J. For. Res. 1: 13-16 (1996)

Agrobacterium-Mediated Transformation of Lombardy Poplar (*Populus nigra* L. var. *italica* Koehne) Using Stem Segments

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Genetically transformed lombardy poplar (*Populus nigra* L. var. *italica* Koehne) plants were regenerated by co-cultivation of stem segments with *Agrobacterium tumefaciens* strain LBA4404 that harbored a binary vector (pBI121) which included genes for β-glucuronidase (GUS) and neomycin phosphotransferase. Successful transformation was confirmed by the ability of stem segments to produce calli in the presence of kanamycin, histochemical and fluorometric assays of GUS activity in plant tissues, and Southern blot analysis.

Key words: *Agrobacterium tumefaciens*, lombardy poplar, *Populus nigra* L. var. *italica* Koehne, shoot regeneration, transformation

Genetic engineering has the potential to allow the selective improvement of a single trait in forest trees without the loss of any of the desired traits of the parental line. Using such techniques, we can overcome the difficulties associated with the breeding of long-lived perennials. Poplar has been successfully exploited as a model plant in attempts to introduce foreign genes into woody plants as a consequence of its rapid growth, the small size of its genome and ease of tissue culture. Transformed poplar plants have been regenerated both by *Agrobacterium tumefaciens*-mediated transformation (Fillatti *et al.*, 1987; De Block, 1990; Klopfenstein *et al.*, 1991; Confalonieri *et al.*, 1994) and by electric discharge particle delivery (McCown *et al.*, 1991).

The lombardy poplar (*Populus nigra* L. var. *italica* Koehne) is one of the most common species in *Populus* and it has been planted worldwide for landscaping and windbreaks. However, no studies of *A. tumefaciens*-mediated transformation of lombardy poplar have been documented. In this study, we established a simple and reliable procedure for the regeneration of transgenic poplar plants. Stem segments were the most effective recipients for *A. tumefaciens*-mediated transformation of lombardy poplar.

Materials and Methods

1 Plant material

Shoot cultures derived from peeled twigs of mature lombardy poplar (Mohri and Miura 1992; Azuma *et al.*, 1993) were maintained on a medium that contained Murashige and Skoog's basal salts (Murashige and Skoog, 1962), Gamborg's B_5 vitamins (Gamborg *et al.*, 1968) and 3% (w/v) sucrose (MSB5S medium) supplemented with 0.8% (w/v) agar and 0.5 mg/*l* 3-indolebutyric acid (IBA). Shoot cultures were incubated at 25°C under cool white fluorescent light (80 $\mu E \cdot m^{-2} s^{-1}$, 16 h photoperiod) and subcultured every two months.

2 Bacterial strain

The disarmed *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) harboring a binary vector (pBI121, Clontech Laboratories, Inc., Palo Alto, CA) with genes for β-glucuronidase (GUS) and neomycin phosphotransferase was used in the transformation experiments. *A. tumefaciens* was grown overnight at 28°C in liquid Luria-Bertani medium (Sambrook *et al.*, 1989) in the presence of 25 mg/*l* kanamycin and 300 mg/*l* streptomycin. The overnight culture was diluted with liquid MSB5S medium to 5×10^8 cells/ml for transformation of poplar tissue.

3 Transformation

Leaf, stem, petiole and root segments from poplar shoot cultures were incubated for 30 min in the diluted culture of *A. tumefaciens* (Horsch *et al.*, 1985). Tissues were then blotted with sterile filter paper and incubated for two days on MSB5S medium (pH 5.8) supplemented with 0.3% (w/v) Gelrite (Scott Laboratories, Inc., Carson, CA) and 200 mM acetosyringone (Aldrich Chem. Co., Milw., WI). Each segment was washed three times with liquid MSB5S medium and then once with liquid MSB5S medium plus 500 mg/*l* cefotaxime (Sigma, St. Louis, MO). The tissues were blotted with sterile filter paper and planted on selective callus-induction medium [MSB5S medium containing 150 mg/*l* kanamycin, 500 mg/*l* cefotaxime, 500 mg/*l* carbenicillin, 0.5 mg/*l* 2,4-dichlorophenoxyacetic acid and 0.25% (w/v) Gelrite]. After calli have been allowed to develop for four weeks, they were excised from tissue segments and transferred to selective shoot-regeneration medium [MSB5S medium supplemented with 150 mg/*l* kanamycin, 500 mg/*l* carbenicillin, 2 mg/*l* trans-zeatin, 0.2 mg/*l* 6-benzyladenine and 0.3% (w/v) Gelrite]. Rooting of shoots was achieved in selective rooting medium [MSB5S medium that contained 150 mg/*l* kanamycin, 500 mg/*l* carbenicillin, 0.5 mg/*l* IBA, 0.02 mg/*l* α-naphthaleneacetic acid and 0.8% (w/v) agar].

