AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF POPULUS DELTOIDES LEAF SECTIONS

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Abstract. Several factors, including Agrobacterium tumefaciens (At) exposure times and concentrations, were varied in efforts to increase transformation efficiency. Leaf sections of Populus deltoides clone C175, collected from shoot cultures, were inoculated with At strain LBA4404 carrying binary vector pBI121. Included in the vector were the selectable marker gene (NPTII) for kanamycin (K) resistance and the reporter gene (uidA) for beta-glucuronidase production. Transformants were identified by selection on medium containing 50 mgK/L and confirmed by histochemical staining for uidA expression. Exposure to At for 120 min proved more effective than shorter times, and elevated concentrations gave more transformants than lower ones. Long exposure times and high concentrations, however, tended to reduce shoot formation. Selection of putative transformants with 50 mgK/L proved workable, but this level clearly inhibited regeneration. The selection process was therefore modified to include culture on nonselective medium for 14 days before transfer to selective medium. This gave higher transformation frequencies than otherwise obtained, apparently a result of transformed calli enlarging and organizing sufficiently to develop on selective medium.

Keywords: Cottonwood, Poplar, Organogenesis, uidA Gene, Gene Transfer.

INTRODUCTION

Populus species and hybrids are among the fastest growing and most commercially important forest trees in the world. Eastern cottonwood (P. deltoides) (Pd) is especially noted for rapid growth and desirable pulping and papermaking properties. Significant genetic improvement has been obtained via classical selection and breeding, and the species is regenerated and planted vegetatively. More rapid and specific improvement, however, may be obtained by insertion of genes for traits not available in the species.

Genetic transformation has become almost routine with a variety of dicotyledonous plants, including a number of Populus species and hybrids. Indeed, the genus has proven to be a model for insertion of genes having commercial value (Chandler 1995). Much of this research, however, was performed with taxa other than Pd or its hybrids (e.g. Fillatti et al. 1987). Transformation has also been accomplished with hybrids between Pd and other species; e.g, P. trichocarpa (Parsons et al. 1986, De Block 1990, Wang et al. 1994) and P. niora (Charest et al. 1992, Devantier et al. 1993). Against this background, we sought to devise a transformation protocol for Pd, with the intent of extending it from clones noted for ease of manipulation in culture to elite clones of commercial value.

In our earlier work (Stephens and Dinus 1994), a gene for enhanced auxin synthesis (Klee et al. 1987) was inserted into a model Pd clone (C175). Transformation frequencies, however, were low, and transgenic plants were not recovered. Accordingly, research was continued with a benign marker gene, the

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uidA reporter gene, to improve protocol efficiency. The present report describes results from three experiments in which several factors hypothesized as important to transformation efficiency were evaluated. Included were: preincubation treatments (Fillatti et al. 1987, Tsai et al. 1994, Confalonieri et al. 1994), At exposure time, and At concentration (Confalonieri et al. 1994). Effectiveness of two antibiotics for clearing cultures of residual At contamination and of culture on nonselective medium for a short time before transfer to selective medium were also tested. Adjustment of these several factors resulted in increased transformation frequencies and recovery of transgenic calli, primordia, and shoots.

MATERIALS AND METHODS

Plant Materials. Three experiments were conducted to evaluate factors influencing genetic transformation and regeneration of transgenic plants from eastern cottonwood clone C175 (Dr. S.G. Ernst, University of Nebraska-Lincoln). This model clone, easily manipulated in culture, was maintained in continuous shoot cultures to supply leaf explants over the long term.

Transformation and Regeneration. A leaf section system developed by Uddin et al. (1990), and modified for transformation by Shorter (1991) was used for transformation and regeneration. Procedures used in research reported here have been described by Dinus (1992) and Dinus and Stephens (1994). Five leaf sections were used per petri plate or replication; numbers of plates varied among experiments and treatments as indicated below.

Transformation was done with At Strain LBA4404 containing the binary vector pBI121. Included in the vector were the selectable marker gene (NPTII) for kanamycin (K) resistance coupled to the NOS promoter and the uidA reporter gene linked to CaMV35S (Clonetech Laboratories Inc.). At was cultured in YM broth (Lin 1994) at 30°C for three days, sedimented by centrifugation at 2500 rpm for 5 min, resuspended in standard leaf section medium, and diluted to the concentrations used for transformation.

