High-frequency transformation of cottonwoods (genus Populus) by *Agrobacterium rhizogenes*

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Abstract: Many species of Populus, particularly cottonwoods of sections Aigeiros and Tacamahaca, remain recalcitrant to genetic transformation. We demonstrate that transgenic trees can be readily produced in several poplar genotypes using wild-type *Agrobacterium rhizogenes.* Hairy roots were produced in a variety of clones that included *Populus trichocarpa* Torn & A. Gray, *Populus deltoides* Bartr. ex Marsh., P. *trichocarpa x P. deltoides* hybrids, and Populus *alba* L. x Populus *grandidentata* Michx., some of which were otherwise recalcitrant to regeneration or transformation. The frequency of hairy roots ranged from 17 to 92%; nearly all hairy roots could be regenerated into transgenic plants. Among 18 transgenic lines that were confirmed by Southern analysis and grown into small trees, 5 showed severe dwarfism and other phenotypic abnormalities, while the majority grew normally. *Agrobacterium rhizogenes* appears to be an effective means for transformation of diverse poplar genotypes and may be useful in genotypes where disarmed strains of *Agrobacterium* or regeneration protocols are inadequate to produce transgenic plants.

Resume : Plusieurs especes du genre Populus, et plus particulierement celles des sections Aigeiros et Tacamahaca, demeurent refractaires a la transformation genetique. Les auteurs demontrent que des arbres transgeniques peuvent titre obtenus facilement pour plusieurs genotypes de peuplier a l'aide dune souche sauvage de *Agrobacterium rhizogenes.* Des racines avec poils absorbents ont ete obtenues pour un nombre important de clones representatifs des especes *Populus trichocarpa* Torr. & A. Gray et *Populus deltoides* Bartr. ex Marsh., ainsi que des hybrides de P. *trichocarpa x P. deltoides* et de Populus *alba* L. x *Populus grandidentata* Michx., dont certains etaient refractaires a la regeneration ou a la transformation. La frequence a laquelle des racines avec pods absorbents furent obtenues variait de 17 a 92% et presque toutes les lignees de racines avec pods absorbents pouvaient titre regenerees en plantes transgeniques. Parmi les 18 lignees transgeniques qui furent confirmees par analyse de type Southern et qui se sont developpees jusqu'au stade de pent arbre, 5 ont affiche un nanisme severe ainsi que d'autres anormalites phenotypiques, alors que la majorite des lignees se sent developpees normalement. L'utilisation de *A. rhizogenes* apparait done titre un moyen efficace pour transformer divers genotypes de peuplier. Cette strategie pourrait titre utile pour la production de plantes transgeniques dens le cas des genotypes pour lesquels la transformation par des souches desarmees de *Agrobacterium* on les protocoles de regeneration s'averent inadequats. [Traduit par la Redaction]

Introduction

Poplar species are important for biomass, pulp and paper, and solid wood products, and commercial plantation area is growing rapidly in the United States. Genetic engineering can complement conventional breeding efforts by providing means for insertion of genes that confer desired traits not readily available in sexually accessible gene pools. In contrast with sexual breeding, it allows new genes to be added while the desired genotypes of clones are preserved and can therefore reduce the time required to produce an elite line. For genetic engineering to be commercially viable, transformation systems are needed that can efficiently produce transgenic trees from diverse genotypes.

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Poplars have been transformed in many laboratories. However, the majority of work has been confined to a few model hybrids of aspen (reviewed in Jouanin et al. 1993). Cottonwoods of sections Aigeiros and Tacamahaca arc usually recalcitrant to regeneration and transformation. There has been only one published report of transgenic cottonwoods (Populus *trichocarpa* Torr. & A. Gray *x Populus deltoides* Bartr. ex Marsh.) established in soil (De Block 1990). One possible explanation for its recalcitrance to transformation is the lack of an efficient regeneration system (De Block 1990), which often requires customizing regeneration conditions to specific genotypes (Hen et al. 1995) to promote cell competence for regeneration (Coleman and Ernst 1990). An alternative to genotype- specific manipulation may be to take advantage of the morphogenetic effects of genes transferred by *Agrobacterium rhizogenes.*