4 Histochemical and fluorometric assays of GUS activity

Histochemical and fluorometric assays of GUS activity in transformed poplar were performed as described by Jefferson *et al.* (1987). For the histochemical assay, leaves of

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transformed poplar were incubated overnight at 37°C in a solution of 0.1 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc) and 50 mM sodium phosphate buffer (pH 7.0). The distribution of GUS activity was examined after the extraction of chlorophyll with ethanol. For the fluorometric assay, leaf discs of transformed poplar plants were homogenized in a microtube with a motor-driven, handmade glass rod in the presence of a small quantity of sea sand (Yamamoto *et al.*, 1994). After centrifugation, aliquots of the supernatant were used for the assay with 4 mM 4-methyl umbeliferyl-β-D-glucuronide (MUG) as the substrate. The concentration of protein was determined by the method of Bradford (1976) with γ-globulin as the standard.

5 Isolation and analysis of DNA

Genomic DNA was extracted from transformed poplar plants as described by Murray and Thompson (1980). DNA was digested with *Eco* RI and *Bam* HI and subjected to electrophoresis in a 0.8% (w/v) agarose gel. DNA fragments were transferred to a Biodyne B nylon membrane (Pall, East Hills, NY) by the alkaline transfer method (Reed and Mann, 1985). The membrane was prehybridized at 42°C overnight in 50 mM Tris-HCl (pH 7.5), 6x SSC, 50% (v/v) formamide, 1% (w/v) SDS, 10% (w/v) dextran sulfate and 100 mg/ml denatured salmon testis DNA. The labeled DNA probe was added directly to the membrane in the prehybridization mixture. After hybridization at 42°C for 16 h, the membrane was washed twice for 15 min with 2x SSC and 0.1% (w/v) SDS at room temperature and then three times at 60°C for 15 min each with 0.1x SSC and 0.1% (w/v) SDS. Then it was exposed to x-ray film (Kodak XOMAT-AR5) at −80°C for 24 h. The *Eco* RI-*Bam* HI fragment (the GUS gene fused to the *nos* terminator) of pBI121 was used as the DNA probe after labeling with [α-^{32}P]-dCTP (110 TBq/mmol, Amersham, Buckinghamshire, England) in the Multiprime DNA labeling system from Amersham.

Results and Discussion

1 A suitable tissue for transformation of lombardy poplar

Various tissues of lombardy poplar were examined for their suitability for *A. tumefaciens*-mediated transformation (Table 1). Stems were the best tissue for such transformation. Leaf, petiole and root segments produced few or no calli on the selection medium. Kanamycin-resistant calli derived from stem segments were obtained on the selection medium within two weeks after transformation. The presence of acetosyringone and the pH of the transformation medium had no effects on the efficiency of the tranformation of lombardy poplar (data not shown). By contrast, the efficiency of *A. tumefaciens*-mediated transformation of *Paulownia fortunei* has been shown to be increased remarkably by the presence of acetosyringone and to depend on the pH (4.5 to 5.0) of the transformation medium (Mohri, T., unpublished results). These results are consistent with previous findings by Godwin *et al.* (1991), who reported that the effects of acetosyringone and pH on *A. tumefaciens*-mediated transformation of herbaceous plants vary among plant species.

Table 1 Selection of suitable tissue for effective transformation of lombardy poplar.

