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Regeneration of transgenic loblolly pine (*Pinus taeda* L.) from zygotic embryos transformed with *Agrobacterium tumefaciens*

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Abstract Embryos of 24 open-pollinated families of loblolly pine (*Pinus taeda* L.) were used as explants to conduct in vitro regeneration. Then, *Agrobacterium tumefaciens* strain GV3101 harboring the plasmid pPCV6NFHygGUSINT was used to transform mature zygotic embryos of seven families of loblolly pine. The frequency of transformation varied among families infected with *A. tumefaciens*. The highest frequency (100%) of transient β -glucuronidase (GUS)-expressing embryos was obtained from family 11-1029 with over 300 blue spots per embryo. Expression of the GUS reporter gene was observed in cotyledons, hypocotyls, and radicles of co-cultivated mature zygotic embryos, as well as in callus and shoots derived from co-cultivated mature zygotic embryos. Ninety transgenic plants were regenerated from hygromycin-resistant callus derived from families WO3, 8-1082 and 11-1029, and 19 transgenic plantlets were established in soil. The presence of the GUS gene in the plant genome was confirmed by polymerase chain reaction, Southern blot, and plant DNA/T-DNA junction analysis. These results suggest that an efficient *A. tumefaciens*-mediated transformation protocol for stable integration of foreign genes into loblolly pine has been developed and that this transformation system could be useful for future studies on transferring economically important genes to loblolly pine.

Keywords Genetic transformation · β -Glucuronidase gene · *Pinus* (transformation) · Plant DNA/T-DNA junction analysis · Transgenic pine · Zygotic embryo

Abbreviations BA: N⁶-benzyladenine · 2,4-D: 2,4-dichlorophenoxyacetic acid · GUS: β -glucuronidase · IBA: indolebutyric acid · *aph(3')*II: promoterless neomycin phosphotransferase II gene · PCR: polymerase chain reaction

Introduction

Genetic transformation in conifers has the potential for the selective improvement of individual traits in elite clones while maintaining the existing combination of genes responsible for the superior phenotype (Charest and Michel 1991; Birch 1997; Walter et al. 1999). At present, although considerable research effort has been devoted to the genetic engineering of conifer species (Sederoff et al. 1986; Klimaszewska et al. 1997), progress has lagged behind advances made in angiosperm forest trees and herbaceous crops due to the recalcitrant nature of woody perennials to manipulation in vitro (Wenck et al. 1999). Among gene-transfer methods reported, the biolistic technique appears to be the most widely used technique for gene transfer into conifers. Transient expression in conifers after biolistic gene transfer has been reported for *Pinus radiata* (Walter et al. 1994), *Picea abies* (Walter et al. 1999), *Picea glauca* (Moench) Voss (Ellis et al. 1993), *Picea mariana* (Mill.) BSP (Tian et al. 1997), *Pinus taeda* (Stomp et al. 1991), and *Pseudotsuga menziesii* (Mirb.) Franco (Goldfarb et al. 1991). To date, stable transformation of conifers using biolistics has been achieved for *Picea abies* (Walter et al. 1999), *Larix laricina* (Klimaszewska et al. 1997), *Picea glauca* (Ellis et al. 1993), *Picea mariana* (Charest et al. 1996), and *Pinus radiata* (Walter et al. 1998). However, this very useful direct gene-transfer method may result in the integration of multiple copies of the transgene, possibly leading to gene silencing (Meyer 2000).

Agrobacterium-mediated transformation has been one of the favoured methods for introduction of foreign genes into higher plants (Birch 1997). The high efficiency

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of *Agrobacterium*-mediated transformation can be used for saturating the genome with insertional mutations to establish relations between sequence data and gene function by reverse genetics (Mathur et al. 1998). Novel techniques such as "Agrolistic" transformation (Hansen and Chilton 1996) and "SAAT" (sonication-assisted *Agrobacterium*-mediated transformation; Trick and Finer 1997) may improve the efficiency of DNA transfer into plant cells and integration of foreign genes into the plant genome. Inoculation with *Agrobacterium* has resulted in tumour development in numerous coniferous species (Sederoff et al. 1986; Loopstra et al. 1990; Stomp et al. 1990). Transient expression of the GUS gene by *Agrobacterium*-mediated transformation has been reported for *Pinus halepensis* Mill (Tzfira et al. 1996), *Pinus taeda* (Wenck et al. 1999), and *Pinus pinea* (Humara et al. 1999). However, regeneration of transgenic plants via co-cultivation with *Agrobacterium* has been restricted to *Larix decidua* (Huang et al. 1991), *Pinus strobus* (Levee et al. 1999), *Picea abies* (Wenck et al. 1999), and *Larix kaempferi* × *L. decidua* (hybrid larch) (Levee et al. 1997).

