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An *Agrobacterium tumefaciens* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*)

Abstract We describe a protocol for *Agrobacterium tumefaciens*-mediated transformation of hybrid cottonwoods (Populus sections Tacamahaca Spach. and Aigeiros Duby). The protocol has allowed routine transformation of several economically important cottonwood hybrids (Populus trichocarpa Torr. & Gray x *P. deltoides* Bartr. ex. Marsh. and *P. deltoides* x *P. nigra* L.) that were previously difficult to transform. The procedure was applied to 11 different hybrid cottonwood genotypes and one *P. deltoides* genotype using kanamycin as the selection agent. Additional experiments showed a very strong interaction between auxin preculture and the effectiveness of various cytokinins for induction of shoot organogenesis. The data also demonstrated the superiority of Agrobacterium strain EHA105 over C58 and LBA4404 for T-DNA transfer based on transient assays with a reporter gene.

Key words Poplar ~ Populus ~ Aspen ~ Cottonwood Transformation ~ Agrobacterium

Abbreviations AS: Acetosyringone ~ BA: 6-Benzyladenine ~ C*IM:* Callus induction media GUS: G-Glucuronidase ~ I*BA:* Indole-3-butyric acid *MES:* 2-[N-morpholinol]ethanesulfonic acid ~ MS: Murashige and Skoog (1962) ~ N*AA:* Naphthalene-acetic acid ~ NPTII: Neomycin phosphotransferase gene ~ RIM: Root induction media ~ SIM: Shoot induction media - TDZ: Thidiazuron ~ 2,4-D: 2,4-Dichloro-phenoxyacetic acid ~ 2iP: N~-(2-isopentenyl) adenine

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Introduction

Asexual transformation methods provide a means for introducing new traits that are difficult to obtain via traditional breeding and allow modification of valuable clones without the genetic recombination that occurs during sexual reproduction. Transformation requires that DNA be inserted into plant cells, incorporated into chromosomes, and expressed in cells that can be induced to regenerate into plants. Although the transfer of DNA into plant cells via Agrobacterium, biolistics, and other physical methods is now routine (reviews in Han et al. 1996; Jouanin et al. 1993; Kim et al. 1997), coupling transformation with the selection of transgenic cells and plant regeneration is still difficult and costly in many economically important species, thereby limiting the use of transgenic crops (Birch 1997).

Hybrid cottonwoods, aspens, and other poplar species (genus Populus) are highly valued by the pulp and paper industry for their fast growth and high quality fiber (Stealer et al. 1996). Although poplars have been transformed for research purposes far more than any other species of forest tree, many economically important genotypes and species remain difficult to transform (Han et al. 1996). Highly efficient transformation systems for poplars have been reported by several laboratories (e.g., Leple et al. 1992; Tsai et al. 1994; Tzifra et al. 1996, 1997; Fladung et al. 1997), however much of the work has been restricted to a few model hybrids and species of section Leuce (aspens and white poplars), many of which had been selected for ease of transformation. Transformation of cottonwood species (sections Tacamahaca Spach and Aigeiros Duby) has been uncommon by comparison and has usually involved one or a few genotypes (e.g., DeBlock

1990; Confalonieri et al. 1994, 1995; Huang et al. 1994; Wang et al. 1996; Heuchelin et al. 1997). Although biolistic and protoplast-based physical DNA transfer have been employed, *Agrobacterium* is the predominant and preferred gene transfer system for poplars (Kim et al. 1997).

We have conducted a number of studies over the last several years on the transformation of hybrid cottonwoods employed by forest industries in the northwestern United States and Canada (primarily *Populus trichocarpa* x *P. deltoides)* and have succeeded in generating over 400 lines of transgenic plants. We have studied a large number of factors involved in *Agrobacterium* transformation, including those affecting the efficiency of DNA delivery into the targeted cells and the regeneration of transgenic plants. Specific variables studied included *Agrobacterium* strain, explant source, genotype, physiological condition for regeneration, and phytohormone type and combination. Because of the large number of factors and the combinations thereof that can affect transformation, and the requirement for rigorous replication of results from *in vitro* culture studies for reliable inferences, only the transformation protocol itself and the results of a few replicated experiments are presented here . The experiments were chosen to provide general guidelines for the development of transformation systems in poplar.

