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Agrobacterium-mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants

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Abstract

A method for *Agrobacterium*-mediated transformation of hybrid poplar (*Populus alba* × *P. grandidentata* cv. 'Crandon') suspension cultures and regeneration of transformed plants is described. Transformants were recovered when suspension cultures were inoculated with *Agrobacterium tumefaciens* at a density of 10^7 colony-forming units ml^{-1}, cocultivated for 48 h, and plated to cellulose acetate filters on Woody Plant Medium containing 4.5 μM 2,4-dichlorophenoxyacetic acid and 250 mg l^{-1} cefotaxime. Levels of cefotaxime greater than 250 mg l^{-1} were unnecessary for control of residual bacteria and inhibited callus growth. Transgenic plants were regenerated by culturing the transformed callus on media containing 0.11 to 27 μM thidiazuron. In contrast to thidiazuron, N^6-benzyladenine had a negative effect on shoot regeneration; the callus became necrotic when we attempted to induce shoots with concentrations of 1.1 to 8.9 μM, and growth was inhibited when concentrations of 0.11 or 0.22 μM were used to regenerate callus from suspension cultures. Following cocultivation of poplar suspension cultures, we recovered transgenic plants containing the maize transposon *Ac*, and callus containing an insect toxin gene from *Bacillus thuringiensis*.

Abbreviations: BA – N^6-benzyladenine, CIM – callus initiation medium, CaMV – cauliflower mosaic virus, cfu's – colony-forming units, HPT – hygromycin phosphotransferase, MS – Murashige and Skoog medium (Murashige & Skoog 1962), NPT-II – neomycin phosphotransferase-II, PAR – photosynthetically active radiation, PCR – polymerase-chain-reaction, TDZ – thidiazuron, WPM – Woody Plant Medium (Lloyd & McCown 1980), 2,4-D – 2,4-dichlorophenoxyacetic acid

Introduction

Transgenic hybrid poplars have been produced using *Agrobacterium*-mediated transformation of leaves (Fillatti et al. 1987) and stems (De Block 1990), and by microprojectile-mediated transformation of stems, cultured nodules, and protoplast-derived cells (McCown et al. 1991). A suspension culture transformation system might have several advantages for inserting genes into poplar, including the ability to screen large numbers of potentially transformed cells, effective inhibition of residual *Agrobacterium* following cocultivation, and high transformation frequencies because rapidly dividing suspension cultures may be particularly prone to stable integration of foreign DNA (An 1985; Okada et al. 1986).

A protocol for *Agrobacterium*-mediated transformation of poplar suspension cultures was developed by evaluating strains of *Agrobacterium*, methods of cocultivation, media for regenerating callus from suspension culture, selection regimes, and shoot regeneration media. We used suspension culture transformation to insert both the *Ac* (*Activator*) transposable element from maize, and an insect toxin gene from *Bacillus thuringiensis* (*Bt*), into hybrid poplar callus. We regenerated transgenic plants from the *Ac*-containing callus via organogenesis, but in the *Bt* transformation experiment, we were unable to regenerate plants from either transformed or untransformed callus.

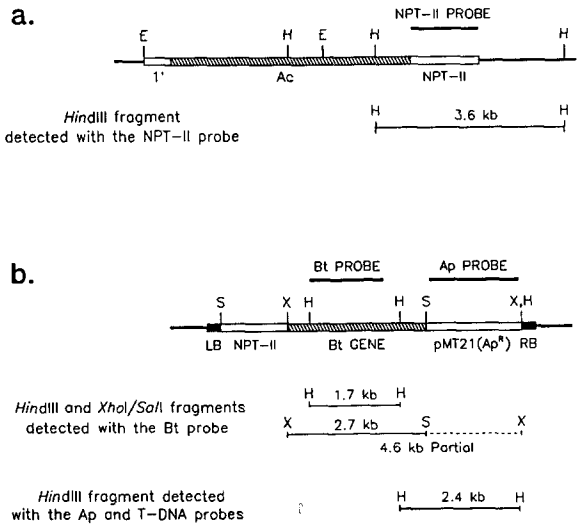

Fig. 1. Restriction site maps of plasmid vectors used to transform hybrid poplar. Panel (*a*); T-DNA region of the cointegrate Ti plasmid vector pGV3850HPT::pKU3. The *Activator* transposable element from maize (*Ac*) was inserted into the untranslated leader of the NPT-II gene downstream of the *Agrobacterium* 1' promoter. The HPT gene and the T-DNA borders are not shown. Panel (*b*); T-DNA region of the binary vector pTV4AMVBTS. RB and LB are the right and left T-DNA borders, pMT21 (Ap^R) is an ampicillin-resistant plasmid, E is *Eco*RI, H is *Hin*dIII, S is *Sal*I, and X is *Xho*I.

Materials and methods

Bacterial strains and plasmids

An *Agrobacterium* cointegrate transformation vector was used to insert the *Ac* transposable element into poplar. This vector (pGV3850HPT::pKU3) contains both an *Ac*/NPT-II gene fusion and a hygromycin phosphotransferase (HPT) gene within the T-DNA borders (Fig. 1, Baker et al. 1987). The HPT gene, which confers resistance to the antibiotic hygromycin, is under the control of the nopaline synthase (*nos*) promoter and polyadenylation signal (van den Elzen et al. 1985). The cointegrate vector was maintained in *Agrobacterium tumefaciens* strain C58C1*rif*. Overnight cultures were grown in MGL medium (An 1986) containing 100 mg l^{-1} carbenicillin.