Loblolly pine (*Pinus taeda*) is an economically important forest tree most widely planted in the southeast United States. Although several studies have demonstrated transformation of cell cultures and tissues (Sederoff et al. 1986; Wenck et al. 1999), there is no report on *A. tumefaciens*-mediated transformation and expression of the β -glucuronidase (GUS) reporter gene in mature zygotic embryo cultures and regenerated plants of loblolly pine. In this study, we report the *Agrobacterium*-mediated transformation and stable expression of the GUS reporter gene using mature zygotic embryos of loblolly pine as explants.

Materials and methods

Plant materials

Mature seeds collected from 21 different genotypes of open-pollinated parent trees of loblolly pine (*Pinus taeda* L.) were obtained from Tree Improvement Program (TIP), North Carolina State University, and mature cones of 4 families, including 1 family present among the 21, were obtained from International Paper Company (IPC, Bainbridge, Ga., USA), in October 1998 (see Table 1), and stored in plastic bags at 4 °C before they were used for tissue culture. Seeds were disinfected by immersion in 70% (w/w) ethanol for 30 s and in household bleach for 10–15 min, followed by four to five rinses in sterile deionized water. Mature zygotic embryos were aseptically removed from the megametophytes and placed horizontally on a solid callogenesis medium in MAGENTA GA7 Vessels with LIFEGUARD Vented Lids (Gibco-BRL) or Petri dishes (100 mm diameter, 15 mm deep; Fisher Scientific). Embryos of all 24 genotypes were used as explants to conduct in vitro regeneration following a previously established protocol (Tang et al. 1998). Mature zygotic embryos of seven genotypes were selected and used for transformation experiments after culturing on pre-treatment medium consisting of TE medium (Tang et al. 1998) supplemented with 10 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg/l N⁶-benzyladenine (BA), and 4 mg/l kinetin for 1–3 weeks to assure successful aseptic culture.

Table 1 Callus induction and shoot regeneration in loblolly pine (*Pinus taeda*). For callus induction, each treatment was replicated three times, and each replicate consisted of 30 embryo explants. For adventitious shoot induction, each treatment was replicated three times, and each replicate consisted of 30–50 pieces of callus 0.5 cm×0.5 cm in size. Values represent the mean percentage (\pm SD) of embryos giving rise to callus, and callus pieces giving rise to buds

Genotype	Seed source	Callus formation (%)	Bud regeneration (%)
8-1082	TIP ^a	61.5 \pm 1.3	77.2 \pm 1.1
8-1064	TIP	50.3 \pm 1.2	48.6 \pm 0.9
WO3	IPC ^b	58.7 \pm 6.5	46.6 \pm 1.3
7-100	IPC	77.7 \pm 1.2	46.3 \pm 2.2
11-1029	TIP	51.6 \pm 9.4	45.5 \pm 1.3
7-1046	TIP	34.4 \pm 1.7	37.9 \pm 1.8
7-1040	TIP	37.8 \pm 1.2	37.8 \pm 2.3
1-1323	TIP	70.1 \pm 0.9	28.5 \pm 1.4
3-1039	TIP	62.4 \pm 1.0	26.0 \pm 1.2
1-1062	TIP	52.2 \pm 1.0	18.9 \pm 0.5
3-1047	TIP	71.3 \pm 1.2	18.5 \pm 1.5
7-1037	TIP	39.6 \pm 1.5	18.4 \pm 1.2
7-1011	TIP	27.1 \pm 2.4	18.4 \pm 0.9
7-56	IPC	71.2 \pm 1.1	18.3 \pm 1.2
8-1020	TIP	37.2 \pm 1.1	18.3 \pm 1.0
3-1050	TIP	51.8 \pm 1.3	17.7 \pm 0.7
8-1039	TIP	45.1 \pm 1.1	15.9 \pm 0.2
3-1058	TIP	19.9 \pm 0.2	13.6 \pm 2.1
7-56	TIP	41.8 \pm 1.3	12.0 \pm 0.7
11-1056	TIP	52.2 \pm 0.9	9.1 \pm 0.9
11-1017	TIP	51.2 \pm 1.1	8.6 \pm 1.2
1-1015	TIP	49.3 \pm 1.1	6.4 \pm 2.3
1-1024	TIP	47.2 \pm 1.1	6.4 \pm 0.2
11-1103	TIP	18.2 \pm 0.9	5.4 \pm 1.4
7-2	IPC	57.4 \pm 0.8	5.4 \pm 1.1

