly higher than those found in blue cell clusters, indicating that these cells were also transformed.

Gene Expression in Regenerated Plants

Somatic embryos induced from transformed lines reacted positively for GUS in the histochemical assay (Fig. 1D), with the exception of those from line 2H3. Blue-staining embryos were often observed developing from unstained proembryogenic masses. Expression of GUS was evident throughout most of the somatic embryo; the radicle end of the somatic embryo, where attached to the proembryogenic masses, remained unstained. This pattern of GUS expression was con-
Figure 1. Regeneration of transgenic yellow-poplar plants from embryogenic cell cultures. A, Fraction of suspension culture passing through 140 μm mesh (x225). B, Five-month-old yellow-poplar plant transformed with pBI121.1 DNA (x0.25). C–E, Histochemical localization of GUS activity: C, suspension culture (x30); D, somatic embryos (x40); E, shoot tip from regenerated plant (x30). p, Proembryogenic mass; s, stipule; a, shoot apex.
sistent among somatic embryos from independently transformed lines.

Five-month-old transgenic yellow-poplar plantlets (Fig. 1B) were assayed histochemically for GUS. Strong enzyme activity was detected in leaves, particularly in the blade (Fig. 1E). Shoot apices, and the stipules that surrounded them, demonstrated GUS activity. In contrast, roots from transformed plants stained weakly for GUS (not shown).

The levels of GUS and NPT II expression were assayed quantitatively in roots and leaves from plants regenerated from three transformed lines (Fig. 3). The expression of genes encoding both GUS and NPT II was found in roots and leaves of all transgenic plants assayed. The amount of GUS activity varied between plants of different lines but was greater in leaves than in roots by a factor ranging from 4 to 16. In contrast, the level of the NPT II enzyme was always greater in roots than in leaves. The ratio of NPT II levels of roots to leaves was between 1.2 (line 2H7) and 3.3 (line 2H2).

DISCUSSION

Embryogenic suspension cultures of yellow-poplar were transformed by microprojectile bombardment and regenerated into plants that expressed two genes from the transforming DNA in both root and shoot tissues. Previous efforts to introduce pBI21.1 DNA biologically into unfractionated yellow-poplar suspension cultures resulted in transient expression of GUS, but not the recovery of kanamycin-resistant stable transformants (31). Sieving the cultures through 140-μm mesh enriched for small clusters of meristematic cells

| Table I. Gene Expression in Independently Transformed Lines of Yellow-Poplar Callus |
|---------------------------------|---------------------------------|
|                                | NPT II Level             | GUS Activity         |
| Callus line                     | pg/mg protein | 4-methylumbelliferone/min/mg protein |
| 14 × 108-3 (control)            | 41.4 ± 11.8       | 8.7 ± 6.2           |
| 2H7                            | 399.3 ± 32.3       | 1542.3 ± 379.2      |
| 1A3                            | 569.6 ± 21.8       | 572.0 ± 13.9        |
| 1A1                            | 573.2 ± 0.3        | 1341.7 ± 29.0       |
| 2H3                            | 690.1 ± 14.3       | 9.5 ± 2.5           |
| 2D5                            | 815.3 ± 15.1       | 536.0 ± 19.8        |
| 2H2                            | 817.9 ± 23.8       | 1706.0 ± 91.5       |
| 2H4                            | 957.4 ± 10.2       | 439.0 ± 53.2        |
| 3B2                            | 1454.8 ± 44.1      | 1297.7 ± 157.3      |
| X-Gluc reaction                |                  |                    |
| Positive (blue)                | 173.4 ± 51.7       | 2696.4 ± 349.1      |
| Negative (white)               | 563.0 ± 266.4      | 3229.2 ± 404.4      |

* Average of two ELISA determinations ± se.  
  b Average of three fluorometric assays ± se.  
  c Suspension cell clusters (line 2H2) reacting positively (blue) and negatively (white) with X-gluc were separated and assayed for NPT II and GUS expression.

Figure 2. Southern blot analysis of the GUS gene in calli of transformed yellow-poplar lines. A, Genomic DNA (10 μg/lane) digested with HindIII and EcoRI. B, Genomic DNA (10 μg/lane) digested with EcoRI. Lane a, Nontransformed 14×108–3; lane b, 1A3; lane c, 2H2; lane d, 2H4; lane e, 2H7; lane f, 3B2; lane g, 1A1; lane h, 2H3; lane s, HindIII-digested λ-DNA standards (mol wt in kilobases). Lanes 1 and 10, genome equivalent of 1 and 10 copies of the GUS gene based on a haploid genome size of 0.8 pg.
identical with those used to synchronize somatic embryogenesis of yellow-poplar (23). The use of this fraction as a target for direct DNA transformation may have increased the transformation efficiency by enriching for cells competent to integrate foreign DNA (24). Alternatively, antibiotic selection may have been made more efficient by the small size of the target tissue, which allowed direct contact with the kanamycin-containing medium. Selection pressure was maintained by subculturing transformed calli into liquid medium with a level of kanamycin that suppressed the growth of nontransformed cells, but did not interfere later with the induction or development of transformed somatic embryos.

Despite stringent antibiotic selection, in many cases, putatively transformed yellow-poplar cultures did not demonstrate uniform GUS expression when assayed histochemically with the substrate X-gluc. A heterogeneous pattern of GUS staining was also observed in maize and rice callus stably transformed by plasmids carrying the GUS gene and selectable markers (7, 8, 13, 20). The lack of GUS expression in this assay has been attributed to the absence of the gene in some cells, differential expression of the gene, or to variable penetration of the substrate (7). Harris et al. (8) separated blue and white sectors of X-gluc-treated maize callus and demonstrated by fluorometric assays that white and blue sectors had similar levels of GUS activity. Likewise, in transformed yellow-poplar suspensions, cell clusters that did not react positively with X-gluc were shown to express both GUS and NPT II. Factors other than gene expression must, in some cases, be responsible for negative results in the histochemical assay for GUS in cultured cells (e.g. antioxidants). The lack of histochemically detectable expression of GUS in some cells would complicate the analysis of a transient expression assay for this enzyme.

Transgenic yellow-poplar somatic embryos exhibited GUS expression in histochemical assays, indicating that substrate penetration was not a problem in differentiated tissues. As the somatic embryos matured into plantlets, there was preferential expression of GUS in leaves over roots. Higher levels of GUS activity were found in leaves than in roots of rice (2) but not tobacco (3, 11) transformed with CaMV 35S-GUS constructions. The CaMV 35S promoter possesses domains that confer tissue-specific and developmentally regulated expression (3) that may be recognized differently in plants evolutionarily divergent from tobacco, such as rice and yellow-poplar, a member of the Magnoliaceae. On the other hand, the nos promoter directs similar patterns of gene expression in transgenic yellow-poplar and tobacco. An et al. (1) measured higher levels of chloramphenicol acetyltransferase activity in roots than in leaves of tobacco plants transformed with a nos-chloramphenicol acetyltransferase construction. Similarly, in yellow-poplar the nos-NPT II gene was preferentially expressed in roots.

In addition to yellow-poplar, microprojectile bombardment of embryogenic suspension cultures has proven successful for the production of transgenic plants of cotton (6), maize (7), and soybean (19). This method is a promising approach to the transformation of tree species, and woody plants in general, because it obviates the development of a protoplast regeneration system and it reduces the likelihood of regenerating chimeras by allowing selection pressure to be applied stringently in liquid culture.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Hazel Wetzstein and Dr. Wayne Parrott for critical reading of the manuscript.

LITERATURE CITED

β-glucuronidase as a versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907