Plant Molecular Biology Reporter **22:** 1–9, September 2004

Publish by Abstract

Agrobacterium-Mediated Transformation of the Genome-Sequenced Poplar Clone, Nisqually-1 *(Populus trichocarpa)*

CAIPING MA, STEVEN H. STRAUSS, and RICHARD MEILAN*

Department of Forest Science, Oregon State University, Corvallis, OR 97331-5752

Abstract. The US Department of Energy recently released a 6.8X draft of the genome sequence for Nisqually-1, a genotype of black cottonwood (*Populus trichocarpa*). To improve its utility for functional genomics research, having an efficient means for transformation and regeneration is necessary. To examine several parameters known to affect the transformation rate, we cocultivated leaf disc and stem explants with a strain of *Agrobacterium tumefaciens* harboring a binary plasmid vector containing genes for both neomycin phosphotransferase (*NPTII*) and β-glucuronidase (*GUS*). Shoot regeneration from stem explants was observed in the presence of kanamycin when thidiazuron was incorporated in the selection medium. Transformation efficiency was influenced by the level of thidiazuron to which explants were exposed during the early stages of shoot induction. Histochemical assays revealed expression of the *GUS* gene in leaf, stem, and root tissues of transgenic plants. Polymerase chain reaction confirmed the presence of both selectable marker and reporter genes in all lines that stained positive for β-glucuronidase activity. By use of our modified protocol, transgenic plants were recovered within 6 mo at an efficiency of 6%, adequate to produce a large number of transgenic events with modest effort.

Full text†: This manuscript, in detail, is available only in the electronic version of the *Plant Molecular Biology Reporter.*

Key words: *Agrobacterium tumefaciens,* genetic transformation, Nisqually-1, *Populus trichocarpa*

Abbreviations: GUS, β-glucuronidase; IBA, indole-3-butyric acid; 2iP, N^6 (2-isopentenyl) adenine; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; *NPTII,* neomycin phosphotransferase gene; PCR, polymerase chain reaction; WPM, woody plant medium; X-gluc, 5-bromo-4-chloro-3-indolyl-β-glucuronic acid.

*Author for correspondence. Present address: Department of Forestry and Natural Resources, 715 W. State Street, Purdue University, West Lafayette, IN 47907-2061, USA; e-mail: rmeilan@fnr.purdue.edu; fax: 765-494-9461.

†Editor's note: Although the scientific content of this manuscript has been reviewed, the full-text Web document has not been edited in detail.

Introduction

The genus *Populus* (poplars, cottonwoods, and aspens) has many advantages as model system for molecular biology of forest trees. These include rapid growth, ease of cloning, small genome, facile transgenesis, and tight coupling between physiological traits and biomass productivity (Bradshaw et al., 2000). A genotype of *Populus* is the third plant species for which the entire genome sequence is known. This female clone of black cottonwood (*Populus trichocarpa*) has come to be known as Nisqually-1, named for the river in Washington along which it was found growing in 1995 (Winstead, 2002).

To fully exploit the utility of this clone as a tool for functional genomic research, an efficient method with which to recover transgenic plants is needed. In this paper, we describe an improved *Agrobacterium tumefaciens*–mediated system for transforming and regenerating Nisqually-1.

Materials and Methods

Plant culture

Microcuttings of Nisqually-1 were initially cultured on hormone-free woody plant medium (WPM; Lloyd and McCown, 1981; M1 in Table 1). Shoot cultures were maintained on this medium and grown at 25 °C under a 16-h photoperiod. Light was provided by fluorescent tubes (TL70, F25T8/TL735; Philips) at a photon flux density of 45 $\mu E\ m^{-2}s^{-1}$. In vitro–grown, 40- to 50-day-old poplar plantlets served as explant sources for the studies reported here.

Binary vector and Agrobacterium strain

The binary vector pGUS-INT (provided by C. Wang and G. Tuskan, Oak Ridge National Laboratory, Oak Ridge, Tenn), containing an *NPTII* gene under control of the nopaline synthase promoter and a *GUS* gene regulated by the CaMV 35S promoter (Guilley et al., 1982), was introduced into *A. tumefaciens* C58/pMP90, a disarmed derivative of the nopaline C58 strain (Koncz and Schell, 1986), by the freeze-thaw method (Holstein et al., 1978).

Media

All media were autoclaved at 120 °C for 20 min. Vitamins, growth regulators, and antibiotics were filter-sterilized and added to the media after autoclaving. All media were adjusted to a pH of 5.6-5.8 before autoclaving except Luria-Bertani (LB) and induction medium (IM) for *Agrobacterium,* which were adjusted to pH 7.0 and 5.0, respectively. The composition of the various media types used is shown in Table 1.