The soil bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* are the two species widely used to transform higher plants, including poplars. Both systems rely on a similar transformation mechanism. However, an important difference between the two species is that transgenic plants can be regenerated from roots induced by wild-type *A. rhizogenes,* while plant cells transformed with *A. tumefacierrs* carrying wild-type Ti (tumor-inducing) plasmids cannot be regenerated into plants. Plants regenerated from Ri (root-inducing)-transformed roots,

however, display the "hairy-root syndrome" (described in Tepfer 1984) caused by the T-DNA (transferred DNA)-borne genes (Schmulling et al. 1988; Gaudin et al. 1994). Transformation with a disarmed *A. tumefaciens* strain produces transgenic plants without such abnormal phenotypes; however, with disarmed *A. tumefaciens* species a precise regeneration selection system must be developed for each plant species or genotype. Although efficiency varies widely, *A. rhizogenes* has been used to produce transgenic trees in *Eucalyptus* (Macrae and van Staden 1993), *Larix* (Huang et al. 1991; Shin et al. 1994), *Malus* (Lambert and Tepfer 1992), Populus (Brasileiro et al. 1991; Charest et al. 1992; Pythoud et al. 1987), and *Robinia* (Han et al. 1993). Pythoud et al. (1987) reported genetic transformation of hybrid cottonwood *(P. trichocarpa x P. deltoides)* clone H11 using *A. rhizogenes*. They were able to regenerate shoots from callus tissues derived from the hairy roots, but did not propagate the shoots in soil or report on shoot morphology.

The abnormal growth often observed in plants transformed with *A. rhizogenes* may be used to advantage in some instances. Careful phenotypic testing of transgenic trees may identify transformants with increased growth rates and larger root masses (Han et al. 1993; Tepfer 1984). The adaptive significance of extensive root development may include improved anchorage, increased interactions with soil microorganisms, and stronger drought tolerance. Apple trees infected with *A. rhizogenes* withstood drought better than uninfected trees (Moore et al. 1979). The greater production of root exudates and water uptake expected from large root systems may increase soil remediation by direct uptake and plant and microbial degradation.

In the present study, we describe an efficient transformation system for a recalcitrant but economically important kind of hybrid cottonwood *(P. trichocarpa x P. deltoides)*. The Ri-transformed poplar trees were regenerated and established in soil, stable incorporation of T-DNA genes into the poplar genome is demonstrated by Southern blot analysis, and phenotypic effects of the expression of Ri T-DNA genes in the transgenic trees are documented.

Materials and methods

Bacterial strains

Four bacterial strains were used. *Agrobacterium rhizogenes* RI000 has the chromosomal background of *A. tumefaciens* strain C58 into which the Ri plasmid of pRiA4b has been conjugated (White et al. 1985). The strain 81240 is disarmed by removal of the core TL- and TR-DNA (White et al. 1985). Strain 81600 is similar to 81000, but carries the cosmid pTVK291 (Jin et al. 1987), which contains the virulence region from the "supervirulent" plasmid, pTiBo542 (Hood et al. 1984). 81601 is the same as 81600 except that the neomycin phosphotransferase gene *(nptll)* from the bacterial transposon Tn5 is inserted into the *HindIII fragment* 21 of the Ri plasmid (Sinkar et al. 1988x). The bacteria were grown overnight at 28°C on Luria-Bertani medium (LB; contains 10 g/L Bacto-trypton, 5 g/L Bacto-yeast extract, and 10 g/L NaCI, adjusted to pH 7.0). The bacteria were then collected by centrifugation and resuspended in LB medium supplemented with 50 mM acetosyringone and 0.1 M galactose.

Tissue culture and transformation

The protocols described for transformation (Han et al. 1993, 1994) and for tissue culture (Han et al. 1995) were used in this study, with minor modifications. *Agrobacterium rhizogenes* strains used as transformation agents are listed in Table 1. Greenwood stems (about 5 mm in diameter) were sterilized in 30% Clorox solution for 15 min followed by five washes with sterile distilled water. Intemodal stem discs (5 mm thick) were cut and soaked for several minutes in an overnight culture of *A. rhizogenes (Aboo = 1.0)* and placed on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) without growth regulators (MSO). After 3 days of co-cultivation, the explants were washed in sterile distilled water three times and transferred to either MSO or MS supplemented with 1 pM indolebutyric acid (IBA) containing 300 mg/L Timentin® (SmithKline Beecham Pharmaceuticals, Philadelphia, Pa.) to kill residual bacteria. The tissue was maintained on the same medium for at least 3 weekly subculture periods for hairy root induction. For shoot regeneration, hairy roots from the clone 53-246 induced by the strain 81601 were cultured on shoot induction medium (MS + 5 ItM 6-benzylaminopurine + 10 1tM a-naphthaleneacetic acid) with 100 mg/L kanamycin. Shoots regenerated from stem internodes of clone 53-246 that were not treated with *Agrobacterium* were used as negative controls throughout the experiments.

