Mol Gen Genet (1987) 206:192–199

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Agrobacterium mediated transformation and regeneration of *Populus*

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Summary. A plant transformation and regeneration system has been developed for *Populus* species. Leaf explants, from stabilized shoot cultures of a *Populus* hybrid NC-5339 (*Populus alba* × *grandidentata*), were co-cultivated with *Agrobacterium tumefaciens* on a tobacco nurse culture. Both oncogenic and disarmed strains of *A. tumefaciens* harboring a binary vector which contained two neomycin phosphotransferase II (NPT II′) and one bacterial 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (*aroA*) chimeric gene fusions were used. Shoots did not develop when leaf explants were co-cultivated with the binary disarmed strain of *A. tumefaciens*. However, transformed plants with and without the wild type T-DNA were obtained using an oncogenic binary strain of *A. tumefaciens*. Successful genetic transformation was confirmed by NPT II′ enzyme activity assays, Southern blot analysis and immunological detection of bacterial EPSP synthase by Western blotting. This is the first report of a successful recovery of transformed plants of a forest tree and also the first record of insertion and expression of a foreign gene of agronomic importance into a woody plant species.

Key words: Poplar – Transformation – Agrobacterium

Introduction

The ability to genetically engineer forest tree species will be particulary useful in view of the factors limiting genetic improvement such as the large size of mature plants and the long sexual generation times (Nelson and Haissig 1984; Sederoff and Ledig 1985). One prerequisite for the application of recombinant DNA technology to forest tree species is the development of gene transfer systems. Methods such as microinjection (Crossway et al. 1986) and direct DNA uptake (Paszkowski et al. 1985; Fromm et al. 1986) have been used to introduce foreign genes into herbaceous crop species; however, the most effective method of gene transfer utilizes the natural infection mechanism of the crown gall disease causing organism, *Agrobacterium tumefaciens* (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estralla, 1983). During the natural infection cycle of *A. tumefaciens*, bacterial T-DNA is integrated into the host plant chromosome, resulting in the production of tumors on plants (Chilton et al. 1980). The tumor inducing genes can be deleted and replaced by heterologous genes without affecting the ability of *A. tumefaciens* to transfer T-DNA to plants (DeGreve et al. 1982). Co-cultivation of these modified *A. tumefaciens* strains with protoplasts, suspension cells, or explant tissue can lead to the isolation of transformed plants lacking oncogenic traits. We therefore set out to develop a transformation system for a hybrid poplar clone, *Populus alba* × *grandidentata* (NC-5339) using *A. tumefaciens* as a vector.

There are a number of characteristics that make *Populus* NC-5339 ideal for transformation studies. Firstly, *Populus* is an important forest tree species worldwide. It is a fast growing deciduous hardwood, cultivated primarily for pulp production. A major factor limiting the establishment and management of short rotation *Populus* plantations is the lack of a broad spectrum herbicide which effectively controls weeds (Akinymiju et al. 1982; Hansen and Netzer 1985; Nelson and Haissig 1986). Production of a *Populus* variety resistant to such herbicides would thus be economically attractive. Introducing the chimeric gene for glyphosate tolerance (Comai et al. 1985) into *Populus* hybrids offers a unique opportunity for weed control. Secondly, *Populus* has been known to be a natural host for *A. tumefaciens* for over ten years. Wild type strains such as strain 27 (Keen et al. 1970) and strain AT181 (Sciaky et al. 1978) have been isolated from galls on *Populus* sp. More recently, *Populus* was reconfirmed as a host for *A. tumefaciens* by demonstrating that T-DNA sequences were present in gall tissue (Parsons et al. 1986) However, to date *A. tumefaciens* has not been successfully used as a vector to transfer foreign genes of agronomic importance into *Populus* plants. Thirdly, *Populus* is a member of a genus of forest trees which are amenable to manipulation in vitro. Shoot cultures of *Populus* can be maintained in vitro and used for clonal propagation thus providing a sterile source of explant material for bacterial co-cultivation experiments. In addition large numbers of propagules can be obtained rapidly for experimental or commerical purposes (Christie 1978; McCown 1985).

In this paper we describe a system for the transfer and expression of foreign genes in *Populus* NC-5339 plants. Using a binary oncogenic strain of *A. tumefaciens* the mutant *aroA* gene, which encodes for an 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase less sensitive to the herbicide glyphosate, was introduced into *Populus* NC-5339 plants. The stable integration and the expression of the *aroA* gene was confirmed by Southern and Western blot analysis. This is the first report of the simultaneous transformation and regeneration of a forest tree species.

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Materials and methods

Plant culture and regeneration. Shoot cultures were established from a poplar hybrid clone, *Populus alba* × *Populus grandidentata* (NC-5339), as described by McCown (1985). Murashige and Skoog minimal organics medium with sucrose (30 g/l), and Phyto-agar (0.6%) (Gibco) was used both for inital culture and subsequent subcultures. The pH of the medium was adjusted to 5.6 with potassium hydroxide solution prior to autocalving for 20 min at 121° C. Magenta (Chicago), GA7 culture vessels, containing 50 ml of medium, were used to maintain shoot cultures. This medium was also used for rooting poplar shoots.