Plant transformation protocol

When the standard poplar transformation protocol for aspen (Leplé et al., 1992) was largely unsuccessful, we modified it on the basis of results of the experiments described below to develop the following protocol for Nisqually-1:

Table 1. Composition of media for various aspects of plant regeneration.

Medium	Component	Type/Source	Amount
Luria-Bertani medium	Bacto-tryptone		16 g/L
	Bacto-yeast extract		8 g/L
	NaCl		5 g/L
Induction medium	Basal medium	Murashige and Skoog (MS)	
	Vitamins	MS	
	Acetosyringone		50 µM
	Galactose		10 mM
	2-(*N*-morpholino)-ethanesulfonic acid		1.28 mM
Wash solution	Basal medium	½ MS	
	Vitamins	MS	
	α-naphthaleneacetic acid (NAA)		1.0 µM
	6-benzylaminopurine (BAP)		1.0 µM
	N^6 (2-isopentenyl) adenine (2iP)		1.0 µM
	Ascorbic acid		250 mg/L
	Timentin		200 mg/L
M1	Basal medium	Woody plant medium (WPM)	
	Vitamins	WPM	
	Amino acid	Calcium gluconate (G-4625; Sigma)	1.3 g/L
	Sugar	Sucrose	20 g/L
	Gelling agents	Agar (Sigma)	3.0 g/L
		Phytagel (Sigma)	1.1 g/L
CIM1	Basal medium	MS	
	Vitamins	MS	
	Amino acid	L-glutamine	200 mg/L
	Sugars	Sucrose	30 g/L
	Gelling agents	Phytagar (Gibco)	3 g/L
		Phytagel (Sigma)	2 g/L
	Growth regulators	2iP	5 µM
		NAA	10 µM
CIM2	CIM1 plus:		
	Antibiotics	Kanamycin	50 mg/L
		Timentin	200 mg/L
SIM1	Basal medium	MS	
	Vitamins	MS	
	Amino acid	L-glutamine	200 mg/L
	Sugars	Sucrose	30 g/L
	Gelling agents	Phytagar (Gibco)	3 g/L
		Phytagel (Sigma)	2 g/L
	Growth regulator	Thidiazuron	0.2 µM
	Antibiotics	Kanamycin	100 mg/L
		Timentin	200 mg/L
SIM2	SIM1 plus:		
	Growth regulator	Thidiazuron	1.0 µM
	Antibiotics	Kanamycin	100 mg/L
		Timentin	200 mg/L

Table 1. (*concluded*).

Medium	Component	Type/Source	Amount
SIM3	SIM2 plus:		
	Growth regulator	Thidiazuron	0.6 µM
SIM4	SIM2 plus:		
	Growth regulator	Thidiazuron	0.01 µM

CIM, callus-induction medium; SIM, shoot-induction medium

1. *Agrobacterium* cells carrying a binary vector are grown for 24 h in LB medium (Table 1) supplemented with the appropriate antibiotics on an orbital shaker at 28 °C and 250 rpm.
2. The cells are pelleted by centrifugation at 3,500 rpm (1,992*g*) for 30-40 min and resuspended in *Agrobacterium* IM (Table 1) at an A_{600} of 0.5-0.6.
3. Internodal stem segments (3-4 mm in length) and leaf discs (4 mm in diameter) are wounded with multiple fine cuts and inoculated by swirling in an *Agrobacterium* suspension for 1 h.
4. The inoculated explants are cocultivated on CIM1 (Table 1) at 22 °C in darkness for 2 d.
5. Explants are washed 4 times in sterile water deionized by reverse osmosis and 1 time with wash solution (Table 1).
6. Explants are cultured for 21 d in the dark on CIM2 (Table 1).
7. Explants are cultured on SIM2 or SIM3 (Table 1) for 20 d in light.
8. Explants are then transferred onto SIM4 (Table 1) and subcultured every 3-4 wk.
9. Regenerated shoots are further screened for kanamycin resistance by rooting on half-strength Murashige and Skoog (1962) (MS) medium supplemented with 0.5 µM indole-3-butyric acid (IBA) and 25 mg/L kanamycin.
10. After approximately 30 d, the elongated, rooted shoots are micropropagated on the same medium or WPM lacking kanamycin (M1, Table 1).

For experiment 1, explants were cultured on CIM2 for 21 d in darkness (through step 6 above) and then transferred to SIM1 (Table 1), grown under lighted conditions, and subcultured onto fresh plates containing the same type of media every 34 wk (in lieu of steps 7 and 8 above). Steps 9 and 10 were not necessary because no shoots were recovered from experiment 1 (see Results).