Growth of transgenic poplars

Shoots regenerated from hairy roots were multiplied and rooted in a Magenta G-7 box (Sigma Co.) containing 50 mL of shoot induction medium with kanamycin. Four ramets per independent transgenic clone were potted (25 cm diameter pot) as in Han et al. (1990) and grown in a greenhouse. Measurements were taken after 5 months of growth; trees were then pruned.

Southern blot analysis

Total plant DNA was isolated from young leaves harvested from potted plants, as in Bradshaw and Stettler (1993). Isolated DNA (3 pg/lane) was digested with *HindIIl and* transferred to a Zeta-probe blotting membrane by DNA alkaline blotting as specified by the manufacturer (Bio-Rad). The blots were hybridized in roller bottles (Robins Scientific) with approximately 5 x 106 cpm/mL (1 cpm (count per minute) = 0.0167 Bc) "P-labeled probe in 0.5 M Na2HP04, pH 7.2, 7% SDS, 10 mM EDTA, and 0.5% nonfat dry milk at 65°C overnight and washed according to Church and Gilbert (1984). Autoradiography was done at-70°C with a single intensifying screen. For probes we used either the *HindIII fragment* 16 (TL-DNA right junction fragment; Han et al. 1993), TL-DNA (pFW302; White et al. 1985), or TR-DNA (pFW41; Huffman et al. 1984).

Statistical analysis

All data were processed using analysis of variance (ANOVA) from the GLM procedure in SAS (SAS Institute Inc. 1990). The data for each clone were separated by Duncan's multiple range test where ANOVA indicated statistical significance.