Materials and methods

In vitro shoot cultures were established using young stems from poplars maintained in a growth room or greenhouse and following general conditions described previously (Han et al. 1995). Stem internodes (including petiole segments) and leaf blades (disks) from either in vitro- or potted, growth room-grown poplars were used for transformation. The temperature of the growth room was maintained at 25 "C, with continuous light from cool-white fluorescent lamps.

The binary vector pV-LEGT02 carries GUS, NPTII, and two glyphosate tolerance genes (GOX and CP4; Barry et al. 1992); NPTII and GUS are under the control of a cauliflower mosaic virus 35S promoter. This vector, in Agrobacterium strain ABI, was used throughout the protocol optimization process, except when testing Agrobacterium strains C58 (pMP90), EHA105, and LBA4404, where we used the binary vector pGUS-INT (C. Wang and G. Tuskan, Oak Ridge National Laboratory). All transformation experiments relied on kanamycin as the selective agent. A variety of NPTII-containing binary vectors were used for related transformation studies, including pAM154 containing APl-GUS and pAM194 containing API-DTA (Mandel and Yanofsky 1995); pART27/CO containing 35S-CONSTANS (Putterill et al. 1995); pBT-9 containing 35S-cry3a (unpublished data); pDWlsl containing 35S-LEAFY (Weigel and Nilsson 1995); and pSK231 containing 35S-AGL20 (M. Yanofsky, personal communication). The basic transformation protocol is shown below.

Tissue culture media

The following culture media were used:
LB: 16 g/I Bacto-tryptone, 8 g/1 Bacto-yeast extract and 5 g/1 NaCI, adjusted to pH 7.0;

1M: MS salts and vitamins+10 μM AS+1(l mM galactose+1.28 mM MES; pH 5.0;
CIM: MS+0.5 ;.μM BA+0.5 μM zeatin+5 wM NAA+S μM 2,4-D+0.3% Phytagar (Gibco BRL)+0.1% Phytagel (Sigma) + 1.28 mM MES; pH 5.8;
WS: 1/2 MS salts and vitamins+250 mg/1 ascorbic acid+l μM NAA+I μM BA+l μM 2iP+500 mg/1 cefotaxime; pH 5.8;
SIM: MS+10 μM BA+10 μM zeatin+l μM NAA+0.3% Phytagar +0.1% Phytagel+1.28 mM MES; pH 5.8.

Transformation steps

1) For growth room-grown materials, greenwood stems or young leaves are sterilized in 20% Clorox solution for 20 min followed by five washes with sterilized distilled water. For tissue culture materials, stem internodes and leaves are harvested every 3-4 weeks.
2) Agrobacterium cells carrying a binary vector are grown overnight at 25 "C in liquid Luria Bertani (LB) medium supplemented with appropriate antibiotics.
3) The cells are collected by centrifugation at 2,560 rpm (1,292 RCF) for 30 min and resuspended in induction medium (IM).
4) The cells are centrifuged as above and resuspended in the IM to the density of OD_{600}=0.3-0.4, induced with shaking (50-100 rpm) for 1 h at room temperature.
5) Explants, with or without pre-culture for 2-7 days on CIM, are soaked for 10-20 min in the bacterial suspension under 0.6-bar vacuum and incubated on a shaker (50 rpm) for 1-2 h at room temperature.
6) The inoculated explants are co-cultivated on CIM at 19-25 °C in the dark for 2-3 days.
7) Explants are washed four times with double-distilled water and once with wash solution (WS).
8) Explants are cultured for 10-30 days in the dark on CIM supplemented with 5(H) mg/1 cefotaxime and 50 mg/I kanamycin.
9) Shoot regeneration is induced on SIM supplemented with 100 mg/I kanamycin for several weeks to months. Explants are subcultured every 2-4 weeks.
10) Regenerated shoots are further screened for kanamycin resistance by rooting on 1/2 MS medium supplemented with 0.5 μM IBA and 25 mg/1 kanamycin. The total length of time from cocultivation to a rooted transgenic plantlet varies widely among clones, ranging from about 4-8 months.