For experiment 2, when the use of SIM1 at step 7 in the above protocol resulted in little improvement, the explants were instead transferred to SIM2 or SIM3 and grown in the light for 20 d before being transferred to SIM4. These explants were subcultured onto fresh SIM4 medium every 3-4 wk until shoots were regenerated from transformed calli (step 8 above). GUS activity was assayed in green calli 20 d after culture on SIM2 or SIM3; 1-2 leaves from each regenerated shoot were assayed 20 d after being transferred to the rooting medium.

Histochemical GUS assay

Histochemical staining of GUS activity was done according to Jefferson et al. (1987). **[Author: Please add Jefferson et al. to reference list. Is it "Jefferson RA, Kavanagh TA, and Bevan MW (1987) GUS fusions: beta-glucuronidase**

as a sensitive and versatile gene fusion marker in higher plants. EMBO 6(13): 3901-7."???] Twenty explants from both stems and leaves were stained 2 d after cocultivation to detect transient *GUS* expression, whereas leaves or whole, rooted, kanamycin-resistant plants were analyzed for stable *GUS* gene expression. Plant materials were stained overnight at 37 °C in a solution containing 2 mM X-gluc (first dissolved in N,N-dimethylformamide), 100 mM phosphate buffer (pH 7.0), and 0.1% Triton X-100. After staining, plant tissues were cleared in 70% ethanol. Samples with any blue foci were scored as positive for GUS activity.

DNA isolation and PCR analysis

Genomic DNA was isolated with the plant DNAeasy Prep Kit (Qiagen) according to the manufacturer's instructions. About 100 ng of poplar DNA was used as template for PCR. Primers for PCR analyses were as follows: NPTII F, ATGCCTGCTTGCCGAATATC; NPTII R, CCAAGCTCTTCAGCAATATCAC; GUS0.01, TGGGCATTCAGTCTG; GUS0.02, GTGATATCGTCCACCCA. To amplify the *NPTII* gene fragment, the reactions were subjected to 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min for 30 cycles; for the *GUS* gene fragment, the conditions were 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min for 30 cycles. PCR products were electrophoresed through 1% agarose gels and stained with ethidium bromide.

Results and Discussion

After 2 d of cocultivation with *Agrobacterium,* most of the explants (>50%) exhibited transient *GUS* expression (Figure 1A and B). In most leaf disc explants, intense GUS activity was recorded at the mid-veins and near cut surfaces (Figure 1B). This is consistent with what has been observed in other poplar genotypes. Callus and adventitious buds eventually formed on each side of micro–cross-sections (5 mm), more so along mid-veins than on other leaf surfaces. Our observations agree with those of Lee-Stadelmann et al. (1989), who found that 4- to 5-mm micro–cross-sections were approximately 25 times more efficient in shoot regeneration than were 1-cm explants.

Our prior experience with poplar and other tree species (e.g., sweetgum) has shown that early selection of transformed calli is critical for recovering transgenic plants. This is required to minimize competition between transformed and nontransformed calli and to reduce the number of "escapes" (false-positives) that regenerate. Therefore, after cocultivation, all explants from these experiments were placed onto CIM2 (Table 1) containing a low level of kanamycin (50 mg/L).

In experiment 1, no explants, neither stem segments nor leaf discs, responded to SIM1 (Table 1). No shoots were produced, and explants turned brown and died after several subcultures. This outcome suggests that the MS salts and the thidiazuron concentration (0.2 μM) were not adequate for induction of shoots in Nisqually-1.

Internodal stem segments from Nisqually-1 performed very well in 3 independent replicates of experiment 2. About 15-21% of the stem explants produced green calli after 2-3 mo of cocultivation (Figure 1C), and the green calli produced