For the *Bt* transformation experiment, we use a binary vector (pTV4AMVBTS) containing both a truncated insect toxin gene from *Bacillus thuringiensis*, and an NPT-II gene for selection of transformed plant cells (Fig. 1, Barton et al. 1987). The *Bt* and NPT-II genes are under the control of the CaMV 35S and *nos* promoters, respectively, and the *nos* polyadenylation signal. Freeze-thaw bacterial transformation (An et al. 1988) was used to transfer the binary vector pTV4AMVBTS into five strains of *Agrobacterium tumefaciens*. C58(pTiC58) contains an armed wild-type C58 Ti plasmid, A281 contains the armed 'hypervirulent' Ti plasmid pTiBo542 (Hood et al. 1986), C58(pTiC58-Z707) is a disarmed version of C58(pTiC58) (Hepburn et al. 1985), EHA-101 is a disarmed version of A281 (Hood et al. 1986), and NT1 lacks the Ti plasmid (Watson et al. 1975). Overnight cultures were grown in MM (Hooykaas 1988) containing 25 mg l^{-1} streptomycin and (for strains containing the binary vector) 100 mg l^{-1} sulfadiazene.

Plant materials and tissue culture media

For each transformation experiment, callus was initiated from *in vitro* shoot cultures of hybrid poplar clone NC-5339 (*Populus alba* L. × *P. grandidentata* Michx. cv. 'Crandon'; Sellmer & McCown 1989) by culturing leaves on solidified callus initiation medium (CIM). CIM is MS or WPM containing 30 g l^{-1} sucrose, 4.5 μM 2,4-D, and 0.22 μM BA, adjusted to a pH of 5.7 to 5.8. Liquid CIM was used for suspension cultures, whereas CIM for plates was solidified with 0.5% agarose (GIBCO-BRL Ultrapure). After 2 to 4 weeks of growth, the callus was used to inoculate

suspension cultures, which were subsequently maintained using weekly transfers of 5 to 10 ml of suspension culture to 50 ml of WPM-CIM in a 250-ml flask. The suspension cultures were grown on a gyratory shaker (120 rpm) at 25°C under cool white fluorescent light (5–10 μmol m^{-2} s^{-1} PAR) using a 16-h photoperiod. Unless otherwise indicated, callus tissue was grown on WPM-CIM in the dark at 25°C, growth regulators were added to the medium prior to autoclaving at 121°C for approximately 20 min, and antibiotics were added after autoclaving. *In vitro* shoot cultures were maintained essentially as described by Russell & McCown (1988). Shoots derived from callus were rooted *in vitro* using shoot elongation medium, and eventually acclimatized to growth in a non-sterile mixture of peat moss and perlite (1:1) in a growth chamber. Shoot elongation medium is shoot culture medium (Russell & McCown 1988) supplemented with 0.01 μM BA.

Callus regeneration from suspension cultures

To develop a system for recovering transformed callus from suspension culture, we tested the effects of 2,4-D BA, and cefotaxime on callus growth. Suspension cultures growing in WPM-CIM were filtered through a 350 μm nylon screen (Small Parts Inc., Miami, FL) and approximately 6 colonies mm^{-2} were plated to cellulose acetate filters (Millipore SMWP, 5.0 μm pore size, 47 mm dia.) on solidified WPM. The plating media consisted of a factorial arrangement of four cefotaxime concentrations and nine growth regulator treatments. The growth regulator treatments were 2.3, 4.5, or 9.0 μM 2,4-D, plus either 0, 0.11, or 0.22 μM BA. Cefotaxime (Calbiochem, La Jolla, CA) was added to a final concentration of 0, 125, 250, or 500 mg l^{-1} free-acid. The final number of treatment combinations was 35 because one of the treatment combinations became contaminated (4.5 μM 2,4-D, 0.22 μM BA, and 250 mg l^{-1} cefotaxime). Three plates were used for each treatment, and callus dry weight was measured 4 weeks after plating. Main effects were tested using the 2,4-D × BA × cefotaxime interaction as the error term. Treatments not significantly different from the best treatment were identified using Tukey's HSD criterion at the $\alpha = 0.05$ level of probability.

Cocultivation and selection of transformants

Transformation with AC

Overnight cultures of *Agrobacterium* (containing pGV3850HPT::pKU3) were grown in medium containing 200 μM acetosyringone (Aldrich). Suspension cultures were filtered through a 500 μm nylon screen and *Agrobacterium* was added to a final density of either 10^6, 10^7, or 10^8 cfu's ml^{-1}. For each density, one flask was cocultivated for 24 h and another for 48 h. After cocultivation, approximately 3, 7, or 14 colonies mm^{-2} were plated to cellulose acetate filters on WPM-CIM containing 500 mg l^{-1} cefotaxime. Four plates were prepared for each plating density.

To identify the transformants, we used two phases of selection on hygromycin. Five days after the cocultivated suspension cultures were plated to the filters, the filters were transferred from medium without hygromycin to medium containing 500 mg l^{-1} cefotaxime and either 0, 7.5, 15, or 30 mg l^{-1} hygromycin B (Sigma). Filters were transferred to new antibiotic-containing medium every 4 to 16 days, and the level of cefotaxime was reduced to 250 mg l^{-1} for the second and all subsequent transfers. For the second phase of selection, we used a single concentration of hygromycin; when colonies were greater than 1 mm in diameter, they were transferred from the filters to agarose plates containing 15 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime. Antibiotic resistant colonies were transferred to WPM-CIM without antibiotics after 1 to 2 months of selection on 15 mg l^{-1} hygromycin.