For experiments on factors affecting transformation, precultured explants were grown on CIM in the dark for 7 days immediately prior to infection with Agrobacterium, then inoculated and selected as described above. A large experiment on the effects of pre-culture on the production of transgenic calli consisted of five repeated trials with three triploid *P. tricho-carpa* x *P. deltoides clones* (24-305, 184-402, and 189-434) and 15-55 leaf and stem explants per clone. In another experiment, three different cytokinins (BA, 10 μM; TDZ, 0.1 μM; and zeatin, 5 μM) were tested for their interaction with pre-culture; these experiments were repeated twice using P. trichocarpa x *P. deltoides clones* 24-305 and 184-402, and the results pooled. Phytohormone concentrations had been chosen based on prior experiments (data not shown). Explant type (leaf discs versus stem segments and petioles) and explant source [young, growth room (< 6 months of age) versus *in vitro*-derived plants] were tested for their effects on transformation efficiency using the same two clones. Finally, we compared octopine (LBA4404), nopaline (C58), and agropine (EHA105) strains for their ability to transform poplar in a transient assay.

Our studies relied on rooting in kanamycin as an indicator of stable transformation. Previous studies using the polymerase chain reaction (PCR), Southern blots, and reporter gene expression have supported this practice. For example, based on GUS-

INT expression Han et al. (1997x) reported an escape rate below 3% for shoots that were selected on 100 mg/1 kanamycin and then rooted in 25 mg/1 kanamycin. Where GUS analyses were used for T-DNA transfer studies, assays were conducted 1 week after inoculation when transient expression is still strong (unpublished studies), following methods described by Jefferson et al. (1987). Data were subjected to analysis of variance (ANOVA) using the GLM procedure in SAS (SAS Institute 1989), and means were compared by Duncan's New Multiple-Range Test (Steele and Torrie 1980) where the GLM-ANOVA analysis showed significance.

Results and discussion

Explant pre-culture

Although we detected no statistically significant differences among pre-culture periods of different durations (0, 1, 3, 5, and 7 days) based on transient GUS expression (data not shown), a 7-day pre-culture significantly increased the number of green calli able to grow under kanamycin selection. Seven days of pre-culture enhanced the recovery of kanamycin-resistant calli several-fold, from a mean of 0.3 (t 0.2)% of explants giving resistant calli in the absence of pre-culture to a mean of 2.0 ($\pm$0.5)% after pre-culture. Because hostcell division is required for successful *Agrobacterium* transformation (Binns and Thomashow 1988), it is not surprising that pre-culture in a high auxin medium often enhances transformation rate (e.g., Mathis and Hinchee 1994; Sangwan et al. 1992). In addition, because there was no effect of pre-culture on transient GUS expression, it is likely that poplars benefit from pre-culture by increasing the competence of cells for DNA incorporation into chromosomes and/or dedifferentiation into callus rather than enhancing DNA transfer to cells. This result also shows that transient expression studies can give misleading results about the importance of transformation procedures needed for the recovery of transgenic tissues.