Antibiotics. Selection of putative transformants was done on medium containing 50 mgK/L. Earlier research with C175 (Shorter 1991) showed that K levels as low as 30 mg/L halt development of nontransformed C175 leaf sections. In the present research, 50 mg/L was used as a safety margin and as recommended by Clonetech Laboratories Inc. Lethal dose assays (Shorter 1991) showed that carbenicillin (CA) concentrations as high as 500 mg/L did not harm C175 leaf sections, and were reasonably effective at clearing cultures of residual At. This concentration was used in the present research until questions arose about efficacy of CA. In response to such questions, cefotaxime (CE) was also evaluated in a lethal dose assay (Dinus et al. 1995). At growth was stopped by 250 mg/L or more, without apparent detriment to C175 leaf sections. Utility in C175 transformation trials, however, was not evaluated until experiments reported herein. All antibiotics were obtained from Sigma Chemical Co., St. Louis, MO. Other changes made to the aforementioned protocols are described below in the context of individual experiments.

Identification and Confirmation of Transformation. Callus, primordia, and/or shoots surviving on selective media were counted as putative transformants. Assays for expression of the uidA gene were performed as per the histochemical methods of Jefferson et al. (1987). Small portions of putatively transformed

calli or leaves were used for assay. Nearly all putative transformants were free of At contamination. The few suspected of being contaminated were rinsed with 70 percent ethanol three times and sterile distilled water twice before assay. When color developed within a few minutes or between plant cells, candidates were not counted as transformed. In sum, plant materials counted as confirmed transformants included only those showing complete expression at the end of the lengthy culture periods noted above. Those showing transient or chimeric expression, or responses due to At contamination, were excluded.

Trial 1. The first experiment evaluated efficacy of the At exposure time (5 min) and concentration (10^9 cfu/mL) used in our earlier research (Shorter 1991). Protocols described above were followed with one exception. Several authors working with *Populus* hybrids and species have incubated explants for 24-48 hr before exposure to At in order to foster explant growth (Fillatti et al. 1987) or to cull unhealthy explants (Tsai et al. 1994). Accordingly, half of all leaf sections used in this trial, regardless of subtreatment, were incubated on standard leaf section medium for 24 hr in darkness prior to At exposure.

Subtreatments are described below. A control (-At-K) was included to verify that leaf sections developed normally (N = 30). To quantify effects of At and the transformation process on regeneration, leaf sections were exposed to At but not K (+At-K) (N = 110). This treatment also was intended to produce putatively transformed shoots for later selection on shoot growth medium containing K. Results from this latter aspect will be reported elsewhere. A +At+K subtreatment was used to assay yields of transformants resulting from selection immediately after exposure to At (N = 110).

Percentages of leaf sections forming callus, primordia, and harvestable shoots were recorded weekly for the first 63 days of culture and at roughly 3 week intervals through 277 days. Putative transformants were assayed for uidA expression at the end of the trial.

Trial 2. The second experiment tested effects of longer At exposure time (30 min) and a lower concentration (10^8 cfu/mL). As a secondary objective, utility of CE for clearing cultures of residual At contamination was compared to that of CA.

Protocols described above were used with one change; half of the leaf sections, regardless of subtreatment, were cultured on medium supplemented with 500 mgCA/L and half on medium containing 250 mgCE/L. As in the foregoing trial, leaf sections were divided among three subtreatments: -At-K control (N = 30); +At-K (N = 150); and +At+K (N = 150).

Percentages of leaf sections forming callus, primordia, and shoots were recorded weekly for the first several weeks to establish that development was proceeding normally. Observations continued at roughly 6-week intervals thereafter. The trial was terminated after 213 days of culture, when putative transformants from the +At+K subtreatments were assayed for uidA expression.

Trial 3. The third experiment compared effects of varying At exposure times (30 versus 120 min) and concentrations (10^7 versus 10^{10} cfu/mL). Subtreatments used in the aforementioned trials were included, and contrasted

with another that provided for culture on nonselective medium for 14 days before transfer to selective medium (+At+KP). The intent was to allow time for development to start, thereby permitting transformed cells to accumulate and differentiate to an extent sufficient to resist the debilitating effects of K and of the dying cells surrounding them.