Transformation with Bt

Suspension cultures were filtered through a 500 μm nylon screen and resuspended in WPM-CIM containing 5 mM MES (4-morpholineethanesulfonic acid), and 50 μM acetosyringone, pH 5.6. The suspension cultures were inoculated with five strains of *Agrobacterium* [C58(pTiC58), C58(pTiC58-Z707), A281, EHA 101, or NT1] containing the binary vector pTV4AMVBTS. Control treatments were inocu-

lated with the same strains of *Agrobacterium*, but lacking the binary vector. The final bacterial density for each treatment was 10^7 cfu's ml^{-1}. After cocultivating for 48 h, the cells were washed and resuspended in WPM-CIM containing 250 mg l^{-1} cefotaxime. The cocultivated cells were plated either 15 or 35 days later. At both times, the suspension cultures were filtered through a 500 μm nylon screen, resuspended in WPM-CIM, and aliquots were plated to WPM containing 4.5 μM 2,4-D, 250 mg l^{-1} cefotaxime, plus kanamycin sulfate (Sigma) or G418 sulfate (Geneticin, GIBCO-BRL).

For the initial plating (15 days after cocultivation), we dispensed approximately 180 colonies mm^{-2} to filters on top of medium containing either 0, 25, 200, or 400 mg l^{-1} kanamycin, or 5, 40, or 80 mg l^{-1} G418. For the second plating (35 days after cocultivation), approximately 20 or 40 colonies mm^{-2} were plated to filters on medium containing either 20 or 40 mg l^{-1} G418. The filters were transferred to fresh antibiotic-containing medium every 4 to 16 days. When the colonies grew larger than 1 mm in diameter, they were transferred from the filters to agarose plates containing the same antibiotics. During the subsequent phase of selection, the colonies on kanamycin were transferred to G418, and the concentrations of G418 were gradually reduced. Four weeks after plating, the colonies remaining on the filters were transferred to antibiotic-free plates by placing the filters upside-down on solidified medium. The colonies that developed on these plates were transferred directly to 10 mg l^{-1} G418. For all colonies, final selection was based on the ability to grow on 10 mg l^{-1} G418.

Shoot regeneration from callus

We attempted to produce shoots from the *Ac* callus lines by transferring the hygromycin-resistant colonies to WPM containing various concentrations of TDZ or BA. Six to eight colonies (4 mm dia.) from four hygromycin-resistant callus lines were transferred to either BA (Sigma) at 0, 1.1, 2.2, 4.4, or 8.9 μM, or TDZ (NORAM Chemical Co., Wilmington, DE) at 0.11, 0.31, 1.0, or 3.1 μM. These plates were maintained under cool-white fluorescent lights (5–10 μmol m^{-2} s^{-1} PAR) using a 16-h photoperiod. The colonies were transferred to shoot elongation medium 4 weeks after the beginning of the experiment, and every 2 to 6 weeks thereafter. At the time of each transfer, the largest shoots were separated and placed on elongation medium. We recorded the percentage of explants with shoots at 9 weeks. Based on the results from the first experiment, we conducted a second experiment that included higher concentrations of TDZ, but omitted the BA treatments. Eight callus lines were transferred to WPM containing TDZ at 0, 0.11, 0.31, 1.0, 3.1, 9.2, or 27 μM.

In three additional experiments, we attempted to produce shoots from colonies transformed with the *Bt* gene. Transformed and untransformed callus lines from the *Bt* experiment were transferred to WPM containing TDZ at 0, 0.11, 0.31, 1.0, 3.1, 9.2, or 27 μM, and subsequently treated as described above. In conjunction with one of these experiments, two *Ac* lines were cultured on WPM containing 1.0 μM TDZ.

Molecular analysis of transformants

Transformation with Ac

Poplar DNA, isolated using the procedure of Wagner et al. (1987), and plasmid DNA from pKU3 were double-digested with *Hin*dIII and *Eco*RI, electrophoresed, and transferred to a nylon membrane using alkaline transfer. A blot of poplar callus DNA was probed with a 506 bp *Eco*RI/*Hin*dIII fragment containing the 1' promoter from pOP4434 (Baker et al. 1987), and a blot of leaf DNA was probed with a 1.0 kb *Bam*HI/*Bg*/II fragment containing the NPT-II gene from SP65*neo*II (provided by K. Barton). The blots were hybridized, washed at high stringency (0.1X SSC, 0.5% SDS final wash), and autoradiographs were exposed at −70°C using intensifying screens.

Transformation with Bt

We used the polymerase-chain-reaction as an initial screen to identify callus lines containing the *Bt* gene. Poplar DNA was isolated from 0.75 g of frozen callus using a mini-prep procedure (Howe 1991), and a *Bt* gene fragment of 704 bp was amplified using *TAq* DNA polymerase (Promega, Madison, WI), 50 ng of poplar callus DNA, and primers complementary to the

coding region of the *Bt* gene (5′-ACGGGAT-TAGAGCGTGTATG-3′ and 5′-AAGGTG-TAAACTGCTCCAGC – 3′). The DNA was amplified using 30 or 40 thermal cycles of 1 min at 95°C, 1 min at 55°C, and 2.5 min at 72°C, followed by a final extension time of 7 min at 72°C. Positive controls included plasmid DNA from pTV4AMVBTS, or DNA from a callus line that had previously tested positive. DNA from a non-cocultivated callus line was used as a negative control. The PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. Presence of the *Bt* gene was confirmed by Southern analysis. DNA from poplar callus, plasmid DNA from pTV4AMVBTS, and DNA from *Agrobacterium* strain C58 (with no binary vector) were digested with *Hin*dIII, or double-digested with X*ho*I and *Sal*I. Southern blots were hybridized with three probes; the *Bt* probe consists of the 5′ portion of the *Bt* coding region isolated from pSP65(5′BT) (provided by K. Barton), the *Ap* probe contains the ampicillin resistance gene from the T-DNA of pTV4AMVBTS, and the wild-type T-DNA probe is a 7.5 kb *Eco*RI fragment from $pTiB_6806$ (provided by L. Moore).