Because the production of transgenic shoots rather than of calli was our aim, we tested whether pre-culture

affected shoot regeneration in response to several growth regulators with cytokinin activity (BA, TDZ, and zeatin). Pre-culture interacted strongly with the growth regulators at the concentrations tested (Table 1). Shoot regeneration frequency and the number of shoots per explant were greatest for TDZ and zeatin when explants were not pre-cultured, conditions in which BA gave a very low rate of shoot regeneration (2% of explants produced shoots). However, after pre-culture, BA was superior to both TDZ and zeatin, producing shoots on 71% of explants compared to 13% and 20% for zeatin and TDZ, respectively. These results show that interactions among sequential growth regulator treatments must be carefully considered when designing regeneration experiments. The results are similar to those of Coleman and Ernst (1990), who found that an auxin pulse modified competence for shoot regeneration in *P. deltoides*. However, our results further show that competence to respond to hormonal signals is differentially affected, suggesting different modes of action for the growth regulators. This is not surprising given their distinct modes of physiological action, particularly the multiple physiological roles of TDZ (e.g., Huetteman and Preece 1993).

Explant source

We compared growth room-grown plants to *in vitro*-grown plants as explant sources for transformation. For the two cottonwood genotypes tested, the rates of callus and shoot production were significantly less with in vitro-derived leaf and stem explants than with growth room-derived explants. Production of kanamycin resistant calli from leaves increased from 2% ($\pm$ 1%) to 8% ($\pm$ 2%) of explants between *in* vitro and growth room sources, and that from stems increased from 7% ($\pm$ 3%) to 21 % (t 4%); shoot production increased from 0 to 2% (t 2%) and from 4% ($\pm$ 2%) to 10% ($\pm$ 3%), respectively. Stems (including petioles) were markedly superior to leaf

Table 1 Influence of pre-culture on the effectiveness of growth regulators for in vitro shoot regeneration from stem internodes of two triploid *P. trichocarpa* $\times$ *P. deltoides* clones (24–305 and 184–402)

Trait	Growth regulators[a]	Without pre-culture[b]	With pre-culture[c]
Percentage of explants producing at least one shoot	BA (10 μM)	2[a] [d]	71[a]
	TDZ (0.1 μM)	77[b]	20[b]
	Zeatin (5 μM)	98[b]	13[b]
Average number of shoots per explant	BA (10 μM)	0.07[a]	2.30[a]
	TDZ (0.1 μM)	2.51[b]	0.52[b]
	Zeatin (5 μM)	3.17[b]	0.12[b]

aTDZ, Thidiazuron; BA, benzyladenine
'Data were collected after 4 weeks of culture on SIM from two independent experiments using 30-40 explants (5 mm long) per treatment in each experiment. SIM composition was the same as that described in the transformation protocol, except with the cytokinin shown in the table replacing those in the standard protocol

`In pre-culture, stem internodes were incubated (MS+10 μM NAA+S p,M 2iP) for 7 days prior to placement on SIM, which was composed as described in the transformation protocol, but with the cytokinin shown in the table in place of that in the protocol)
`Different letters indicate means are significantly different at the 0.05 probability level

blades for regeneration of callus and shoots, a result we have observed frequently (unpublished data).

In vitro-derived materials are generally preferred over field-grown, greenhouse, or growth room materials as explants for cocultivation because they need no surface sterilization, are often more juvenile and thus have superior regeneration capacity, and are less lignified (Civinova and Sladky 1990). However, in vitro-grown explants have usually less hardiness and resistance to desiccation during transformation. The larger growth room-grown cottonwood leaves may have produced more kanamycin-resistant calli and shoots because their larger midveins allowed for more bacterial-host cell contacts; shoot regeneration is observed mainly in the vascular bundles of stems (Han et al. 1995). Incompletely lignified stem sections of greenhouse-grown hybrid cottonwoods have also been efficiently transformed with A. *rhizogenes* (Han et al. 1997b).