Shoot cultures were maintained from shoots orginating from axillary buds every four weeks by excising a shoot, removing the leaves and the apical bud, and placing the cut stem horizontally onto fresh medium.

All cultures were grown in a controlled environment room at 25° C ± 2° C, using cool white fluorescent light 50 micro Einsteins per meter^2 per second (50 $\mu Em^{-2}S$) for 16 h light day cycles. For shoot regeneration from leaf explants the medium (described above) for maintenance of shoot cultures was used with the addition of BA (1.0 mg/l) and zeatin (ZEA) (1.0 mg/l). After autoclaving and cooling to 50° C, carbenicillin (500 mg/l) (a bacteriostatic substance) and kanamycin (60 mg/l), (a selective antibiotic), were added to the medium by filter sterilization as required. The medium was then dispensed into 100 × 15 mm petri dishes (35–40 ml per dish).

Bacterial strains. Agrobacterium tumefaciens strains LBA4404 is a non-oncogenic derivative of Ach5 from which the T-DNA has been deleted (Hoekema et al. 1983). This strain carries an intact *vir* region and can mediate the introduction of any T-DNA present in the bacterium into plants. C58 is an oncogenic, nopaline producing strain of *A. tumefaciens* (Hamilton and Fall 1971). A binary vector plasmid, pPMG85/587 was introduced into strains LBA4404 and C58. This vector plasmid carries a modified T-DNA, with three chimeric genes (Fig. 1). Two of these genes code for neomycin phosphotransferase (NPT II′) which confers resistance to the antibiotic kanamycin (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983), and are spliced to either the octopine synthase promoter (ocs-NPT II′) (Barker et al. 1983) or to the mannopine synthase promoter (mas-NPT II′) (Barker et al. 1983). The third gene confers tolerance to the herbicide glyphosate (Comai et al. 1983) and has been spliced to the mannopine synthase promoter (mas-*aroA*). *A. tumefaciens* was maintained in AB solid minimal medium (Watson et al. 1975), supplemented with 100 mg/l kanamycin in the case of strains containing pPMG85/587. Cultures were restreaked every fifteen days. For co-cultivation, a single colony from a freshly streaked plate was used to inoculate 5 ml of MG/L broth (Chilton et al. 1974) in a 25 × 150 mm culture tube and incubated overnight. The overnight culture was then diluted to the appropriate concentration (strains C58/587/85 and C58; 2×10^8 cfu/ml; strain LBA4404/587/85: $5–10 \times 10^8$ cfu/ml) using the absorbance of the cultures at 550 nm to measure the bacterial concentration.

Plant transformation. Transformation of *Populus* NC-5339 was obtained by co-cultivating leaf segments with *A. tumefaciens* in the presence of tobacco suspension cells (Horsch et al. 1985). The tobacco feeder plates were prepared 2 days prior to use by pipetting 0.5 ml of tobacco suspension culture onto petri dishes (100 × 25 mm) containing 50 ml of Murashige minimal organics medium (K.C. Biological), supplemented with 2,4-D (0.1 mg/l) kinetin (1.0 mg/l) thiamine hydrochloride, (0.9 mg/l), potassium acid phosphate, (200 mg/l), and Difco Bacto agar (0.8%). After 2 days, a sterile filter paper disc (Whatman 3 mm) was placed on top of the tobacco cells. The filter paper discs were prewashed in distilled water and autoclaved in the tobacco suspension medium (described above) but without the agar.

The tobacco cell suspensions used for the feeder plates were maintained weekly by transfer of 10 ml of cell suspension into 100 ml of fresh liquid medium. The medium used for maintenance of tobacco cell suspensions (described above) is the same medium used for the feeder plates with the agar omitted.

After the tobacco feeder plates were prepared, poplar leaves obtained from sterile shoot culture, were cut into segments (~ 2 cm^2) and preincubated on feeder plates for 24 h at 25° C, under low light conditions (10 $\mu E\ m^2\ s^{-1}$). Leaf segments were then placed into 1–5 ml of an MG/L broth culture of *A. tumefaciens* strain C58/587/85, C58 or strain LBA4404/587/85 diluted to the appropriate concentration. After 30 min, leaf segments were blotted to remove