Results

Callus regeneration from suspension cultures

BA had a consistently negative effect on callus growth, regardless of the concentration of 2,4-D in the medium. The best growth occurred when the suspension cultures were plated to medium containing 2.3 μM 2,4-D and no BA, but this treatment was not significantly better than the other treatments lacking BA (4.5 or 9.0 μM, 2,4-D).

Cefotaxime also reduced callus growth substantially. In those treatments without BA, for example, the colonies grown on 500 mg l^{-1} cefotaxime had less than 40% of the dry weight of the controls. The negative effect of cefotaxime was observed at all concentrations tested; when the colonies were grown on media with 0, 125, 250, and 500 mg l^{-1} cefotaxime (and no BA), the dry-weights averaged 84, 73, 58, and 31 mg respectively.

Evaluation of cocultivation and selection regimes

Cocultivation and selection regimes were evaluated after we identified the transformants using Southern analysis (see Molecular Analysis of Transformants).

Transformation with Ac

No transformants were recovered from the suspension cultures cocultivated for 24 h, but six transformants were recovered from cultures cocultivated for 48 h; five from the treatment inoculated with 10^7 cfu's ml^{-1}, and one from the treatment inoculated with 10^6 cfu's ml^{-1} of bacteria.

For the best cocultivation treatment, all transformants were recovered from colonies plated at the highest density (14 colonies mm^{-2}). At this plating density, transformants were recovered from three of the four hygromycin concentrations used for initial selection (no hygromycin, 15 mg l^{-1}, and 30 mg l^{-1}; Table 1). One transformant was recovered from the 416 colonies that received no initial selection; therefore, the frequency of transformation prior to selection was estimated to be 0.2%.

Transformation with Bt

Based on results from the *Ac* experiment, we used 10^7 cfu's ml^{-1} of *Agrobacterium* and a 48-h cocultivation period for inserting the *Bt* gene. Our best results were obtained when the suspension cultures were cocultivated with C58(pTiC58) and plated 15 days later. We recovered eight independent transformants using this protocol, including two with rearranged *Bt* genes. A single transformant was recovered using the disarmed strain C58(pTiC58-Z707), but no transformants were recovered using A281, EHA 101, or NT1. Following the initial plating, a 'lawn' of cells developed on plates containing either 5 mg l^{-1} G418 or 25 mg l^{-1} kanamycin, and these plates were discarded. Because the selection intensity was also too low using 200 or 400 mg l^{-1} kanamycin (i.e. individual colonies were difficult to identify), these filters were transferred to 40 and 80 mg l^{-1} G418, respectively, 2 weeks after plating. In

Table 1. Summary of results using the best cocultivation protocol from each of two experiments. Poplar suspension cultures were inoculated with 10^7 cfu's ml^{-1} of *Agrobacterium tumefaciens* and cocultivated for 48 hours.

Treatment used for initial selection[1]	Number of surviving colonies		Number of transformed colonies	Percent of surviving colonies that contained foreign DNA	
	Initial selection	Final selection[2]		Initial selection	Final selection[2]
Transformation with *Ac*[3] (using *Agrobacterium* strain C58 containing the cointegrate vector pGV3850HPT::pKU3)					
No antibiotics	416	1	1	0.2	100
7.5 mg l^{-1} hygromycin	25	1	0	0.0	0
15 mg l^{-1} hygromycin	7	2	2	28.6	100
30 mg l^{-1} hygromycin	91	3	2	2.2	67
Transformation with *Bt*[4] (using *Agrobacterium* strain C58(pTiC58) containing the binary vector pTV4AMVBTS)					
No antibiotics	286	1	0	0.0	0
40 mg l^{-1} G418	81	20	15 (6)[5]	18.5	75
80 mg l^{-1} G418	0	0	0	0.0	0
200 mg l^{-1} kanamycin/ 40 mg l^{-1} G418	66	3	2	3.0	67
400 mg l^{-1} kanamycin/ 80 mg l^{-1} G418	51	0	0	0.0	0

[1] For the kanamycin/G418 treatments, the suspension cultures were plated to kanamycin, but transferred to G418 2 weeks later.
[2] Final selection was 15 mg l^{-1} hygromcyin for the *Ac* transformation experiment, and 10 mg l^{-1} G418 for the *Bt* transformation experiment.
[3] The plating density for the *Ac* transformation experiment was approximately 14 colonies mm^{-2}.
[4] For the *Bt* transformation experiment, the cells were plated 15 days after the end of the cocultivation treatment, using a plating density of approximately 180 colonies mm^{-2}.
[5] Only six independent transformants were positively identified from 15 transgenic callus lines (see text).

general, G418 concentrations of 20 to 40 mg l^{-1} were best for the initial phase of selection. The selection intensity was too low using 5 mg l^{-1} G418, and 80 mg l^{-1} G418 was too toxic.

We used the results from the best cocultivation protocol to evaluate differences among the selection treatments (Table 1). The greatest number of transformants was recovered when the cells were plated to 40 mg l^{-} G418. For this treatment, 81 colonies survived the initial phase of selection. After the final phase of selection, molecular analysis indicated that 15 of the 20 colonies that were still alive contained the *Bt* gene, but only 6 of these were independent transformants (see Molecular Analysis of Transformants). In summary, our best results were obtained when suspension cultures were cocultivated for 48 h with C58(pTiC58), then plated 15 days later on 40 mg l^{-1} G418 at a density of 180 colonies mm^{-2}.

We also recovered 286 colonies from the plates without antibiotics (i.e. no initial selection), but

none was transformed. Therefore, the frequency of transformation prior to selection was below 0.35%.