Protocols were the same as those used earlier, except for changes described immediately above. Leaf sections were divided among 13 treatment combinations as follows: -At-K (N = 15); +At-K, 4 combinations of exposure times and concentrations (N = 15 per combination); +At+K, 4 combinations (N = 30 per combination); and +At+KP, 4 combinations (N = 30 per combination).

Percentages of leaf sections forming callus, primordia, and harvestable shoots were recorded weekly for the first few weeks, and at roughly four week intervals through the 91st day of culture. The experiment was then terminated, and putative transformants were collected from +At+K and +At+KP treatments for uidA assay and regeneration.

RESULTS AND DISCUSSION

Trial 1. Results from the first trial showed that incubation on standard leaf section medium for 24 hr before exposure to At did not provoke differential responses. Accordingly, results were averaged over all explants given each subtreatment.

In retrospect, this finding is not surprising. Though such practices are used with some frequency (Tsai et al. 1994, Confalonieri et al. 1994), few data are available to substantiate efficacy of this extra step in transformation protocols. Also, effectiveness of such treatments would seem dependent upon their being sufficiently long to ensure that development not only starts but also gets well underway. The time course of development for control leaf sections (Figure 1) shows that callus formation began in the first few days of culture. Primordia, first visible manifestation of organized meristematic centers, however, appeared 14 to 21 days later. Thus, incubation to ensure that development is not hindered by exposure to At and/or selective medium probably should span the first 14 to 21 days of culture. Such treatment also seems best applied after At exposure but before transfer to selective medium. This would permit transformed cells to multiply before dying cells surrounding them interfere with development. This approach was tested in Trial 3; the outcome is described below.

Development on +At-K leaf sections was delayed relative to those given the control treatment (Figure 1, Table 1). Percentages of explants forming callus and primordia eventually reached control levels, but shoot formation and elongation were delayed and reduced relative to controls. Thus, exposure to At and other aspects of the transformation protocol appear disruptive to regeneration.

Development on explants given the +At+K treatment was slow; callus was not evident until the 56th day of culture. Primordia and shoot formation were inhibited (Table 1) even though fair numbers of calli survived and continued to grow. Midway through the trial, a number of leaf sections showing promise were removed from this medium, cultured on nonselective medium to foster

Figure 1: Percentage of Explants from Trial 1 Having Callus (—●—), Primordia (—■—), and Shoots (—▲—) Through Day 63; A (-At-K) and B (+At-K)

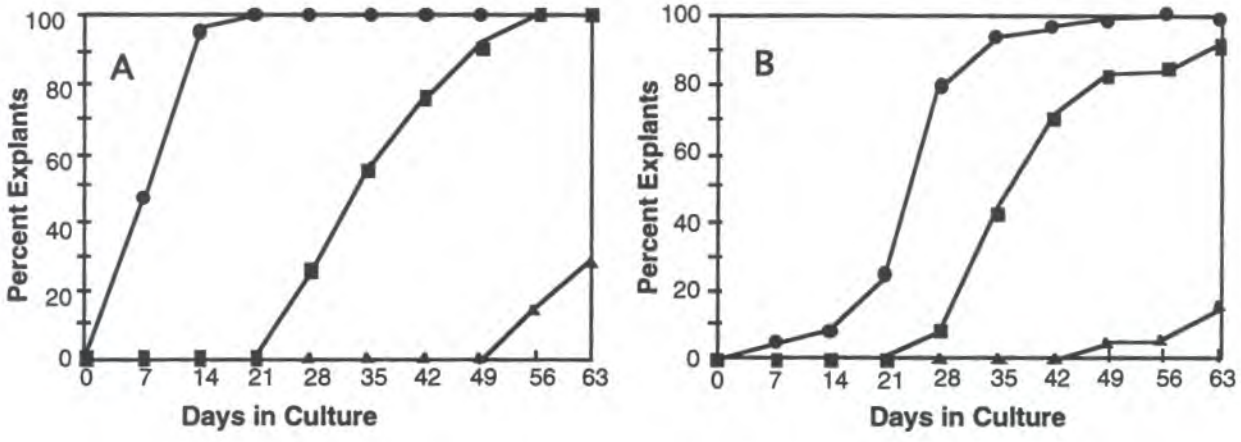

Table 1: Development on C175 leaf sections 277 days after exposure to At (10^9 cfu/mL) for 5 min and cultured on medium supplemented with 500 mgCA/L.