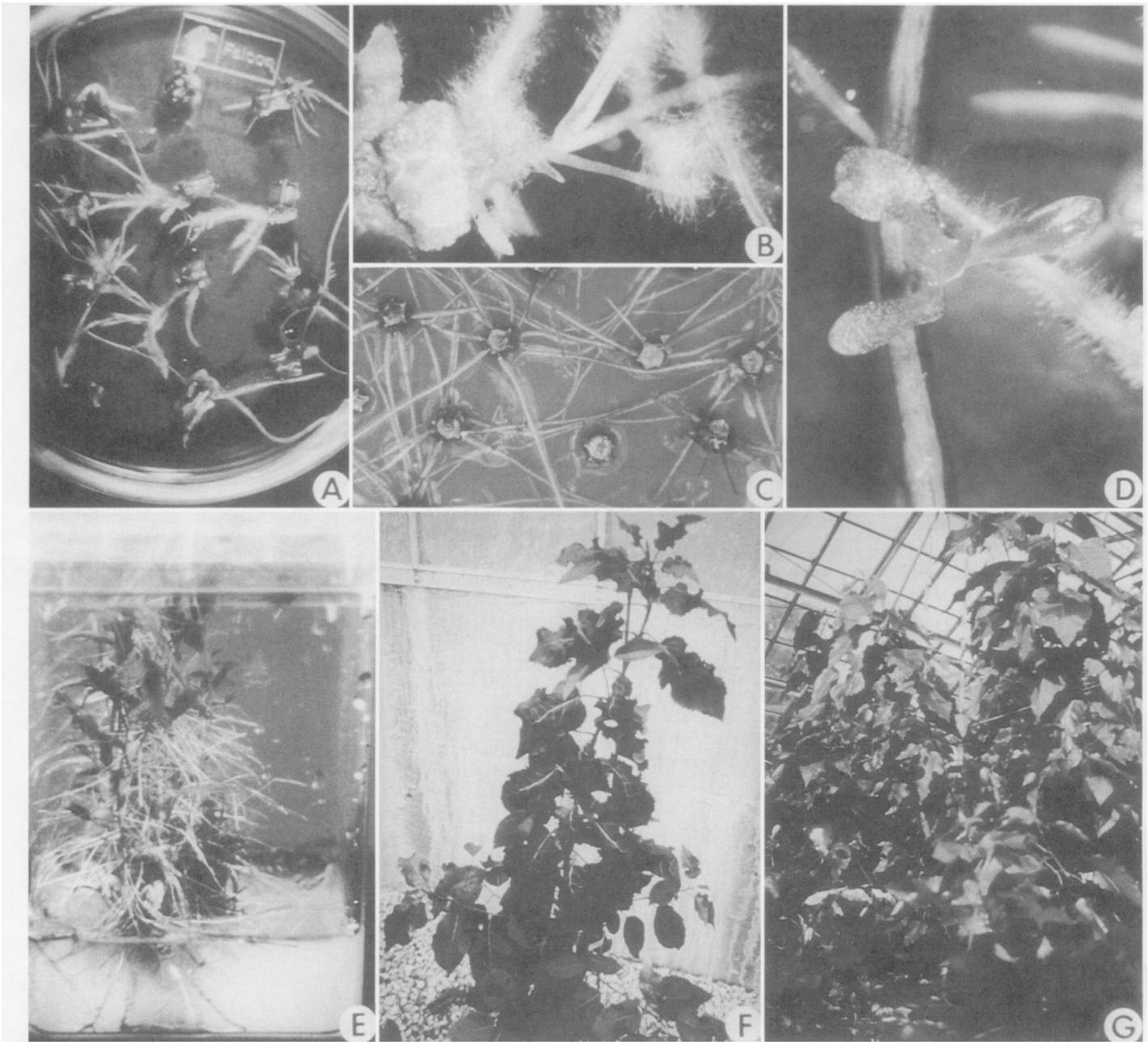
Results

Transformation

Hairy roots started to form on the cut surface of stem discs 7 days after inoculation with A. *rhizogenes.* These roots differed from normal roots in the following ways: (I) roots were initiated in the presence of little or no auxin in the culture medium; (2) hairy roots arose on the cut surface from cells that were transformed with A. *rhizogenes,* while normal roots were induced on the periphery of the stem segments in response to exogenously supplied auxin that diffused into the stem tissue from the medium (Figs. l A, 113, and IC); and (3) hairy roots developed a large number of root hairs and lateral branches (Figs. 1 A and 1 B).

Hairy root formation frequency varied from 17 to 92%, depending on plant genotype (Table 1). Infection with the wildtype strain 81000 transformed the hybrid cottonwood clone

Fig. 1. (A) Hairy root formation on stem segments after 1-3 weeks of inoculation. (B) Hairy root showing a unique morphology such as branching and abundant root hairs. (C) Normal adventitious roots induced by auxin treatment, showing no significant branching or root hairs. (D) Shoot regeneration from a hairy root. (E) Transgenic shoot cultures have tendency to produce aerial root mass. (F) Transgenic tree with altered phenotype such as dark green, wrinkled leaves. (G) Transgenic trees with normal phenotype.



24-305 (17%), but the infection with the supervirulent strain 81600 substantially increased transformation frequency (92%). The disarmed strain 81240 produced no hairy roots after infection. Populus *deltoides* clone ILL129, which did not respond to ordinary tissue culture manipulations for regeneration, produced hairy roots from 19% of explants. Spontaneous shoot regeneration (7%) was also observed with this clone when inoculated with 81601.

The hairy root tissues (longer than 2 cm) induced by 81601 from the clone 53-246 were transferred to shoot induction medium. Shoot regeneration was observed on roots 20-35 days later (Fig. 1 D). Nineteen of 75 hairy roots (25%) produced transgenic shoots in the presence of 100 mg/L kanamycin. All of the resulting shoots were successfully rooted in either MSO or MS medium supplemented with 1 pM IBA in the presence of 100 mg/L kanamycin.

Phenotypic consequences of transformation

Hairy root regenerants easily produced aerial roots in tissue culture (Fig. l E), and their roots showed plagiotropism in soil. They tended to form densely packed, horizontally radiating roots near the surface of the soil. About 10% of all transgenic

Table 1. Hairy root formation frequency.