Shoot regeneration from callus

A total of five shoot regeneration experiments were conducted. In the first experiment, colonies transformed with *Ac* were tested on BA and TDZ. When the colonies were transferred to BA, a few buds and some tiny shoots were produced, but no shoots could be kept alive. In contrast to BA, seven of eight *Ac* lines produced healthy shoots on TDZ. Because the percentage of explants with shoots was similar among the shoot-producing lines, it appeared that the single line that failed to produce shoots was qualitatively different from the rest, perhaps due to some genetic or epigenetic change. Because of its anomalous performance, this line was omitted from the results presented in Fig. 2. A 250-fold range of TDZ concentrations (0.11 to 27 μM) was effective at producing viable shoots from the *Ac* lines (Fig. 2). The best treatment (0.31 μM TDZ) produced shoots from 70% of the explants when averaged over both experiments.

Although we tested the *Bt* callus lines using the same TDZ treatments that were successful with the *Ac* lines, we failed to produce any shoots from any of the *Bt* lines in three separate experiments. When two *Ac* lines were included as positive controls in one of these experiments, however, both of them produced shoots.

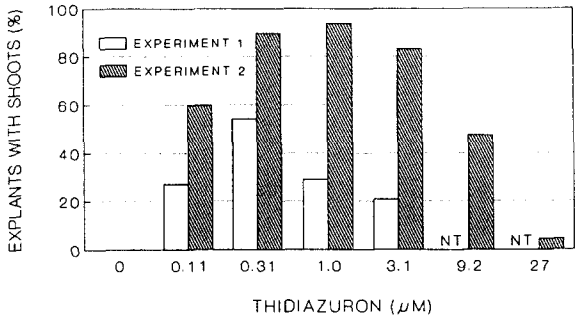

Fig. 2. Percentage of callus explants that produced shoots on Woody Plant Medium containing thidiazuron. NT indicates that the 9.2 and 27 μM treatments were not tested in the first experiment.

Molecular analysis of transformants

Transformation with Ac

Eight hygromycin-resistant callus lines remained following the final phase of selection, six of which contained foreign DNA based on Southern analysis. Plants were regenerated from five of the transformed lines (discussed above), and Southern analysis showed that all of the plants contained foreign DNA. Representative results from three of the hygromycin-resistant lines are presented in Fig. 3a; two of the hygromycin-resistant lines (*Ac*1 and *Ac*4) are transformed, but *Ac*3 shows no evidence of foreign DNA.

Transformation with Bt

We used results from PCR analysis to discard antibiotic-resistant lines that did not contain the *Bt* gene based on two criteria. The first criterion was the absence of the diagnostic 704 bp fragment following amplification with *Bt*-specific primers. The second criterion was the presence of a faint 'spurious band' of approximately 400 bp that was amplified from both untransformed callus and callus lines containing the *Bt* gene (Strauss et al. 1991). The presence of the spurious band was used to rule out general failure of the PCR reaction, thereby eliminating false-negative results.

Following the final phase of selection, we recovered 24 callus lines from the best cocultivation treatment (summed over all selection treatments, Table 1). Based on PCR analysis, however, only 17 of these lines contained the *Bt* gene. Using a *Bt* gene probe, we subsequently tested 16 of the PCR-positive lines for the presence of a 2.7 kb *Xho*I/*Sal*I fragment and a 1.7 kb *Hin*dIII fragment that are diagnostic for the *Bt* gene (Figs. 1, 3b, and 4b; one line was not tested because of insufficient tissue). Based on these analyses, we concluded that 14 lines contain intact *Bt* genes (Fig. 4b shows 15 lines with the 1.7 kb *Hin*dIII fragment; 14 transformants from the best cocultivation protocol, plus *Bt*38, which was transformed using C58(pTiC58-707)). Two additional lines contain *Bt* genes that are rearranged. The 1.7 kb *Hin*dIII fragment is missing from *Bt*139, but we did detect a slightly larger fragment (Fig. 4b). For *Bt*6, we detected