Treatment	Percent of Leaf Sections Forming		
	Callus	Primordia	Shoots
-At-K	100	100	94
+At-K	100	97	9
+At+K	38	0	0

development, and then returned to selective medium. They may not have survived if left on selective medium. As a result, percent of +At+K explants forming callus (Table 1), and therefore percent putative transformants (Table 2), is inflated by roughly 9 percentage points.

As noted above, all +At+K calli that survived through end of the trial were regarded as putative transformants (Tables 1 and 2). Only 5 percent showed expression of the uidA gene, and only 1 percent were rated as confirmed transformants. Thus, this short AT exposure time did not produce high frequencies of lasting transformation despite the relatively high At concentration, a finding in line with other recent investigations in which, depending on species and explant, exposure times ranged from 20 (Confalonieri et al. 1994) to 240 min (Wang et al. 1994).

Trial 2. Findings from the second trial, an effort to increase transformation rates via a longer At exposure time, mirrored those of the first trial. Frequencies of control explants forming callus, primordia, and shoots reached 100 percent quickly, regardless of CA and CE treatment.

Performance of +At-K leaf sections was similar to that of controls (Table 3), except that shoot formation was somewhat lower. Though small, this differential response confirms that At exposure and/or the transformation process reduces regeneration potential. Differences between responses to CA and CE, however, were nominal.

Table 2: Putative and confirmed transformation frequencies as affected by At exposure time (ET) and concentration (Conc).

At ET and Conc. (min. and cfu/ml)		Transformation, Percent of Available Leaf Sections Putative	Confirmed
Trial 1: 5, 10^9		38	1
Trial 2: 30, 10^8,	CA	13	1
	CE	19	1
Trial 3: 91 Days On Selective Medium			
30, 10^7		10	0
10^{10}		37	0
120, 10^7		36	10
10^{10}		27	10
14 Days on Nonselective + 77 Days On Selective Medium			
30, 10^7		27	10
10^{10}		33	10
120, 10^7		43	3
10^{10}		47	13

Explants given the +At+K treatment also formed callus and primordia with some frequency (Table 3), higher than in the first experiment. Shoot formation was also higher. No differences were apparent between responses to CA and CE.

Frequency of putative transformants averaged 16 percent; CA and CE produced similar outcomes (Table 2). All transformants, putative and confirmed, were calli; none of the primordia or shoots surviving on selective medium were transgenic. Only 3 percent of surviving calli showed uidA expression, and only an average of 1 percent were counted as confirmed transformants. These few transformants were distributed equally between CA and CE treatments. Thus, increasing At exposure from 5 to 30 min, and slightly reducing concentration to a level considered desirable by other workers (e.g., Confalonieri et al. 1994) did not raise transformation frequencies above levels noted earlier.

Regardless of subtreatment, differences between CA and CE treatments were minor, thereby confirming that CE does not interfere with regeneration from C175 leaf sections (Table 3). Indeed, when all subtreatments are considered, CE may have had a slight advantage in that numbers of putative transformants were slightly higher than for CA, an outcome possibly associated with lesser interference by residual At.

Residual At contamination was not as severe a problem in this experiment as in our earlier research. Midway through the present experiment, percentages of contaminated explants ranged from 43 to 60 percent, with +At+K explants most affected. Contamination levels were similar on CA and CE medium. With time, however, the margin between antibiotics widened,

Table 3: Development of C175 leaf sections 213 days after exposure to At (108 cfu/mL) for 30 min and cultured on media supplemented with 500 mgCA/L or 250 mgCE/L.

Treatment	Percent of Leaf Sections Forming		
	Callus	Primordia	Shoots
Carbenicillin			
-At-K	100	100	100
+At-K	97	94	82
+At+K	13	3	3
Cefotaxime			
-At-K	100	100	100
+At-K	100	99	100
+At+K	19	7	1

particularly in +At+K subtreatments. Within this subtreatment, 57 percent of explants cultured on CA experienced At contamination at one time or another as opposed to only 39 percent of those given CE. Taken together, such findings confirm those from dose/response assays (Dinus et al. 1995), and demonstrate the utility of using CE in the future.