Bacterial strains*	Plant genotype†	No. of explants	Explants producing hairy roots (%)‡
R1000	TD: 24-305	60	17
R1240	TD: 24-305	60	0
R1600	TD: 24-305	60	92
R1601	T: 93-968	41	22
R1601	D: ILL129	42	19
R1601	TD: 53-242	39	23
R1601	TD: 53-246	46	44
R1601	TD: H11	33	85
R1601	AG: NC-5339	48	69

*R1000, *A. tumefaciens* strain C58 + pRiA4b; R1240, disarmed R1000; R1600, R1000 + pTVK291 (carries the virulence region of a supervirulent plasmid pTiBo542); R1601, R1600 + *nptII* gene.

†T, *P. trichocarpa*; D, *P. deltoides*; TD, *P. trichocarpa* × *P. deltoides*; AG, *Populus alba* L. × *P. grandidentata* Michx. Clones 53-246, 93-968, and ILL129 were obtained from Dr. Toby Bradshaw at University of Washington, Seattle, Wash., U.S.A.; H11 and NC-5339, from Brent McCown at University of Wisconsin, Madison, Wis., U.S.A.; and 24-305, from Dr. Brian Stanton at the James River Corporation, Camas, Wash.

‡Results from single experiment.

trees had dark green, wrinkled, and thick leaves (Fig. 1F). The wrinkled leaf morphology appeared and disappeared several times during tree development. Six of 18 hairy root regenerants had stunted growth. These dwarfed trees had short internodes and abundant lateral branches. Some of the lateral branches were vinelike, tended to grow horizontally, and grew faster than the apical leader. Applications of 2.5 mM GA3 (gibberellic acid; Sigma Co.) to the apical buds of these trees resulted in increased shoot growth. Two-thirds of the hairy root regenerants showed apical dominance and were morphologically indistinguishable from the normal plant (Fig. 1G). One regenerant (line 6D9, carrying only TR-DNA) was significantly taller than normal. All transgenics had fewer branches than did normal plants with the exception of one, whose number was not significantly different from normal (Table 2). In the summer of 1995 we planted these transgenic trees in the field for the study of transgene expression over time.

Southern blot analyses

Southern blot analysis using the right border sequences of TLDNA as a probe showed that all four transgenic trees analyzed had multiple insertions of TL-DNA into the poplar genome (Fig. 2). Of 15 transgenic lines analyzed by Southern blot analyses using either TL- or TR-DNA sequences as probes, 13 lines had both TL- and TR-DNA sequences, while two (clones 6D9 and 820) had only TR-DNA sequences. These two clones (6D9 and 820) showed the greatest height growth. Eight transgenic lines carrying both TL- and TR-DNA sequences were used in growth measurements. Only 2 of the 8 lines, however, showed stunted growth.

Discussion

The phenotype of hairy root transgenic plants ranges from normal (Huang et al. 1991) through a variety of altered phenotypes, including larger root masses (Hart et al. 1993; Tepfer 1984), greater growth rates (Strobel and Nachmias 1985; Strobel et al. 1988), wrinkled leaves (Christy and Sinclair 1992), and increased drought tolerance (Moore et al. 1979). Two-thirds of our transgenic cottonwoods showed no obvious hairy root phenotypes when established in soil, while one-third had severely stunted shoot growth.

Dwarfism was also observed among transgenic tobacco plants overexpressing the rolA gene (Dehio et al. 1993) and potato plants overexpressing the rolC gene (Schmulling et al. 1993). Leaves of those plants contained 20-60% less GAS than control plants. The GA3 application reduced the severity of phenotypic alteration induced by the rolA transgene but not by the rolC transgene (Schmulling et al. 1993). Promoter activities of both rolB and rolC differed from that of rolA in transgenic tobacco (Schmulling et al. 1988). In all trees tested, the dwarfism of our cottonwood clones appeared to be reversed by GA3 application. This suggests that initially they had low gibberellic acid concentrations because of the overexpression of the rolA gene. The lack of dwarfism among most of our transformants, even when they contained both TL- and TRDNA sequences, may have resulted from differential transfer or expression of individual rol genes among independent transformants.

We observed dark green, wrinkled leaves in about 10% of the transgenic poplars. This phenotype varied in expression during development, with no obvious environmental cue, when the trees were maintained in a greenhouse. The wrinkled leaves tended to appear in the young leaves on the leader but not on the branches. Expression of the rolA transgene was responsible for dark green, wrinkled leaves in transgenic potato (Schmulling et al. 1993).