Agrobacterium strains

We studied the influence of three commonly used disarmed strains of *Agrobacterium* on rate of T-DNA transfer, measured via the number of GUS-expressing foci (i.e., blue spots) 1 week after transformation (Table 2). In the highly susceptible hybrid Leuce clone 717, strain did not have a significant effect; however, the rate of GUS expression was three- to tenfold higher than that observed in the cottonwoods. In both of the cottonwoods, EHA105 showed a statistically significant increase in the rate of T-DNA transfer; it was approximately tenfold greater in 24-305 and twofold greater in 184-402. C58 and LBA4404 were not significantly different from one another. Fluorometric GUS assays on a sample of these materials closely paralleled these results (data not shown).

Virulence of *Agrobacterium* strains varies widely among plant hosts (Bush and Pueppke 1991; Davis et al. 1991; Hobbs et al. 1989) and is particularly important for the transformation of recalcitrant species. Most

transgenic poplars have been produced using nopaline strains of *Agrobacterium;* transformation with an octopine strain, such as LBA4404, has often not been successful (Fillatti et al. 1987; Pythoud et al. 1987). Among the three strains (AchS, A281, and C58), C58 was the most virulent on *P. nigra* leaf discs (Confalon-ieri et al. 1994). However, our results suggest that EHA105 may be superior to C58 for some recalcitrant poplars.

Transformation protocol

The transformation protocol we developed for routine use includes some of the factors tested above and other factors known to be important in other species but that we have not yet shown to be important in cottonwoods. The method still needs to be tested on a wider variety of genotypes and optimized to enable more rapid production of transgenic shoots. This work is ongoing. Nonetheless, the protocol appears to be effective on a wide variety of genotypes that we have tested to date, averaging 2% of explants giving rise to a transgenic shoots and roots (Table 3). This includes one of two genotypes of *P. deltoides* studied and a number of *P. trichocarpa* x *P. deltoides* hybrids. All of the diploid hybrids tested, and two of the triploids (24-305 and 184-402), have been used in commercial plantations in the Pacific Northwest. The diploid hybrid clone OP367, a *P. deltoides* x *P. nigra* clone that was not successfully transformed in earlier experiments (Table 3), has now been successfully transformed at a rate of 9% (19 lines from 224 explants cocultivated) with construct pBT-9 in strain C58 under kanamycin selection. Most of the cottonwood clones shown in Table 3 have also been transformed to the point of rooting in kanamycin with other *Agrobacterium* strains and binary vectors in studies with other constructs. This includes the strains ASE, C58, and EHA105; the clones 17-50, 19-53, 24-305, 50-197, 18402, and 18934; and the plasmids pDW151, pAMi94, pAM154, pART27/CO, pSK231, and pBT-9 (unpublished data). Thus, the protocol is robust for different constructs and is repeatable over time and with different students and technicians assisting with in vitro culture. A slightly modified version has also been used successfully with glyphosate selection during callogenesis and early stages of shoot induction (1-2 months) using the CP4 selectable marker gene (unpublished data).

The present transformation system is based on indirect organogenesis, where shoot regeneration follows a period of callus induction and involves several weeks or months of unorganized growth prior to the appearance of shoots. It differs only modestly, primarily in some of the phytohormones employed and media amendments, from the protocol for transformation of the Leuce hybrid 717 presented by Leple et al. (1992). Our system was employed following a number of attempts at direct organogenesis, where shoots are immediately induced

Table 2 Effects of *Agrobacterium* strain on transformation as assayed by number of foci with distinct histochemical GUS expression on explants 1 week after cocultivation

| | Clone | | |
Strain	717[a]	24–305	184–402
C58	27.6[b ns]	0.3[b]	4.4[b]
EHA105	30.3[ns]	2.8[a]	10.7[a]
LBA4404	11.7[ns]	0.1[b]	3.3[b]

x717, *Populus tremula* x *P. alba*; 24-305 and *184-402, P. trichocarpa* x *P. deltoides*
'Histochemical GUS assay was conducted using data pooled from three independent experiments, 5 explants per experiment. Different letters indicate means are significantly different at the 0.05 probability level. ns, Not significant

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