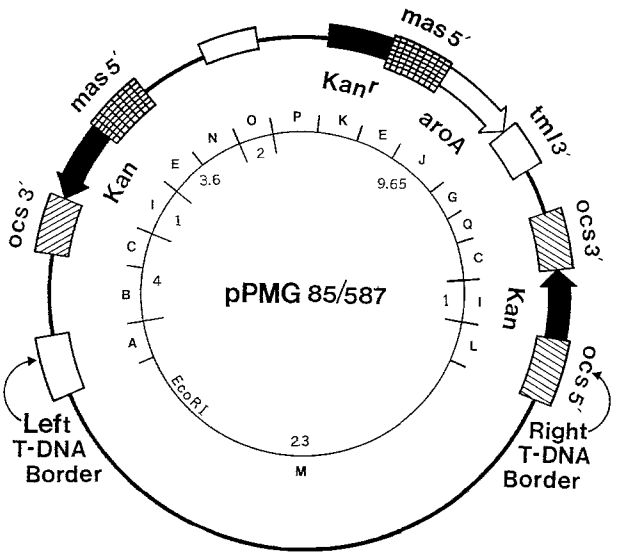

Fig. 1. Genetic map of pPMG85/587. The *inner lettered circle* defines each genetic element. A, left T-DNA border (625 to 2,212; Barker et al. 1983); B, pUC-CAT 2.4 kb (Buckley 1985); C, octopine synthase (*ocs*) 3′ region from *Sma*I (11,207) changed to *Xho*I, to *Eco*RI (12,823); I, NPT II′ from Tn*5* 1 kb; E, mannopine synthetase (*mas*) 5′ region from *Xho*I (21,476) to *Cla*I (20,128); N, pACYC184 *Sal*I to *Eco*RI 2.2 kb; O, *Eco*RI 2 kb fragment from T-L DNA of pRiA4; P, pACY184 *Eco*RI to *Sal*I 1.5 kb; K, NPT II′ from pUC4K *Sal*I fragment; J, *aroA* gene from pPMG38 *Bam*HI to *Sal*I 1.3 kb; G, *tml* 3′ region from *Sma*I (11,207) changed to *Xho*I, to *Bam*HI (9,062); Q, pACY184 *Bam*HI to *Sal*I 0.3 kb; L, *ocs* 5′ region, including the right T-DNA border, from nucleotide (13, 643) to *Xho*I (15,208); M, pRK290 *Sal*I to *Bgl*II, 20 kb. Plasmid pPMG85, encompassing C to Q clockwise, was described previously (Comai et al. 1985). Plasmid pCGN587, C to B clockwise, was constructed by Vic Knauf (unpublished results). *Eco*RI digestion sites are marked in the *inside circle* with the size of the fragments marked in kilobase pairs

excess suspensions and replaced onto the feeder plates for 48–96 h of co-incubation with the bacteria. Leaf segments were subsequently transferred to regeneration medium containing carbenicillin (500 mg/l) and kanamycin (60 mg/l or 0 mg/l), as described above. Shoots were excised when they were approximately 1–2 cm long and propagated from axillary buds as described above.

The rate of shoot regeneration from *Populus* NC-5339 leaf explants was determined by scoring the number of explants which regenerated shoots and by counting the number of shoots developing from each of these explants. This data was collected from two separate experiments where each treatment was comprised of a minimum of three plates (nine explants per plate). The mean percent of explants regenerating shoots and the mean number of shoots regenerating per explant were calculated. An arc-sine transformation was performed on the percent values for each plate prior to averaging. Standard deviations and standard errors were calculated for each mean (Sincich 1982). To determine if there were significant differences in the regeneration rates from control (not co-cultivated) and co-cultivated explants, a one tailed t-test about a population mean was conducted (Sincich 1982).

Molecular analysis. The neomycin phosphotransferase II (NPT II′) enzyme confers resistance to the antibiotic kanamycin and allows for direct selection of transformed plant tissue. Activity of the enzyme NPT II′ in leaves and shoots of transformed and control plants was measured using the method of Reiss et al. 1981, modified by the addition of a final wash. The P-81 ion exchange paper was rinsed three times in a proteinase K solution (1.0 µg/ml) at 90° C to reduce nonspecific binding of $^{32}PO_4$ in phosphorylated proteins. To determine if the wild type T-DNA from strain C58 was being expressed, nopaline assays were conducted on leaf samples from both A nd B-type shoots. Nopaline assays were carried out as described by Otten and Schilperoort (1978).

Western blot analysis. Western blot analysis of the bacterial EPSP synthase enzyme encoded by the *aroA* gene was conducted using the protocol described by Comai et al. (1985), except for the initial preparation of tissue extracts. Leaf tissue (0.5 g) was ground in liquid nitrogen with 0.2 g of polyvinylpyrrolidone polyclar AT, and suspended in 2.0 ml of 0.1 *M* Na citrate buffer, pH 5.6, containing 10 mM EDTA, 150 mM NaCl, 0.05% Nonidet P-40, 25 mg/ml bovine serum albumin, 10 mM dithiothreitol, 10 mM thiourea, and 10 µM leupeptin.