Transgenic trees with normal phenotypes suggest that the rol transgenes may have been silenced in those trees after the hairy root formation-regeneration process. Sinkar et al. (1988b) observed frequent reversion of hairy root phenotypes in tobacco. Their Southern and Northern blot analyses suggested that the revenant phenotypes were due to the transcriptional inactivation of T-DNA genes. Gene silencing in transgenic plants is widely known (Finnegan and McElroy 1994). Brandle et al. (1995) reported that co-suppression of a transgene was triggered by the common agronomic practice of seedling transplantation in field-grown transgenic tobacco. Several different genetic mechanisms appear to be involved (Matzke and Matzke 1995), often associated with multiple copies of transgenes (Flavell 1994) and DNA methylation.

We report high transformation efficiency for hybrid cottonwood, which has shown recalcitrance in our transformation experiments with A. *tumefaciens*. The main obstacle in transformation appears to be targeting and isolating regenerable cells. Actively dividing cells are more susceptible to *Agrobacterium* infection (Akama et al. 1992), and competence for transformation is associated with the timing of cell division at the wound site (Braun and Mandle 1948). Therefore, transformation frequency may be improved by increasing the number of regenerable cells that are in active cell division. *Agrobacterium rhizogenes* may "naturally" condition the plant cells for transformation-regeneration by providing plant hormones to promote cell division in cells surrounding the wound site. Conditioning may also result from morphogenic potential in hairy root tissues. For example, inoculation of *P. deltoides* clone ILL129 with *A. rhizogenes* strain 81601 resulted in high root

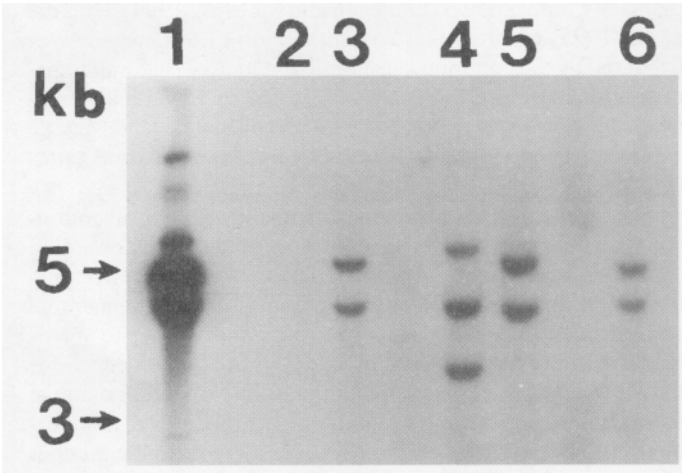
Table 2. Growth of *Agrobacterium rhizogenes* transgenic poplars after 5 months in the greenhouse.

Transgenic clones*	Height (cm)	Stem diam. (mm)	Leaf length (mm)	Leaf width (mm)	No. of leaves	Branch length (mm)	Branch diam. (mm)	No. of branches
6Z7	9*d*	3*e*	49*d*	25*c*	7*f*	0*b*	0*b*	0*c*
6RZ1	10*d*	4*e*	55*d*	28*c*	8*ef*	0*b*	0*b*	0*c*
6D1	11*d*	3*e*	52*d*	23*c*	7*f*	0*b*	0*b*	0*c*
R6-30	13*d*	4*e*	56*d*	35*c*	6*f*	0*b*	0*b*	0*c*
6R24	25*d*	4*e*	73*d*	51*c*	12*e*	0*b*	0*b*	0*c*
6D13	112*c*	9*d*	222*c*	142*ab*	18*d*	230*a*	3.2*a*	2.5*bc*
6R9	131*bc*	11*abcd*	273*ab*	163*ab*	30*ab*	247*a*	2.9*a*	15.7*a*
6D14	131*bc*	10*bcd*	241*abc*	132*b*	25*bc*	195*a*	2.7*a*	3.0*bc*
6D12	134*bc*	11*abcd*	226*bc*	135*b*	27*bc*	188*a*	2.4*a*	3.8*bc*
6R26	137*bc*	10*bcd*	248*abc*	138*ab*	27*bc*	248*a*	3.2*a*	6.0*b*
R6-31	143*b*	11*abcd*	258*abc*	161*ab*	25*bc*	228*a*	3.0*a*	3.5*bc*
6D5	145*b*	10*bcd*	239*abc*	139*ab*	27*bc*	125*ab*	2.1*a*	5.3*b*
R1991	146*b*	11*abcd*	261*abc*	159*ab*	30*ab*	243*a*	2.5*a*	5.5*b*
6R18	147*b*	11*abcd*	239*abc*	136*ab*	28*bc*	168*a*	2.4*a*	3.3*bc*
6D16	152*ab*	12*ab*	261*abc*	160*ab*	24*c*	158*a*	2.2*a*	2.5*bc*
6R25	153*ab*	11*abcd*	265*abc*	155*ab*	29*bc*	223*a*	3.1*a*	4.0*bc*
6R20	154*ab*	12*ab*	277*a*	168*a*	27*bc*	283*a*	3.0*a*	5.0*b*
6D9	173*a*	13*a*	270*abc*	165*ab*	30*ab*	188*a*	2.4*a*	3.5*bc*
Control	139*b*	12*ab*	260*abc*	155*ab*	34*a*	277*a*	3.3*a*	13.7*a*

