

***Agrobacterium* – mediated Transformation of Paulownia (*Paulownia fortunei*)**

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Abstract

Genetically transformed calli of paulownia (*Paulownia fortunei*) were generated at high frequency after co-cultivation of petiole segments with *Agrobacterium tumefaciens* strain LBA4404 that harbored a binary vector (pBI121) which included genes for GUS and NPT II. The effects of acetosyringone, carbon sources and pH on the transformation of paulownia were examined. The presence of acetosyringone in the co-cultivation medium enhanced the induction efficiency of kanamycin-resistant calli. Sucrose was most suitable carbon source in the medium. The pH of the medium had no significant effect on the efficiency at pH 5 to 8. Successful transformation was confirmed by histochemical analysis of GUS activity in kanamycin-resistant calli, and by the detection of NPT II gene in the genome. Kanamycin-resistant calli induced from petiole segments had the ability of regeneration, however, the frequency of regeneration of shoot was very low.

Key words: *Agrobacterium tumefaciens*, paulownia, *Paulownia fortunei*, transformation, woody plant.

Abbreviations

BA, 6-benzyladenine; GUS, β -glucuronidase; IBA, 3-indolebutyric acid; NAA, α -naphthaleneacetic acid; NPT II, neomycin phosphotransferase II; PCR, polymerase chain reaction; x-gluc, 5-bromo-4-chloro-3-indolyl glucuronide; zeatin, trans-zeatin.

Genetic engineering has the potential to allow the selective improvement of individual traits 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, which need a long time to produce progeny. However, many difficulties have been encountered in attempts to regenerate transgenic woody plants, and appropriate regeneration systems have not yet been established in many cases. The production of transgenic broad-leaved trees, excluding fruit trees, has been limited to only a few genera, which include *Populus* (Fillatti *et al.*, 1987; De Block, 1990; Mohri *et al.*, 1996), *Liquidambar* (Sullivan and Lagrimini, 1993), *Robinia* (Han *et al.*, 1993; Igasaki *et al.*, 2000), *Betula* (Mohri *et al.*, 1997), *Eucalyptus* (Mullins *et*

al., 1997), *Santalum* (Shiri and Rao, 1998) and *Pittosporum* (Kondo *et al.*, 2002).

Paulownia (*Paulownia fortunei*) is a fast growing species of hardwood tree that is native to East Asia and it is of great commercial value. However, to our knowledge, no studies on the transformation of paulownia had been documented until we have explored microprojectile bombardment-mediated DNA transfer in paulownia (Mohri and Shinohara, 1996). In this reports, we examined the optimal conditions for the *A. tumefaciens*-mediated transformation of paulownia.

Shoot cultures derived from mature seeds were maintained on a medium that contained Murashige and Skoog's basal salts (Murashige and Skoog, 1962), Gamborg's B5 vitamins (Gamborg *et al.*, 1968) and 3% (w/v) sucrose (MSB5S medium) supplemented with 0.8% (w/v) agar and 0.5 mg l⁻¹ IBA. Shoot cultures were incubated at 25 °C under fluorescent lamps (30 μ Em⁻² s⁻¹, 16-h photoperiod) and subcultured every 2 months.

The disarmed strain *A. tumefaciens* LBA4404 (Hoekema *et al.*, 1983) that harbored a binary vector (pBI121; Clontech Laboratories, Inc., Palo Alto, CA) with genes for GUS and NPTII 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⁻¹ kanamycin and 300 mg l⁻¹ streptomycin. The overnight culture was diluted with liquid MSB5S medium to 5 × 10⁸ cells ml⁻¹ for transformation of paulownia tissue.