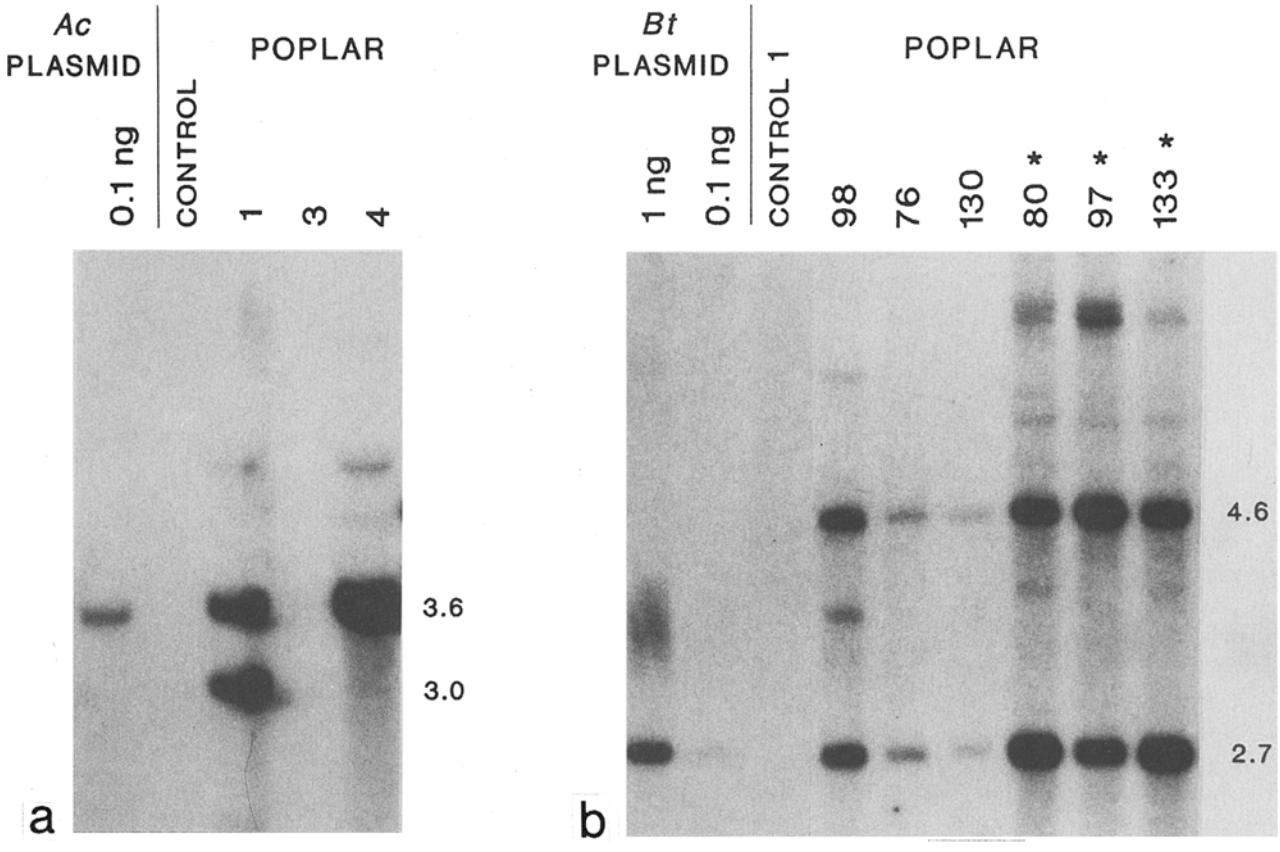

Fig. 3. Southern analyses of hybrid poplar tissues containing the *Ac* transposable element from maize and an insect toxin gene from *Bacillus thuringiensis* (*Bt*). Panel (*a*); DNA from the plasmid pKU3 and DNA from poplar leaves (9 μg) were double-digested with *Hin*dIII and *Eco*RI and hybridized with an NPT-II gene probe. Lane 1 (*Ac* plasmid) contains DNA from pKU3 mixed with DNA from untransformed poplar, lane 2 (Control) contains DNA from untransformed poplar, and lanes 3 to 5 contain DNA from poplar lines *Ac*1, *Ac*3 and *Ac*4. *Ac*3 is an untransformed line that survived antibiotic selection, where *Ac*1 and *Ac*4 contain foreign DNA. The 3.0 kb fragment in *Ac*1 may result from *in planta* excision of the *Ac* element from the T-DNA. Panel (*b*); DNA from the binary vector pTV4AMVBTS and DNA from poplar callus (5 μg) were double-digested with *Xho*I and *Sal*I and hybridized with a *Bt* gene probe. Lanes 1 and 2 (*Bt* plasmid) contain DNA from pTV4AMVBTS mixed with DNA from untransformed poplar, lane 3 (Control 1) contains DNA from untransformed poplar, and lanes 4 to 9 contain DNA from transformed poplar lines *Bt*98 to *Bt*133. The 2.7 kb band is an *Xho*I/*Sal*I fragment that spans the *Bt* gene, and the 4.6 kb band is an *Xho*I fragment that results from incomplete digestion of the *Sal*I restriction sites (see Fig. 1). Fragment sizes are given in kilobase pairs.

no *Hin*dIII fragments, but we did detect a fragment with an anomalous size when the *Bt* probe was hybridized to the *Xho*I/*Sal*I blot (data not shown). The structure of the *Bt* gene in *Bt*129 is unknown because it was not tested using Southern analysis.

We also used a wild-type T-DNA probe and tests for auxin-independent growth to identify which callus lines contained wild-type T-DNA in addition to the *Bt* gene. The T-DNA probe detected two *Hin*dIII fragments in DNA isolated from *Agrobacterium*, and similar fragments were detected in a number of transgenic callus lines (2.0 and 3.4 kb, Fig. 4a). Both diagnostic frag-