Note: Means with different letters are significantly different at the 0.01 probability level.

*All transgenic lines were derived from *P. trichocarpa* × *P. deltoides* clone 53-246, and control was nontransgenic shoot regenerated from stem internode of the same clone.

Fig. 2. Southern blot analysis of transgcnic poplars. total poplar DNA was digested with HindIII, size-fractionated (in kilobases (kb)) on agarose gel by electrophoresis, and transferred to Zeta-probe membrane (Bio-Rad). The blots were hybridized with szp-labeled HindIII border fragment (H-16) of TL-DNA of A. *rhizogenes* 81601 (White et al. 1985). Autoradiograms of blots are shown: lane 1, total DNA (10 ng) of A. *rhizogenes* 81601; lane 2, normal poplar (3 Itg); lanes 3-6, transformants 6D1, 6D8, 6D11, and 6824 (3 pg each), respectively. Each hybridizing band indicates the incorporation of a copy of TL-DNA.



and shoot regeneration. Without inoculation this particular clone was incapable of regeneration in our tissue culture studies (Han et al. 1995). *Agrobacterium rhizogenes* has been the only vector able to give rise to transformed plants in some recalcitrant tree species (Han et al. 1993; Shin et al. 1994).

There are at least three possible means of taking advantage *of A. rhizogenes* for transformation of recalcitrant species without encountering phenotypic abnormalities. One is to place the *rol* genes under an inducible gene expression system and have them expressed only during the transformation process. A number of systems for inducible gene expression have now been described in plants (Roder et al. 1994; Men et al. 1996). Another option is to use co-transformation, where two separate T-DNAs contained within a single bacterium are transferred into plant genomes. Binary vectors derived from *A. tumefaciens* can be efficiently transferred into plant cells when they are placed in a virulent strain *of A. rhizogenes. A* high frequency *of* co-transfer *of* wild-type and binary vector T-DNAs is generally obtained: 43-60% in alfalfa (Simpson et al. 1986; Sukhapinda et al. 1987), 39-85 % in *Brassica* (De Block and Debrouwer 1991), 86 % in *Stylosanthes* (Manners and Way 1989), and 70-80% in tobacco (Hamill et al. 1987; Hamamoto et al. 1990). As demonstrated in the present study, these hairy roots can often be induced to regenerate into transgenic plants. Under some conditions, the T-DNAs from the binary and wild-type plasmids integrate into unlinked sites and can be segregated during sexual reproduction. Moreover, in a population *of* co-cultivated cells, not all will be transformed with both the wild-type and binary T-DNA, potentiating their separation during regeneration. For example, when co-transformed tomato tissues were cultured on a medium supplemented with appropriate growth regulators and antibiotics, they produced callus instead *of* hairy roots, from which phenotypically normal transgenic plants could sometimes be obtained (Shahin et al. 1986). Finally, our results suggest that a useful proportion *of* transgenic poplar lines produced with *A. rhizogenes* transformation show no discernible phenotypic effect; whatever the mechanism, it may be possible to simply screen

a large number of transgenic lines for those that remain free of abnormalities.

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