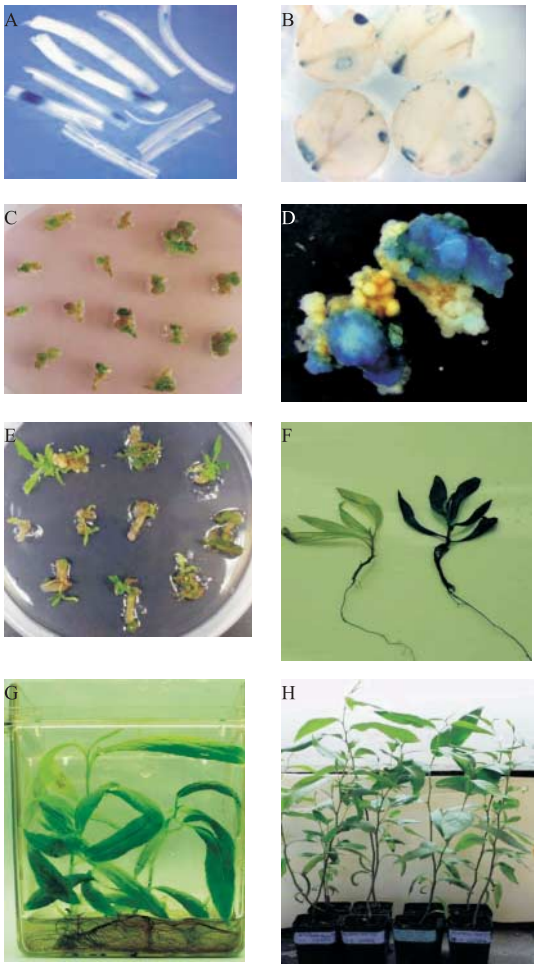

Figure 1. Transformation of black cottonwood *(Populus trichocarpa)* Nisqually-1. A, B: transient expression of β-glucuronidase *(GUS)* gene in stem (A) and leaf (B) explants, 2 d after inoculation. C: Green calli derived from stem, after 20 d of culture on SIM3 (Table 1). D: Stable GUS expression in transgenic calli. E: Shoot regenerated from stem explants, after 30 d of culture on SIM4 (Table 1). F: stable *GUS* expression in transgenic plantlet (right); left, untransformed (control) plant. G: Transformants grown on root-selection media. H: Transgenic plants growing vigorously in soil in a greenhouse, 4 mo after being transplanted.

on selection medium were confirmed to be transgenic on the basis of GUS activity assays (Figure 1D). However, all leaf discs turned brown and died on selection medium, presumably the result of a suboptimal callus-induction medium.

Thidiazuron, a cytokinin-like compound, has been used extensively for shoot organogenesis and somatic embryogenesis in a variety of plant species (Howe et al., 1994; Noël et al., 2002; Huchinson et al., 1996; Sriskandarajah et al., 1994). Optimizing the level and duration of thidiazuron exposure is important for producing healthy shoots at a high frequency. An intermediate thidiazuron

Table 2. Effect of different shoot-induction media on the efficiency with which Nisqually-1 was transformed by use of p35S-GUS-INT.

Shoot-Induction Medium Used	No. of Explants Inoculated	No. of Green Calli Formed (%)	No. of Regenerated Transgenic Shoots Showing *GUS* Expression (%)
SIM2	212	32 (15)	8 (3.8)
SIM3	227	48 (21)	13 (5.7)

GUS, β-glucuronidase gene.

concentration (0.6 μM) resulted in a higher transformation efficiency (Table 2). It was previously shown that when explants were kept too long on media supplemented with high concentrations of thidiazuron, shoot formation either was delayed or exhibited hyperhydricity or other morphological abnormalities (Huetteman and Preece, 1993). With Nisqually-1, explants were placed on shoot selection media containing 0.6 or 1.0 μM thidiazuron for 20 d before being transferred to the same medium within which the thidiazuron was reduced to 0.01 μM. Transgenic shoots started to regenerate within 1 wk of being transferred to a medium containing the lowest level of thidiazuron, and more shoots were produced as explants were subcultured (every 3-4 wk) on the same medium (Figure 1E).

Leaf explants of Nisqually-1 required different types and levels of growth regulators for induction of callus than did stem explants from this clone. About 20% of leaf discs cultured on callus-induction medium (MS) containing 1.0 μM 6-benzylaminopurine (BAP) and 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) produced *GUS*-positive calli but failed to regenerate shoots on the several types of shoot-induction media supplemented with different levels of thidiazuron (data not shown).

All *GUS*-positive shoots rooted easily under selection on half-strength MS root-induction medium containing 25 mg/L kanamycin and 0.5 μM IBA (Figure 1G). About 4 roots were produced per shoot. These rooted plantlets were then transplanted into potting mix and transferred to the greenhouse, where they grew vigorously (Figure 1H). Nontransgenic control shoots did not produce any roots and eventually died on root-selection medium (data not show).

PCR with *GUS*- and *NPTII*-specific primers provided a rapid means of screening regenerants. Fragments of the predicted size were amplified for both transgenes in all tested lines, thereby confirming the stable integration of tDNA into the plant's genome (Figure 2).

In summary, the transformation and regeneration system described here is simple and effective, allowing routine introduction of genes into an important poplar genotype. Given the efficiency achieved with our protocol, it is possible for one person to produce hundreds of independent lines of transgenic Nisqually-1 within a 6-mo period.