Tissue segments from paulownia 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 2 days on MSB5S medium (pH 5.8) supplemented with 0.3% (w/v) Gelrite (Scott Laboratories, Inc., Carson, CA) and 150 μM acetosyringone (4-acetyl-2,6-dimethoxyphenol; Aldrich, Milwaukee, WI). Each segment was washed twice with liquid MSB5S medium and then once with liquid MSB5S medium plus 500 mg l⁻¹ cefotaxime (Sigma, St. Louis, MO). The tissues were blotted with sterile filter paper and planted on selective callus-induction medium [MSB5S medium containing 50 mg l⁻¹ kanamycin, 500 mg l⁻¹ cefotaxime, 500 mg l⁻¹ carbenicillin, 1 mg l⁻¹ zeatin, 1 mg l⁻¹ BAP, 0.1 mg l⁻¹ NAA and 0.3% (w/v) Gelrite]. After calli had been allowed to develop for 4 weeks, they were excised from tissue segments and transferred to selective shoot-regeneration medium [MSB5S medium supplemented with 50 mg l⁻¹ kanamycin, 250 mg l⁻¹ cefotaxime, 1 mg l⁻¹ zeatin, 1 mg l⁻¹ BAP, 0.1 mg l⁻¹ NAA, 0.15% (w/v) Gelrite and 0.4% (w/v) bacto-agar (Difco Laboratories, Detroit, MI)]. Rooting of shoots was achieved in selective rooting medium [MSB5S medium that contained 50 mg l⁻¹ kana-

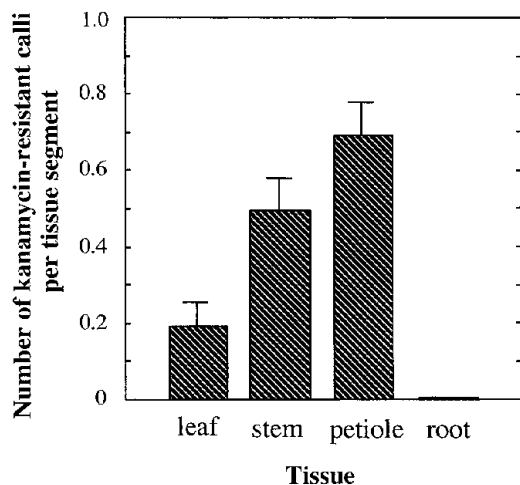


Fig. 1 Selection of suitable tissue for the most efficient transformation of paulownia. A total of 42 tissue segments was co-cultivated with *A. tumefaciens* on medium that contained 150 μM acetosyringone and 3% (w/v) sucrose at pH 5.8. The numbers of kanamycin-resistant calli were examined 30 days after induction of calli. Values of calli induced per tissue segment are means ± SE of five replicate experiments.

mycin, 250 mg l⁻¹ carbenicillin, 0.5 mg l⁻¹ IBA, 0.02 mg l⁻¹ NAA and 0.8% (w/v) agar].

Histochemical and fluorometric assays of GUS activity in transformed birch were performed as described by Jefferson *et al.* (1987). For histochemical assays, leaves of transformed birch were incubated overnight at 37 °C in a solution of 0.1 g l⁻¹ 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.

Genomic DNA was extracted from transformed paulownia calli as described by Murray and Thompson (1980). The primers for PCR (5'AT-GAAAAGCCTGAATC3' and 5'TTATTCCTTGCCCTCG3') were based on the nucleotide sequence of NPT II gene. Conditions for amplification were incubation at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 120 s.

Four different tissue segments of *in vitro* paulownia plants were examined with respect to their suitability for *A. tumefaciens*-mediated transformation (**Fig. 1**). Petioles were identified as the best tissue for such transformation. The proportion of petiole and stem segments that produced kanamycin-resistant calli after transformation was much higher than that of leaf and root segments. This result is inconsistent with previous our reports of the use of stem segments for lombardy poplar (Mohri *et al.*, 1996), leaf discs for Japanese white birch (Mohri *et al.*, 1997) and leaf and stem segments for *Robinia pseudoacacia* (Igasaki *et al.*, 2000). Thus, the best tissue for transformation obviously varies among species of woody plants.

Kanamycin-resistant calli derived from petiole segments were obtained on the selection medium within 4 weeks after transformation. Randomly selected kanamycin-resistant calli were strongly