Tissue	Number of tissue segments	Number of kanamycin-resistant calli	
Leaf	700	0	(0)[a]
Stem	655	1048	(1.6)
Petiole	100	2	(0.02)
Root	40	0	(0)

[a] Values in parentheses indicate average numbers of calli induced per tissue segment.

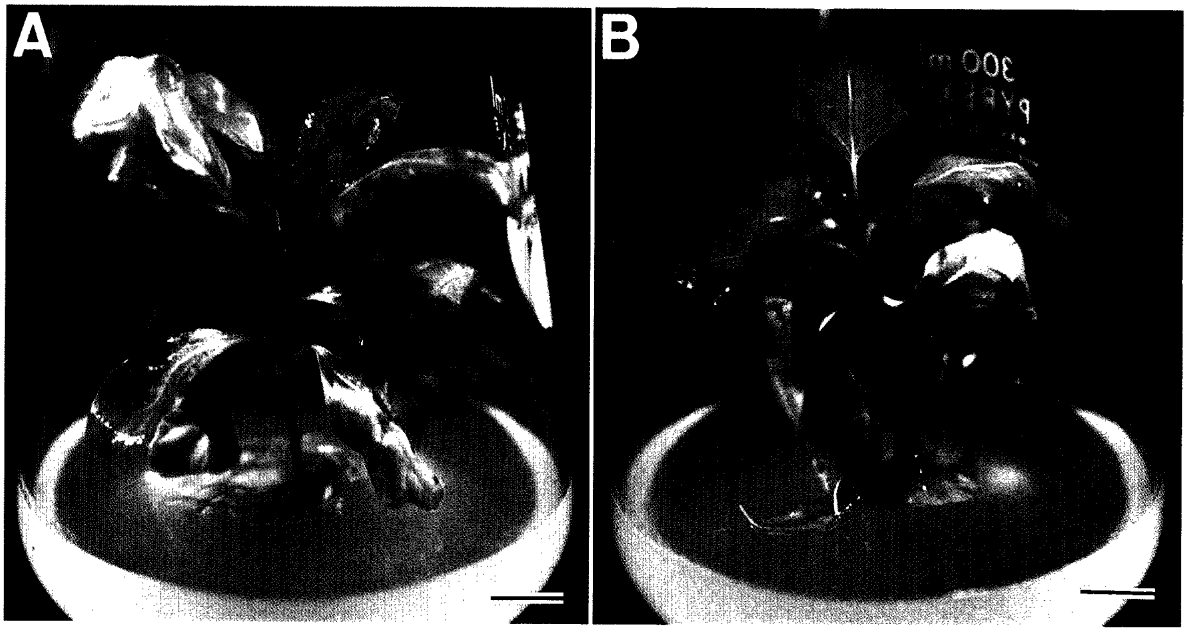

Fig. 1 Regeneration of plantlets. (A), the control poplar used in this study; (B), a transformed poplar plantlet. Bar=1 cm.

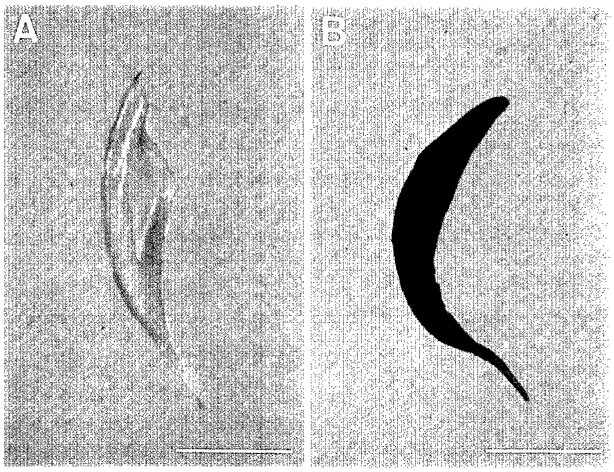

Fig. 2 Histochemical assay of GUS activity in transformed lombardy poplar. (A), a control leaf after staining with x-gluc; (B), a transformed leaf after staining with x-gluc. Bar=1 cm.

Table 2 Expression of GUS activity in transformed lombardy poplar plants.