Acknowledgments

The authors wish to express their gratitude to Dr Toby Bradshaw for providing the initial microcuttings of Nisqually-1. This work was funded by of the Tree Genetic

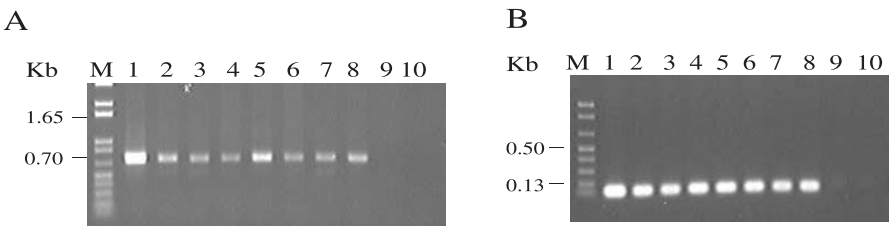

Figure 2. Molecular characterization of putative transformants via PCR. A: Amplification of a specific 700-bp fragment from the β-glucuronidase (*GUS*) gene (A). B: 130-bp fragment amplified from the neomycin phosphotransferase (*NPTII*) gene. M: Molecular weight marker (1 kb plus DNA ladder). Lanes labeled 1-9 contained the following templates in the PCR reaction mixtures: p35S-GUS-INT (positive control); genomic DNA from 7 separate transformation events (lines); and a nontransformed plant (negative control), respectively. Lane 10 was no-template (double-distilled H_2O) control.

Engineering Research Cooperative (http://www.fsl.orst.edu/tgerc/index.htm) and the National Science Foundation I/UCRC program (grant no. 9980423-EEC). It was also partially supported by a grant from the US Department of Energy (award no. 4000023558).

References

Bradshaw HD, Ceulemans R, Davis J, and Stettler R (2000) Emerging model system in plant biology: poplar *(Populus)* as a model forest tree. J Plant Growth Regul 19: 306-313.

Guilley H, Dudley RK, Jonard G, Balaze E, and Richards KE (1982) Transcription of cauliflower mosaic virus DNA: detection of promoter sequences, and characterization of transcripts. Cell 30: 763-773.

Han KH, Meilan R, Ma C, and Strauss SH (2000) An *Agrobacterium tumefaciens* protocol effective on a variety of cottonwood hybrids (genus *Populus*). Plant Cell Rep 19: 315-320.**[Author: Han et al. not cited in text. Please add an in-text citation or delete reference.]**

Holstein M, De Wacek D, Depicker A, Messers E, van Montagu M, and Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens.* Mol Gen Genet 163: 181-187.

Howe GT, Goldfarb B, and Strauss SH (1994) *Agrobacterium*-mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants. Plant Cell Tissue Org 36: 59-71.

Huchinson MJ, Murch SJ, and Saxena PK (1996) Morphoregulatory role of thidiazuron: evidence of the involvement of endogenous auxin in thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium* × *hortoyum* Bailey). J Plant Physiol 149: 573-579.

Huetteman CA and Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Org 33: 105-119.

Koncz C and Schell J (1986) The promoter of the T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204: 383-396.

Lee-Stadelmann OY, Lee SW, Hackett WP, and Read PE (1989) The formation of adventitious buds in vitro on micro-cross sections of hybrid *Populus* leaf midveins. Plant Sci 61: 263-272.

Leplé JC, Brasileiro ACM, Michel MF, Delmotte F, and Jouanin L (1992) Transgenic poplars: expression of chimeric genes using four different constructs. Plant Cell Rep 11: 137-141.

Lloyd G and McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia,* by use of shoot-tip culture. Int Plant Prop Soc Proc 30: 421-427. **[Author: This is given as 1981 in text. Please confirm which year is correct… 1980 or 1981.]**

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497.

Noël N, Leplé JC, and Pilate G (2002) Optimization of in vitro micropropagation and regeneration for *Populus* × *interamericana* and *Populus* × *euramericana* hybrids *(P. deltoides, P. trichocarpa, and P. nigra).* Plant Cell Rep 20: 1150-1155.

Sriskandarajah S, Goodwin PB, and Speris J (1994) Genetic transformation of the apple scion cultivar "Delicious" via *Agrobacterium tumefaciens.* Plant Cell Tissue Org 36: 317-329.

Winstead ER (2002) Tree genome sequence in eighteen months. Genome News Network (GNN). Accessed 30 September 2004 <http://www.genomenewsnetwork.org/articles/02_02/tree_genome.shtml>.