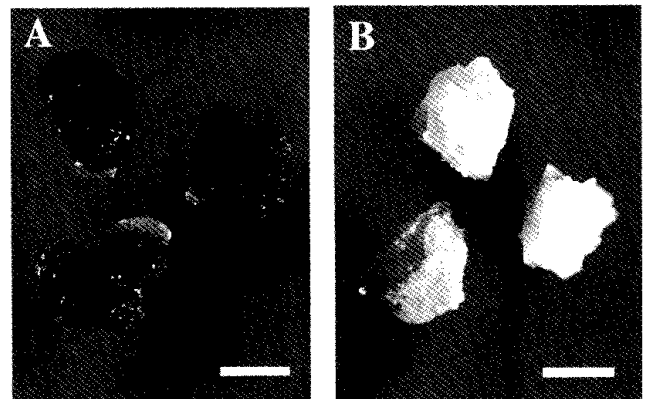


Fig. 2 Histochemical assay of GUS activity in transformed paulownia calli by staining with x-gluc. (A) transformed calli. (B) control calli. Bars = 1 cm

stained at high frequency (96%, 48 out of 50 calli tested) after histochemical staining for GUS activity (Fig. 2). This result suggests that an integrated GUS gene was expressed at high levels under the control of the 35S promoter of cauliflower mosaic virus. Successful transformation of paulownia was confirmed by PCR analysis (Fig. 3). We selected three transgenic calli (L-1, L-2 and L-3), derived from different lines of petiole segment, at random for PCR analysis. PCR analysis showed directly that the NPT II gene had been introduced into the genome of paulownia by the *A. tumefaciens*-mediated transformation. Therefore, petiole segments were used in the subsequent study.

The presence of acetosyringone in the co-cultivation medium increased the efficiency of transformation of paulownia (Fig. 4). The optimal concentration of acetosyringone was 150 μM for such transformation. The efficiency of transformation in the presence of 150 μM acetosyringone was more than three times that in the absence of acetosyringone. A positive effect of acetosyringone on *A. tumefaciens*-mediated transformation has been demonstrated in many plant species. The

efficiency of *A. tumefaciens*-mediated transformation of Japanese white birch was also promoted by the addition of acetosyringone to the transformation medium (Mohri *et al.*, 1997). However, acetosyringone had no effect on the efficiency of transformation of lombardy poplar (Mohri *et al.*, 1996). Our results suggest that the effect of acetosyringone on the *A. tumefaciens*-mediated transfor-

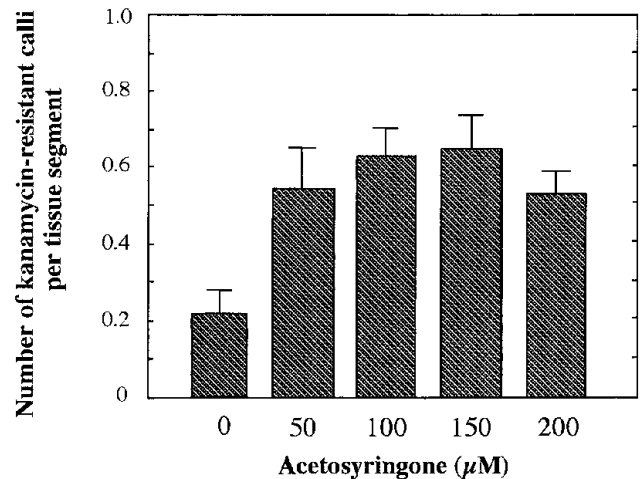


Fig. 4 Effects of acetosyringone on the induction of kanamycin-resistant calli during transformation of paulownia. A total of 21 petiole segments was co-cultivated with *A. tumefaciens* on medium that contained 3% (w/v) sucrose and various concentrations of acetosyringone as indicated, at pH 5.8. Values of calli induced per petiole segment are means \pm SE of five replicate experiments.