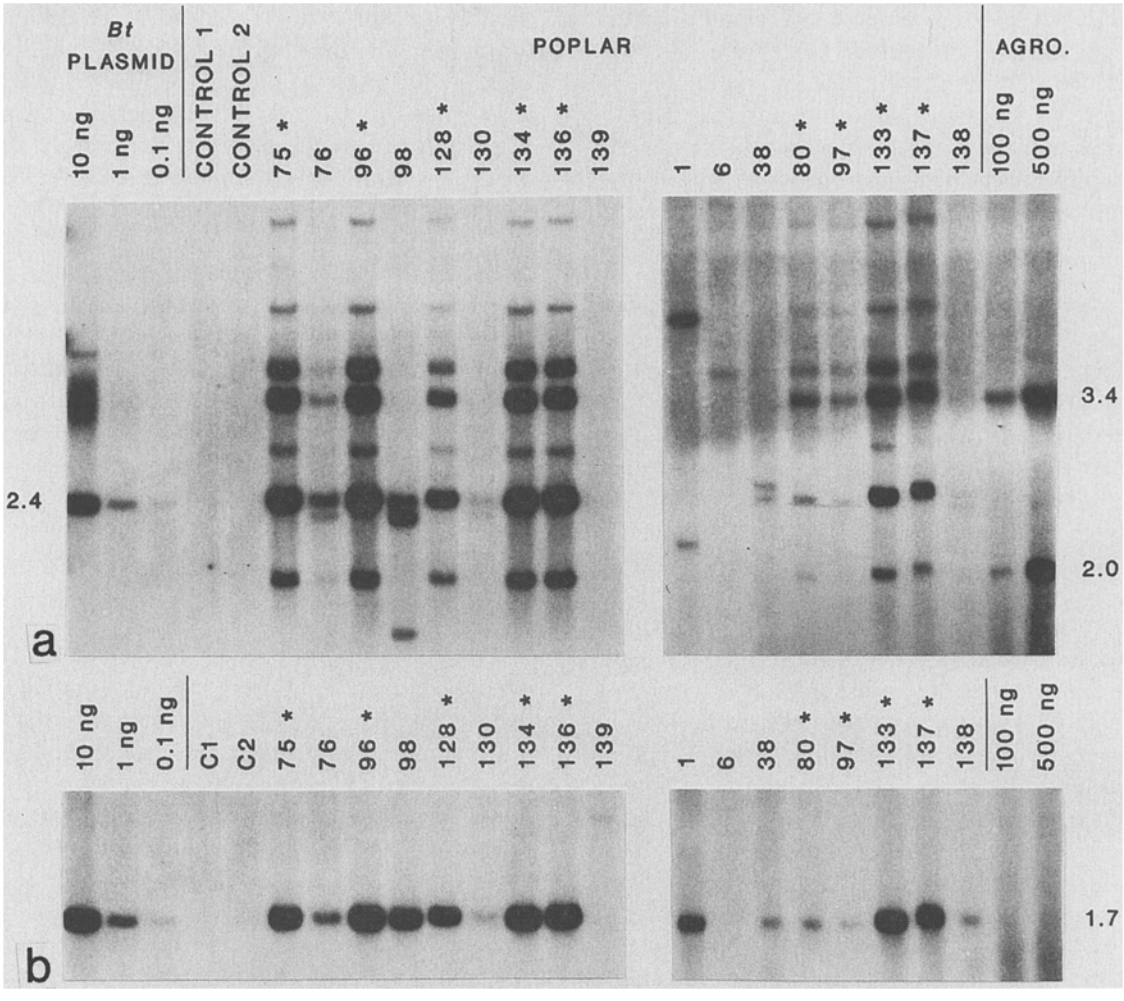

Fig. 4. Southern analyses of hybrid poplar tissues containing an insect toxin gene from *Bacillus thuringiensis* (*Bt*). Panel (*a*); DNA from the binary vector pTV4AMVBTS and DNA from poplar callus (5 μg) were digested with *Hin*dIII and hybridized with a wild-type T-DNA probe from *Agrobacterium tumefaciens*. Panel (*b*); Same blot as in (*a*), but hybridized with a *Bt* gene probe. Lanes 1 to 3 (*Bt* plasmid) contain DNA from pTV4AMVBTS mixed with DNA from untransformed poplar, lanes 4 and 5 (Controls) contain DNA from untransformed poplar, lanes to 6 to 22 contain DNA from transgenic poplar lines *Bt*75 to *Bt*138, and lanes 23 and 24 (Agro.) contain DNA from *Agrobacterium tumefaciens*. All transgenic lines were cocultivated with C58(pTiC58), except for *Bt*38, which was transformed using C58(pTiC58-Z707). The callus lines labeled with asterisks are isogenic (i.e. derived from a single transformed cell, see text). Fragment sizes are given in kilobase pairs.

ments were detected in *Bt*76, and eight of the nine lines identified with asterisks in Fig. 4a. Although the 2.0 kb fragment could not be detected in *Bt*97, this line is clearly identical to other lines that contain the 2.0 kb fragment (see below). Except for *Bt*76, the lines with wild-type T-DNA grew well on medium without auxin (including *Bt*97), suggesting that the auxin-biosynthetic genes from *Agrobacterium* were being expressed.

The T-DNA probe also detected fragments from the binary vector (note the 2.4 kb fragments in lanes 1 to 3, Fig. 4a). These fragments were detected because the T-DNA cloning vector (pBR325) and the binary vector share an ampicillin-resistance gene. During preparation of

the T-DNA probe, some of the cloning vector was apparently co-purified with the T-DNA insert. To determine which fragments in Fig. 4a are derived from the binary vector, we hybridized the blots with the ampicillin-resistance gene from pTV4AMVBTS (*Ap* probe). All of the fragments except the 2.0 and 3.4 kb fragments were detected, indicating that they are derived from the binary vector. Many of the binary vector fragments, however, are not the expected size of 2.4 kb. They could be partial-digestion products, fragments derived from integration of inverted or tandem copies of T-DNA, or other rearrangements commonly associated with *Agrobacterium*-mediated gene transfer (Peerbolte et al. 1986; Gheysen et al. 1987; Jorgensen et al. 1987).

Nine of the transformed lines (those identified with asterisks in Figs 3b, 4) have an identical pattern of restriction fragments. This result was unexpected because some of these fragments are large enough that they must span at least one of the T-DNA borders, and thus should be unique for each transformant (because restriction sites in the adjacent poplar DNA should be different for each individual). Because these lines were derived from the same cocultivation treatment, we concluded that they are isogenic (i.e. derived from a single transformed cell). Except for the nine isogenic lines, the restriction fragment patterns in Fig. 4a are unique for each transformant, suggesting that they arose from independently transformed cells. In summary, we recovered 17 transgenic callus lines from the best cocultivation treatment, but these lines include only eight independent transformants (Table 1). We did not count *Bt*129 as an independently transformed line because it was not tested using Southern analysis, and because it was derived from the same cocultivation treatment as the nine isogenic lines.