	GUS activity[a] (nmol 4-MU[b] mg protein^{-1} min^{-1})
Control	0.04±0.02
Transformant 1	8.08±0.04
Transformant 2	2.36±0.45
Transformant 3	1.13±0.08

[a] Values are means±S.E. of four measurements. [b] 4-MU, 4-methyl umbelliferone.

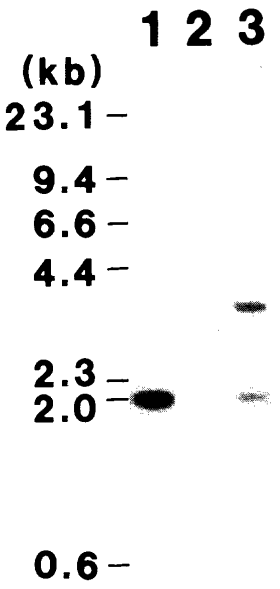

Fig. 3 Detection of an integrated GUS gene(s) in the genome of transformed lombardy poplar. A 2-kb DNA fragment of pBI121, which was used as the DNA probe (3 pg; Lane 1), and DNA from a control poplar (2 μg; Lane 2) and from a transformed poplar (2 μg; Lane 3), after digestion with *Eco* RI and *Bam* HI, were subjected to electrophoresis in a 0.8% (w/v) agarose gel and transferred to a nylon membrane. The GUS gene fused to the *nos* terminator from pBI121 was used as the DNA probe in Southern blot analysis.

2 Regeneration of transgenic lombardy poplar

We have established a system for regeneration of plantlets from leaf protoplasts of lombardy poplar (Mohri and Miura, 1992). Using this system, we were able to regenerate the transgenic poplar from the kanamycin-resistant calli (Fig. 1B). The shoots and roots were induced in individual selection media within four and two weeks, respectively. The frequency of regeneration of shoots and roots were approximately 100 and 60%, respectively, in the presence of kanamycin. The morphological features of the transformed poplar plants did not differ from those of nontransformants. The leaves of the transformants were strongly stained after histochemical staining for GUS activity (Fig. 2B), suggesting that an integrated GUS gene was expressed at high levels under control of the cauliflower mosaic virus 35S promoter. Our success in transformation was confirmed by fluorometric GUS assays and Southern blot analysis.

3 Detection of the GUS gene in the genome of lombardy poplar

The expression of the GUS gene in transformants was analyzed by a fluorometric assay for GUS activity (Table 2). Three transformants derived from different callus lines were randomly selected. The level of GUS activity in transformants was 30- to 200-fold higher than that in controls. The control leaves had very low background activity. These transformants retained the high GUS activity after the subcultures were repeated five times (data not shown). Furthermore, Southern blot analysis showed directly that the GUS gene has been introduced into the poplar genome by *A. tumefaciens*-mediated transformation (Fig. 3). Since the size of the genome of lombardy poplar is unknown, we could not determine the exact number of copies of the inserted GUS gene. A stoichiometry of one copy per transformant was estimated using the 2.1-kb DNA fragment of the GUS-*nos* terminator as the standard, and assuming a haploid genome size of 0.7 pg. No variation in the copy number of the GUS gene was observed among three transformants (data not shown). The variations in GUS activity of the transformants were probably caused by the presumably random integration of T-DNA into the poplar genome and probably reflected the influence of the surrounding genome on the expression of the GUS gene.

To our knowledge, this is the first report of *A. tumefaciens*-mediated transformation of lombardy poplar. We found that stem segments were the most suitable tissue for transformation. This result seems to be somewhat inconsistent with previous reports of the use of leaf discs for *A. tumefaciens*-mediated transformation of the other poplars (Fillatti *et al.*, 1987; De Block 1990; Klopfenstein *et al.*, 1991; Confalonieri *et al.*, 1994). In fact, leaves of lombardy poplar produced no calli in the presence of kanamycin (Table 1). The best tissue for transformation obviously varies among species of woody plants. The present efficient and reproducible transformation system will allow the selective improvement of single traits in the poplar by introduction of economically relevant genes that regulate morphological traits and resistance to insects and disease.

The authors express their gratitude to Dr. Yuzuru Mukai and Mr. Norihiro Futamura for their encouragement and for helpful discussions during the course of this work.

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(Accepted December 6, 1995)