DNA Isolation and southern analysis. DNA was isolated from 1 g of leaf or shoot tissue by the following protocol. Tissue was frozen in liquid nitrogen, pulverized with mortar and pestle and homogenized with a Brinkmann Polytron with seven ten-second pulses in 10 ml of nuclei isolation buffer (10 mM Tris, pH 9.5, 4 mM spermidine, 1 mM spermine, 10 mM EDTA, 80 mM KCl, 0.5 *M* sucrose, 10 mM β-mercaptoethanol, 0.5% Triton X-100) at 0° C. The homogenate was filtered through Miracloth (Calbiochem, Behring) and nuclei were pelleted by centrifugation (5 min, 3,000 × *g*), resuspended in 5 ml of nuclei isolation buffer, recentrifuged (5 min at 300 × *g*) and resuspended in 1.5 ml of the same buffer. Then 1.5 ml lysis buffer (0.1 *M* Tris, pH 9.5, 40 mM EDTA, 2% sarkosyl, and 12 units of proteinase K) was added, gently mixed, and the nuclei were incubated in this solution at 65° C for thirty minutes to one hour. This digested chromatin preparation was then extracted gently with TE (10 mM Tris, 1 mM EDTA)-saturated phenol (pH 7.5) and then with TE-saturated phenol-chloroform (1:1; pH 7.5). The aqueous phase was adjusted to 0.3 *M* with NH_4 Acetate and precipitated twice with two volumes of ethanol. The DNA was then resuspended in 10 mM Tris (pH 8.0), 10 mM NaCl, and stored at 4° C. Isolated DNA was subsequently digested with *Eco*RI, separated by gel electrophoresis, blotted onto nitrocellulose and then hybridized separately to an *aroA* probe, a NPT-II′ probe and a nopaline probe. Procedures for restriction digestion, gel electrophoresis, Southern transfer and hybridization were as described by Maniatis et al. 1982). The probe for the NPT II′ and the *aroA* gene was synthesized by in vitro transcription of plasmid pCGN464 and pCGN1008. These plasmids consist of the NPT II′ gene and the *aroA* gene respectively cloned in pSP64, and SP6-transcription vector (Melton et al. 1984). The protocol recommended by the manufacturer for synthesis and hybridization was followed (Promega Biotec, Madison, WI). The probe for the T-DNA of the pTiC58 was a ^{32}P-labelled fragment, *Hin*dIII-23, (Depicker et al. 1980) carrying the nopaline synthase gene.

Results

Plant regeneration

Regeneration from *Populus* NC-5339 leaf explants was obtained on MS medium supplemented with 1.0 mg/l BA and 1 mg/l Zeatin. Shoots developed at the cut edges of leaf explants 20–30 days after plating. An average of 1–3 shoots developed from 35% of the leaf explants plated (Table 1). The number of shoots increased with the size of the explant and the amount of cut surface. Based on microscopic observations during development, shoots appeared to originate from adventitious meristems rather than somatic embryos. Anthocyanin production was observed at the site of shoot development, just prior to shoot initiation.

Plant transformation

More than 30% of the explants co-cultivated with *Agrobacterium* strain C58/587/85 developed shoots within 3–4 weeks on regeneration medium containing kanamycin at 60 mg/l (Table 1). An average of four kanamycin resistant shoots developed per explant. Growth of control tissue was completely inhibited by kanamycin at 60 mg/l (Fig. 2).

Significant differences in the number of explants regenerating shoots on a non-selective media were observed between explants co-cultivated with *A. tumefaciens* (strains C58 and C58/587/85) and control (not co-cultivated) explants at the 5% and 2.5% levels. Not only did more explants regenerate shoots after co-cultivation (Table 1) but in the case of C58/587/85 four times more shoots regenerated per co-cultivated explant (Table 1, Fig. 2) than control explants. Consistent differences in the average number of shoots regenerating from explants co-cultivated with C58 and control explants were observed; however, these differences were only significant at the 10% level. No differences were noted between the regeneration rate of leaf explants co-cultivated with LBA4404/587/85 and control leaf explants (data not shown). In two experiments using 60 ex-

Table 1. Shoot regeneration from co-cultivated and control leaf explants of *Populus* NC-5339 cultivated on regeneration medium with or without the use of the selective antibiotic kanamycin

	Shoot regeneration from *Populus* leaf explants							
	0 mg/l kanamycin				60 mg/l kanamycin			
	Average % of explants regenerating shoots	Standard error	Average number of shoots per explant	Standard error	Average % of explants regenerating shoots	Standard error	Average number of shoots per explant	Standard error
Control (not co-cultivated)	36.0[a]	8.3	2.9[d]	0.59	0		0	
C58	55.2[b]	4.9	4.1[e]	0.46	0		0	
C58/587/85	59.3[c]	5.7	11.97[f]	1.32	32.0	2.1	4.4	0.90

Pairwise comparisons were made between means using a one tailed t-test for population means
[a] Significantly different from C58 at the 5% level
[b] Not significantly different from C58/587/85
[c] Significantly different from control at the 2.5% level
[d] Significantly different from C58 at the 10% level
[e] Significantly different from C58/587/85 at the 5% level
[f] Significantly different from control at the 5% level