Trial 3. Treatments tested in the third trial yielded definite increases in transformation frequencies (Tables 2 and 4). The trial was terminated after 91 days. Development generally was better than in Trials 1 and 2, and results from them showed little advantage to longer culture periods.

Table 4: Development of C175 leaf sections as affected by varying At exposure times and concentrations after 91 days of culture.

Treatment		Percent of Explants Forming		
		Callus	Primordia	Shoots
At-K: Control		100	100	40
+AT-K: 30 min,	107	100	100	47
	1010	100	100	47
120 min,	107	100	100	67
	1010	100	100	20
+AT+K: 30 min,	10^7	10	3	0
	10^{10}	37	0	0
120 min,	10^7	36	3	3
	10^{10}	27	10	3
+AT+KP: 30 min,	10^7	27	10	0
	10^{10}	33	10	0
120 min,	10^7	43	17	0
	10^{10}	47	10	0

Control explants formed callus and primordia at frequencies similar to those noted above (Tables 1, 3, and 4). Shoot production was reduced relative

to earlier experiments, but explants were healthy and yields were expected to increase beyond those noted at 91 days.

Leaf sections given the +At-K treatment performed similarly to controls (Table 4). Though somewhat inconsistent across treatments, shoot production tended to decline with increasing exposure time and concentration. Concentration appeared to have the greater effect. The fact that shoot production was not greater highlights the need for At exposure times and concentrations that maximize transformation without reducing regeneration.

Explants given the +At+K treatment formed callus, primordia, and shoots at similar or slightly higher frequencies than in earlier trials (Tables 1, 3, and 4). The overall increase in survival and development suggests that greater At exposure times and concentrations produce higher frequencies of transformation, even though they reduce regeneration potential to some extent.

Culture for 14 days on nonselective medium before transfer to selective medium increased numbers of +At+KP explants forming callus and primordia. Although shoot formation did not increase relative to +At+K subtreatments in this and other experiments, overall development was enhanced, and the improvement appears associated primarily with longer exposure time.

Similar trends were apparent for percentages of putative and confirmed transformants (Table 2), and yields from +At+KP subtreatments were greater than those from +At+K subtreatments in this and the other two experiments. In addition, frequencies varied directly with exposure time and concentration, with longer exposure time having the more pronounced effect. Concerns that +At+KP putative transformants would be largely transient and/or chimeric were not realized. When averaged over all subtreatments, the difference between confirmed and putative transformants was only slightly greater for the +At+KP subtreatments.

Collectively, findings from the third trial indicate that longer exposure times (120 min) and modest concentrations (perhaps, 10^{8}) offer much promise for raising transformation efficiency without reducing regeneration potential. That the +At+KP treatment tended to foster development and produced higher frequencies of both putative and confirmed transformants further indicates importance of allowing development to start and proceed for some time before challenging putative transformants with selective medium. This procedure apparently allows transformed calli to form and accumulate meristematic centers sufficiently organized to survive and develop.

Clearly, selection with K is far from ideal, and research on transformation would benefit from availability of a more benign marker gene. Increasing public concern about placing genetically altered trees resistant to antibiotics in the environment emphasizes need for such markers. Until such markers are developed, efficient regeneration of transgenic Pd plants will require some further adjustment of K concentrations in selective media. More importantly, treatments similar to the +At+KP tack used here also seem a workable means to circumvent the barrier posed by selection with K. While this approach resulted in slightly more escapes and partial or transient transformants, overall yields of confirmed transformants were greater and obtained in far less time than with other treatments. Trials to reevaluate

the procedure and to test other time periods on nonselective medium are underway.

Transgenic calli from Trial 3 have been transferred to media designed to force development of shoots. Also, leaves from cultures containing primordia and/or shoots have been harvested for multiplication/regeneration on standard leaf section medium. Shoots have been rooted, and are being multiplied via the leaf section protocol. These materials will be used to again verify transformation via histochemical and polymerase chain reaction assays for uidA gene expression and presence, respectively, and to check for any abnormalities in morphology and growth.

Results are also being used to effect transformation with and to study expression of a gene for enhanced auxin synthesis (Klee et al. 1987) in Pd clone C175, and to extend transformation to elite clones of commercial value.

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