Discussion

We developed a procedure for transforming hybrid poplar suspension cultures using *Agrobacterium tumefaciens*. The greatest number of transformants was recovered when filtered suspension cultures were inoculated with *Agrobacterium* to a final density of 10^7 cfu's ml^{-1} and cocultivated for 48 h. At the end of the cocultivation treatment, the cells were plated to cellulose acetate filters on solidified medium containing cefotaxime. In the first experiment, the suspension cultures were plated immediately after cocultivation and the filters were transferred to medium containing hygromycin 5 days later. In the second experiment, we maintained the cells in suspension culture for 15 or 35 days, then plated them directly to medium containing antibiotics for selection (kanamycin or G418). The latter approach is not recommended because it may lead to the recovery of multiple copies of the same transformant. For example, in the second experiment we recovered nine identical transformants when the cells were plated 15 days after cocultivation. Useful plating densities ranged from 14 to 180 colonies mm^{-2} depending on the antibiotic type and concentration used to identify transformants.

Callus growth following cocultivation was strongly affected by the constituents of the plating medium. Because the colonies in the first transformation experiments grew poorly following plating, we hypothesized that cefotaxime was inhibiting callus growth. We tested the effect of cefotaxime and found that the inhibitory effect of cefotaxime was roughly linear for concentrations up to 500 mg l^{-1}. These results were unanticipated because concentrations as high as 500 mg l^{-1} were reported to have little effect on plant tissues (Pollock et al. 1983; Ellis et al. 1989; Tsang et al. 1989). When we reduced the concentration of cefotaxime from 500 mg l^{-1} to 250 mg l^{-1}, callus growth was acceptable and growth of residual *Agrobacterium* was not a problem. If BA was included in the plating medium, callus growth was also reduced substantially. For this reason, we used 4.5 μM 2,4-D and no BA in the plating medium. BA also showed toxic effects when it was used to induce shoot formation from callus. Although BA is widely used in tissue culture of *Populus* (Park & Son 1988; Russell & McCown 1988; Sellmer & McCown 1989; De Block 1990), toxicity has been reported (Coleman & Ernst 1989).

Our results demonstrate that both the NPT-II and HPT genes can be used as selectable markers in poplar, although they may not be equally effective. Differences in growth and vigor between the transformed and untrans-

formed colonies on hygromycin were striking in the first experiment, but when kanamycin and G418 were used in the second experiment, differences were difficult to detect. Problems using the NPT-II gene as a selectable marker in *Populus* have been noted (Sellmer & McCown 1989; McCown et al. 1991), and the HPT gene is superior to NPT-II in a number of monocot species that have a high tolerance to kanamycin (Dekeyser et al. 1989). The relative efficiency of these selectable markers for poplar transformation should be evaluated. If the NPT-II gene is used as a selectable marker, G418 may be more effective than kanamycin because of its greater toxicity to NC-5339 callus (our observations) and other plants (Sheikholeslam & Weeks 1987; Tsang et al. 1989).

In the first experiment we regenerated transformed plants by culturing callus on medium containing thidiazuron. In a second experiment, however, we could not regenerate plants from either transformed callus, or untransformed controls. Failure to regenerate shoots in the second experiment was not caused by gene insertion because controls that were not cocultivated with *Agrobacterium* were similarly unresponsive. Because we were unable to regenerate shoots from any of the callus lines in the *Bt* transformation experiment, it appears that some genetic or epigenetic change had occurred in the suspension cultures prior to cocultivation.

Although the presence of residual *Agrobacterium* could produce misleading results from PCR and Southern analyses, we concluded that NC-5339 was stably transformed for a number of reasons. First, in the *Bt* experiment we checked for the presence of residual bacteria using PCR. Using the *Bt* gene primers, no positive PCR signals were obtained from the control treatments (callus lines cocultivated with the novirulent strain NT1 containing the binary vector). This result indicates that residual bacteria (or at least the binary vector) had been eliminated during repeated subculturing on medium containing cefotaxime. Second, DNA isolated from the putatively transformed plants and callus lines had restriction fragment paterns that were different from the binary vector and *Agrobacterium* DNA controls (see Figs 3 and 4a for example). In the *Bt* experiment, these unique fragments may be partial-digestion products resulting from methylation of CG dinucleotides (characteristic of plant DNA), transformant-specific T-DNA border fragments, or DNA rearrangements. In the *Ac* experiment, the unique fragments can be explained by *Ac* excision *in planta*.

We initially hypothesized that suspension cultures might have several advantages for transforming poplar, including high transformation frequencies, the ability to screen large numbers of potentially transformed cells, and more effective inhibition of residual bacterial contamination. Using tobacco suspension cultures, An (1985) estimated transformation frequencies of 10 to 50% on a colony basis, with corresponding frequencies of 2 to 10% for individual cells. We estimated transformation frequencies (without selection) of 0.2% and <0.35% in two different experiments. Although these frequencies do not approach those reported for tobacco, optimization of the procedure may improve results. On the other hand, we were able to screen large numbers of potentially transformed cells, and control of residual *Agrobacterium* was not a problem as it has been in systems using larger explants (Sellmer & McCown 1989). Transformation using suspension cultures has a number of disadvantages, however. Using our procedure, production of transgenic plants involves a two-step approach; first, transformed colonies are selected, and second, plants are regenerated from the transformed callus. Although we attempted to simplify the procedure by plating the suspension cultures directly to shoot induction medium, this approach was unsuccessful. Second, antibiotics tend to inhibit callus growth less effectively than they inhibit organogenesis (Ellis et al. 1989), potentially making selection more difficult. Finally, although our transgenic plants appeared normal, the introduction of somaclonal variation may be more likely using suspension cultures, and regeneration of plants from callus is not certain.

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