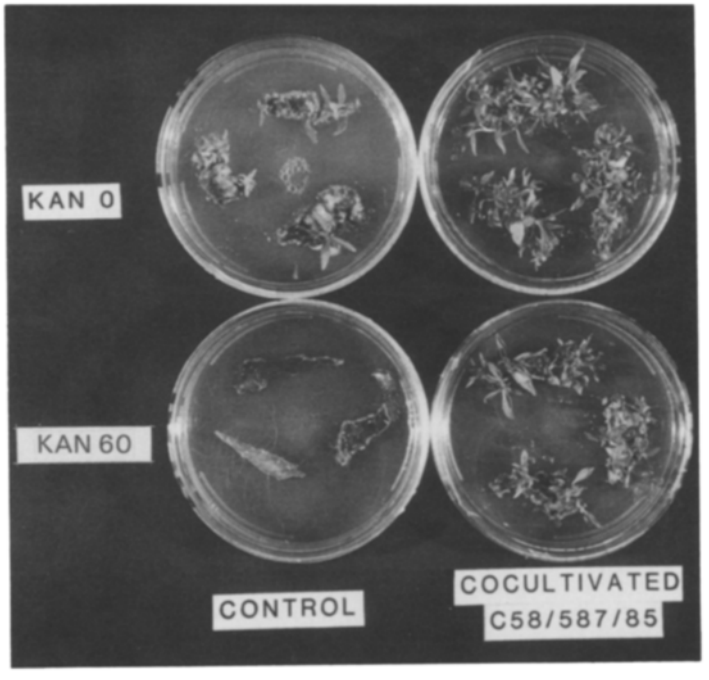

Fig. 2. Differences in the rate of shoot regeneration between co-cultivated and control (not-co-cultivated) *Populus* NC5339 leaf explants in the absence or presence of kanamycin

plants per treatment, kanamycin-resistant shoots were never recovered from explants co-cultivated with the binary, disarmed strain of *A. tumefaciens*, LBA4404/587/85.

Shoots which developed on medium containing kanamycin could be divided into two classes (A and B) based on shoot morphology. The phenotype of the B-Type shoots differed markedly from that of the untransformed *Populus* NC-5339 shoots. B-type shoots grew in a classical teratomic fashion, with thickened stems and greatly reduced leaf surface area per stem, compared to the untransformed controls (Fig. 3). In addition, when B-type shoots were excised and plated onto a rooting medium, a mass of callus formed at the base of the shoot and new B-type shoots, but not roots, developed. In comparison, A-type shoots were visually indistinguishable from untransformed control shoots and they also developed roots within two weeks when excised and plated onto rooting medium.

Nopaline synthase and NPT II′ enzyme assays were conducted on 20 of the B-type and 40 of the A-type shoots previously selected on medium containing kanamycin (60 mg/l). Nopaline synthase activity was detected in all B-type shoots but not in any of the A-type or untransformed shoots (Fig. 4). NPT II′ enzyme activity assays were also conducted on the same shoots from both phenotypic classes (Fig. 5). Eighteen of the 20 B-type shoots and 38 of the 40 A-type shoots tested exhibited NPT II′ enzyme activity whereas samples of untransformed poplar plants did not (Fig. 5).

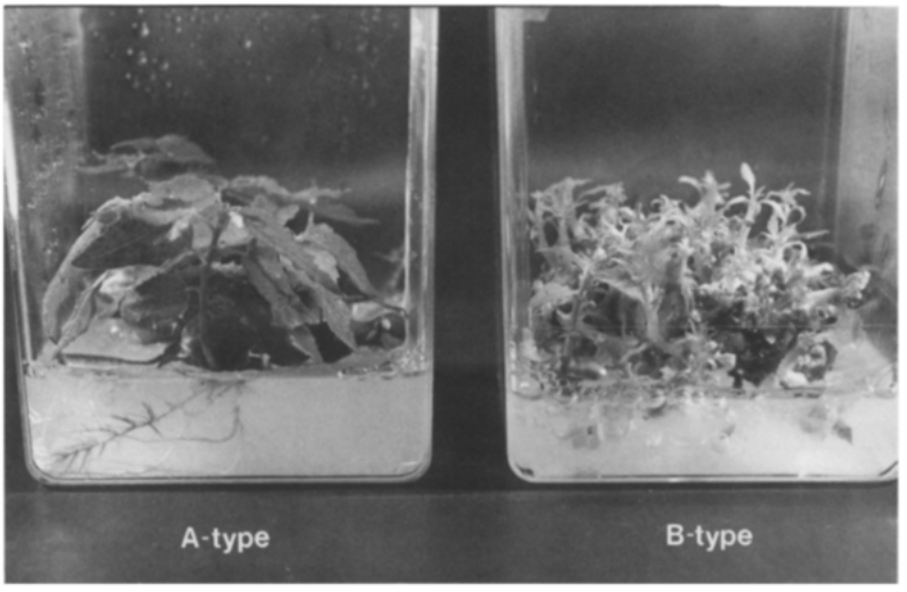

Fig. 3. Transformed *Populus* NC-5339 plants and shoots originating from leaf explant co-cultivations with a binary oncogenic strain of *A. tumefaciens*, C58/587/85. Two distinct shoot morphologies developed: A-type shoots, which are visually indistinguishable from parent plants, do not contain the wild type T-DNA; B-type shoots, which grow in a teratomic fashion, contain wild type T-DNA