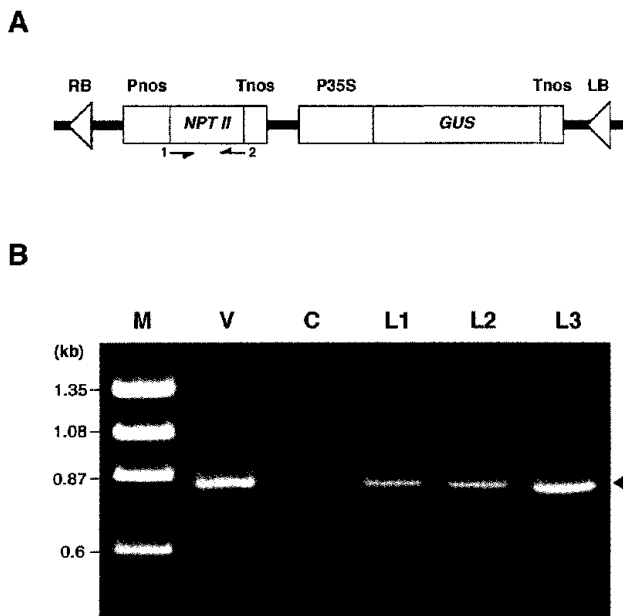


Fig. 3 Detection by PCR analysis of integrated NPT II gene in the genome of transgenic paulownia calli. (A) The primers (arrow 1 and 2) for PCR analysis. Only the T-DNA region of pBI121 is shown. RB, right border of T-DNA; LB, left border of T-DNA; Pnos, promoter of the nopaline synthase gene; Tnos, terminator of the nopaline synthase gene. (B) Detection of the NPT II gene by PCR analysis. The arrow head indicated NPT II gene. M, DNA maker; V, vector; C, control callus of paulownia; L1-L3, kanamycin-resistant calli of paulownia.

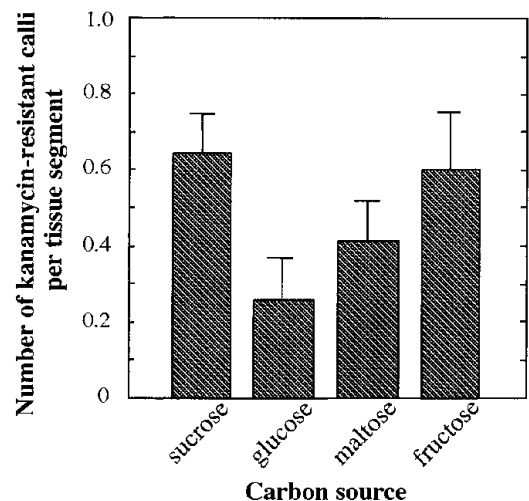


Fig. 5 Effects of carbon sources on the induction of kanamycin-resistant calli during transformation of paulownia. A total of 21 petiole segments was co-cultivated with *A. tumefaciens* on medium that contained 150 μM acetosyringone and 3% (w/v) several carbon sources as indicated, at pH 5.8. Values of calli induced per petiole segment are means \pm SE of five replicate experiments.

mation of broad-leaved trees also varies among species of woody plants.

Sucrose and fructose in the co-cultivation medium had also favorable effects on the efficiency of transformation of paulownia (Fig. 5). Sucrose was most suitable carbon source for the induction of kanamycin-resistant calli. Tissue segments in the presence of glucose exhibited symptoms of chlorosis during 2 days co-cultivation probably because of the damage by the overgrowth of *Agrobacterium*, while tissue segments in the presence of other carbon sources never showed the same phenomenon. This result seems to be somewhat inconsistent with a previous report by Shimoda *et al.* (1990), who found that only a group of aldoses, such as D-glucose, can markedly enhance acetosyringone-dependent expression of virulence genes, and that disaccharides such as sucrose and lactose show no enhancement of virulence gene expression. The difference in the effect of monosaccharides and disaccharides might have been derived from the assay methods in the two studies. Sucrose may be effective on the viability of paulownia tissue segments.

The pH of the co-cultivation medium had no significant effect on the efficiency of transformation of paulownia (data not shown). The number of kanamycin-resistant calli was fewer at pH 4 than at other pH values. *A. tumefaciens* may be unable to infect plant materials efficiently at pH 4.

As described above, we established a simple and reliable procedure for the induction of kanamycin-resistant calli of paulownia. To our knowledge, this is the first report of *A. tumefaciens*-mediated transformation of paulownia. Wei *et al.* (1991) established a system for the regeneration of plantlets from calli derived from the leaf mesophyll protoplasts of *Paulownia fortunei*. Although we used their system with slight modifications, few paulownia shoots were regenerated from kanamycin-resistant calli (1.2%, 6 shoots from 500 calli tested). No roots were regenerated from the above shoots in selective rooting medium. Thus, we have not succeeded in the regeneration of transgenic paulownia plants. When we overcome this problem, the present efficient and reproducible transformation system will allow the selective improvement of single traits in the paulownia by introduction of economically relevant genes that regulate morphological traits and resistance to insects and disease (Mohri *et al.*, 1999, Igasaki and Shinohara, 2000).

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