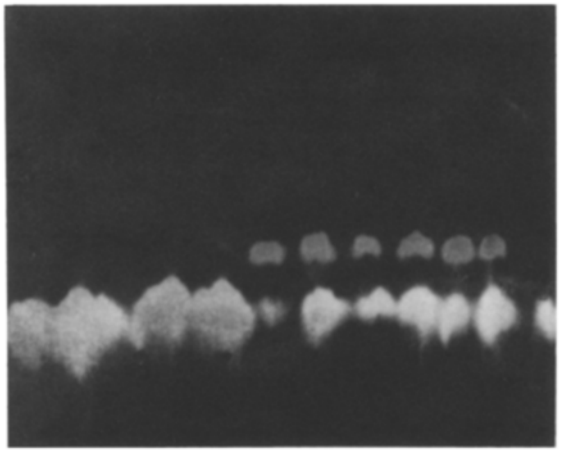

Fig. 4. Nopaline assay run on 100 mg of leaf tissue from transformed *Populus* NC-5339 shoots (A-type and B-type) derived from leaf explant co-cultivations with *A. tumefaciens*, strain C58/587/85. Samples were extracted in acidified methanol and centrifuged. The supernatent was spotted onto whatman 3 mm paper and fractionated by high voltage electrophoresis. The presence of nopaline was evidenced by staining the electrophoretogram with phenanthreneguinone and viewing under UV light. In the positive control, lane 6, the faster migrating spot is nopaline. Lanes 1–5, A-type leaf samples; lanes, 7–10 B-type leaf samples; lane 11, nopaline negative control

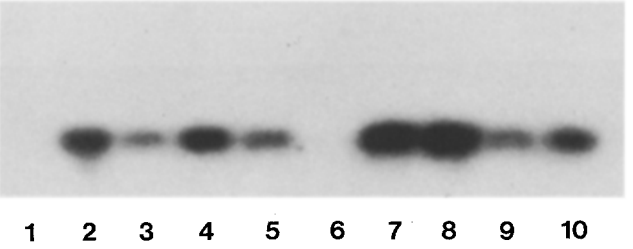

Fig. 5. Detection of NPT II′ enzyme activity in transformed *Populus* NC-5339 plants and shoots. Lane 1, leaf extract from an untransformed *Populus* NC-5339 plant; lane 2, leaf extract from a positive tomato control; lanes 3–10 leaf extracts from A-type shoots

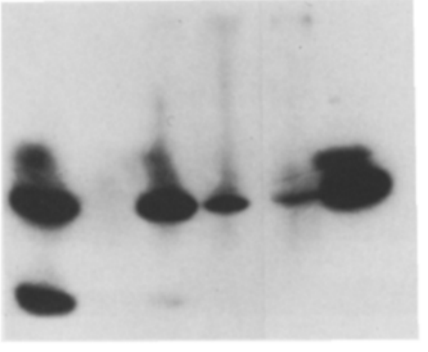

Fig. 6. Detection of the bacterial EPSP synthase protein in transformed *Populus* NC-5339 plants by Western blot analysis. Lane 1, leaf extract from a tomato plant transformed using *A. tumefaciens* strain LBA4404 pPMG587/85; lane 2, leaf extract from a control untransformed *Populus* NC-5339 plant; lanes 3 and 4, leaf extracts from transformed *Populus* NC-5339 plants; lane 5, 250 mg purified EPSP synthase protein added to untransformed *Populus* NC-5339 leaf extract; lane 6, 250 mg purified EPSP synthase protein and no leaf extract

Western analysis

A Western blot analysis was conducted on putative transformants to detect the presence of the bacterial EPSP synthase enzyme. As shown in Fig. 6, lanes 3 and 4, a polypeptide which reacts with the anti-EPSP synthase serum and with the expected molecular weight was present in transformed *Populus* NC-5339 plants. This polypeptide was not found in control untransformed plants. The amount of bacterial EPSP synthase protein observed varied between different transformation events. (Fig. 6, lanes 3 and 4.) Tomato leaves (Fig. 6, lane 1) containing the same *aroA* gene fusion, appeared to produce slightly more EPSP synthase protein per gram fresh weight of leaf tissue than *Populus* NC-5339 (Fig. 6, lane 3). It is possible, however, that the efficiency of protein recovery differed between species and

hence the amount of *aroA* protein observed may not reflect in vitro levels.

Southern analysis

Isolation of DNA from poplar was first attempted by the protocol of Dellaporta et al. (1983). This protocol is effective for other plant species, however, it yielded poplar DNA that was heavily contaminated with gelatinous material, probably polysaccharides, and was not suitable for restriction endonuclease digestion and electrophoresis. The protocol described in the Materials and methods was designed to overcome this problem. It consists of nuclei purification followed by lysis, proteolytic digestion and organic solvent extraction. Using this method we obtained high yields of DNA (20–40 μg/gm fresh weight) which was easily digested and electrophoresed. The size of the DNA obtained is not satisfactory as 10%–20% is in the 5–20 kb range, nevertheless, it was suitable for Southern analysis. Approximately 5 μg DNA was digested with endonuclease *Eco*RI, electrophoresed on 0.7% agarose gels and blotted onto nitrocellulose. The blots were hybridized to RNA probes for NPT II′ and *aroA*, and to a nick-translated probe for the T-DNA of pTiC58. Digestion of the binary plasmid pPMG85/587 with *Eco*RI yields a 1 kb fragment carrying the NPT II′ gene. A fragment of this size was found in all putative transformants (Fig. 7), and not in the control. Similarly, the expected 9.7 kb fragment containing the *aroA* gene was seen in the transformants expressing the *aroA*-EPSP synthase (Fig. 8.). Two transformants 39-3 and 39-115 were *aroA*-negative by Western blotting and did not contain the 9.7 kb fragment. A segment of pPMG58/587 spanning the *mas-aroA* and *mas*-NPT II′ could have been deleted by recombination between the homologous NPT II′-*ocs*-3′ regions of pPMG85//587 (see Fig. 1). If that had occurred, however, we would expect to still find a 4 kb *Eco*RI fragment (Fig. 1, bands B and C) hybridizing to pUC. Such a fragment was not found (data not shown) and neither 39-3 nor 39-115 contain any DNA homologous to pUC. This suggests that transformation in both these plants resulted from an incomplete T-DNA transfer beginning at the right border and ending before the *aroA* gene. If this hypothesis is correct these plants should not contain the left border. Additional bands hybridizing to the probe are visible in both *aroA* and NPT II′ blots. These bands are difficult to explain as their size does not fit those expected from the pPMG85/587 map. The 4.6 kb band in the *aroA* blot is also found in the control and is probably due to spurious hybridization.

Fragment *Hind* 23, located on the right end of pTiC58 T-DNA, was obtained by digesting plasmid p*Hind* 23 (Vic Knauf, unpublished results) with *Hind*III, separating the vector band (pUC18) from the 3.5 kb *Hind* 23 insert by agarose gel eletrophoresis, and electroeluting *Hind* 23 from the gel (Maniatis et al. 1983). Using nick translated *Hind* 23 as probe, a signal corresponding to C58 T-DNA was found in A-10 and A-33 but not in any other poplar samples (data not shown). *Hind* 23 hybridized to a large *Eco*RI fragment (>30 kb) in A-10, and to a ~15 kb fragment in A-33. Heterogeneity is expected in this case as the *Eco*RI fragment spans the right T-DNA border. In summary, Southern analysis of putative transformed poplars confirmed NPT II′ assays, *aroA*-EPSP synthase Western blot analysis, nopaline analysis and phenotypic classification. Considerable variation in copy number of T-DNA was observed. Since the genome size of poplar is not known, we cannot accurately estimate the copy number range. Preliminary comparison to concentration standards (data not shown) suggest the range of 1 copy for 39-3, to 100 copies for A-10, assuming a 1 pg haploid genome size.

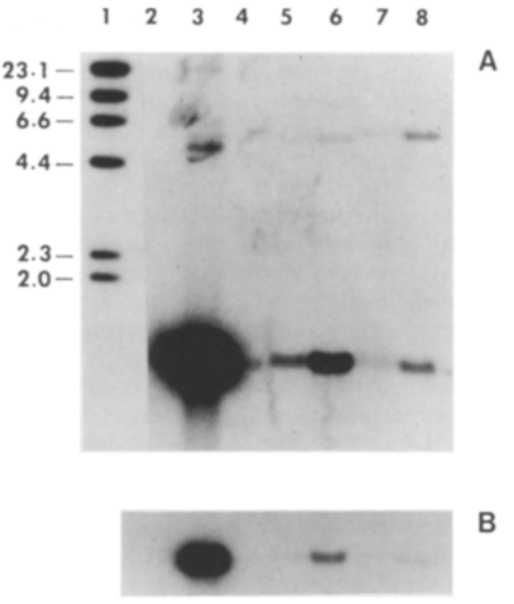

Fig. 7A, B. Southern blot analysis of the NPT II′ gene in transformed poplar. Lane 1, coliphage lambda *Hind*III standard; 2, control poplar; 3 and 4 A-11 and A-33 are transformed poplar lines of the teratoma type; 5 and 6, 75-12 and 75-15 are normal *aroA*-positive; lines 7 and 8, 39-3 and 39-115 are normal, *aroA*-negative lines. The hybridized blot was exposed for **A** 20 h(r) and **B** 4 h(r). Equal amounts of DNA (5 μg) were loaded in each lane, with the exception of lane 4 where 2.5 μg was loaded. The kanamcyin resistance gene from pUC4K (Fig. 1) does not hybridize to the NPT II probe

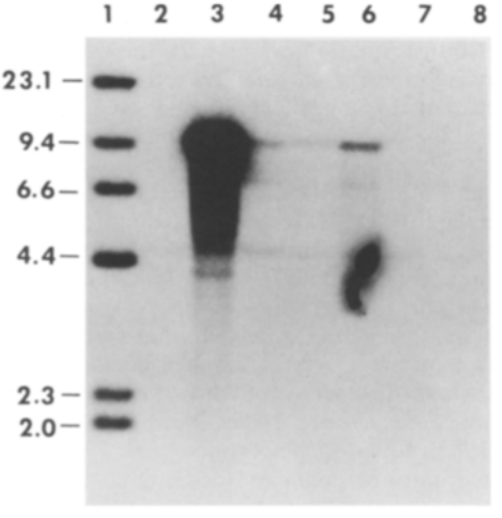

Fig. 8. Southern blot analysis of the *aroA* gene in transformed poplar. Lane 1, coliphage lambda *Hind*III standard; 2, control poplar; 3 and 4 A-11 and A-33 are transformed poplar lines of the teratoma type; 5 and 6, 75-12 and 75-15 are normal *aroA*-positive; lines 7 and 8, 39-3 and 39-115 are normal, *aroA*-negative lines

Discussion

The mutant EPSP synthase gene, which confers tolerance to the herbicide glyphosate, and two selectable NPT II′ genes have been transferred and expressed in *Populus*. This is the first study to report the recovery of transformed plants of a forest tree species, the insertion and expression of an agronomically useful gene in a woody crop, and the successful use of an *Agrobacterium* explant co-cultivation system for a woody species. At present, we are cloning the transformed *Populus* plants which are producing the *aroA* protein to determine the level of tolerance conferred by the introduced glyphosate-tolerance gene.

The transformation system we have described is an explant co-cultivation system which combines three important features: the use of in vitro *Populus* shoot cultures, the use of a specific oncogenic strain of *A. tumefaciens* and the use of a medium which induces shoot development from *Populus* NC-5339 leaf explants. Shoot cultures provide a juvenile, sterile and reproducible source of explant tissue and such source tissue has shown superior growth and regeneration potential when compared to other sources (Smith and McCown 1983). We also found that use of a C58-based binary oncogenic strain of *Agrobacterium* not only mediated the transfer of DNA into *Populus* but also enhanced the rate of shoot proliferation from leaf explants. Since both the binary oncogenic *Agrobacterium* strain C58/587/85 and the unmodified strain C58 enhanced the rate of shoot regeneration it is possible that either the oncogenes of the wild type T-DNA or the *trans*-zeatin (TZS) gene carried within the *vir* region (Regier and Morris 1982) are producing a local hormonal environment that enhances regeneration in conjunction with the hormones in the regeneration medium. Enhanced rates of regeneration may be necessary to detect transformation events since the rate of regeneration is usually decreased after bacterial co-cultivation. The effect of the oncogenic strain may be a species-specific one since the opposite result is observed when tomato cotyledons were co-cultivated with the same strains (C58/587/85 and C58) (Fillatti, unpublished data). Callus developed from the cut edge of tomato cotyledons following co-cultivation with these two strains; however, shoot regeneration was completely inhibited. In most Ti plasmid vector systems the oncogenes that encode for the production of auxin and cytokinin are deleted to ensure that species-specific hormonal regimes required for shoot regeneration can be maintained. Most of these vectors, however, have been developed using *Solanaceous* species such as tobacco (Horsch et al. 1985), tomato (McCormick et al. 1985; Fillatti, unpublished data) or petunia (Horsch et al. 1985) as host tissue. *Populus* seems to be an exception in this regard since the hormones produced by the oncogenes or the TZS gene enhance shoot regeneration when in combination with the hormones in the regeneration medium. It is possible that the delivery of plant hormones via *A. tumefaciens* may become an integral part of future transformation/regeneration systems, as this type of hormonal supplement clearly has a different effect from that provided by the medium. This approach may also have application for the regeneration of recalcitrant plant species. It should be noted that since the hormone genes and the selected marker are on different plasmids, transformants containing only the binary T-DNA can be easily obtained.

Alternative explanations for the failure of leaf explants co-cultivated with the binary disarmed strain LBA4404 pPMG587-85, to develop transformed shoots are possible. This *Agrobacterium* strain may simply not be virulent on *Populus* or the co-cultivation conditions may not be optimal for this strain. In tomato co-cultivation experiments we have observed large decreases in the transformation and regeneration rates when variables such as the co-cultivation time and the bacterial concentrations are not optimized for a particular strain/cultivar combination (Fillatti, unpublished). It is also possible that without the hormones produced by of strain C58, the rate of regeneration is too low to recover transformants.

When binary oncogenic vectors such as strain C58/587/85 are used three insertion events are possible, either or both T-DNAs can be present. However, our system selects against one of these insertion events through the use of selectable NPT II′ genes. By Southern blot analysis we have demonstrated that it is possible to distinguish between the other two types of insertion events by analyzing for nopaline production or by visually assessing the phenotpye of regenerated shoots.

In a recent report Parsons et al. (1986) were successful in obtaining evidence for gene transfer by *Agrobacterium* to cells of a *Populus* hybrid in culture but were unable to recover transformed plants. The possible reasons for this are: (1) stabilized shoot cultures were not employed, (2) the strains of *Agrobacterium* used provided a different hormonal environment from the strains used in our experiments, (3) a different genotype of *Populus* was used (Parsons et al. (1986) used a *Populus trichocarpa* × *P. deltoides* hybrid).

In summary, the transformation and regeneration system developed here is simple and effective, allowing rountine introduction of genes into *Populus*. The success of more recent transformation experiments in our laboratories using other *Populus* hybrid clones (NC-5331, *P. nigra* cv. *betulifolia* × *P. trichocarpa*; NC-5272, *P. nigra* × *P. laurifolia*; NC-11390, *P. maximowiczii* × *P. trichocarpa*) has further confirmed the use of this regeneration and transformation technique on a wide range of *Populus* species. This system will also allow the development of herbicide-tolerant poplar trees, permitting chemical control of weeds, and thus substantially decreasing losses due to competition during the early stages of cultivation.

Acknowledgements. The authors would like to thank Burt Rose for his help with the vector construction, and Richard Michelmore and Robert Goodman for their critical review of the manuscript.

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Communicated by R.B. Goldberg

Received August 20, 1986