^aTree Improvement Program, N.C. State (extracted from cones, dried, stored at 4 °C, in plastic bags)

^bInternational Paper Company (Georgia); cones stored at 4 °C in paper bags

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain GV3101 containing the binary plasmid pPCV6NFHygGUSINT (kindly provided by Professor Csaba Koncz) (Mathur et al. 1998) (Fig. 1) was used for the transformation experiments. Plasmid pPCV6NFHygGUSINT carries a promoterless neomycin phosphotransferase gene (*aph(3')*II), the hygromycin phosphotransferase gene (*hpt*) which confers hygromycin resistance, a GUS gene under the control of the cauliflower mosaic virus 35S promoter, and the terminator from the nopaline synthase gene (*nos*). *Agrobacterium tumefaciens* strain GV3101 was grown overnight at 28 °C in liquid YEP medium (Sambrook et al. 1989) supplemented with 100 mg/l carbenicillin. The overnight culture was used for transformation of mature zygotic embryos. Concentration of bacterial cultures was determined in a MILTON ROY spectronic 1201 at OD_{600 nm}.

Co-cultivation and selection

For ultrasound wounding, the sonication-assisted *Agrobacterium*-mediated transformation technique (SAAT; Trick and Finer 1997) was used. Mature zygotic embryos were sonicated in an ultrasound water bath (Model T-21B; L&R Manufacturing Company, Kearny, N.J., USA) for 0–120 s, and incubated a further 10–15 min without sonication followed by infection. Mature zygotic embryos were then inoculated with *Agrobacterium*

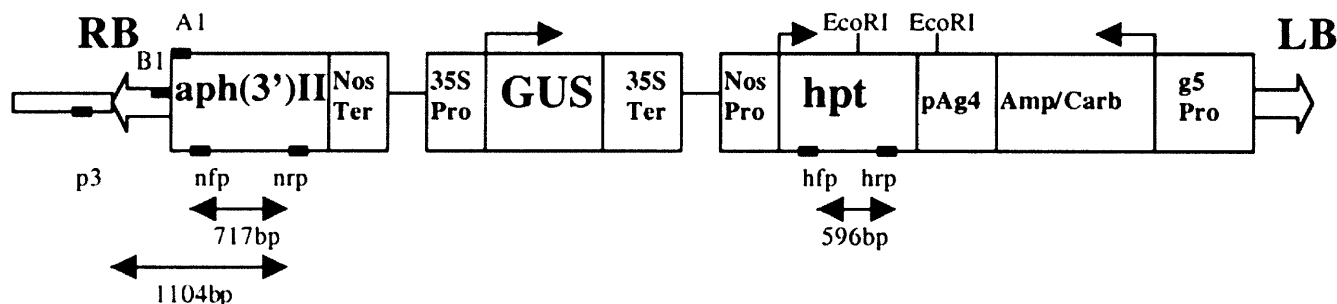


Fig. 1 T-DNA region of pPCV6NFHygGUSINT plasmid. *RB* Right border of T-DNA, *LB* left border of T-DNA, *NosPro* promoter of the nopaline synthase gene, *NosTer* terminator of the nopaline synthase gene, *35SPro* the cauliflower mosaic virus 35S promoter, *35Ster* terminator of the CaMV 35S transcription unit, *GUS* β -glucuronidase gene, *aph(3')II* promoterless neomycin phosphotransferase gene, *hpt* hygromycin phosphotransferase gene, *pAg4* TL-DNA gene 4 transcription terminator, *g5pro* TL-DNA gene 5 promoter. Binding sites of PCR primers P3, nfp, nrp, hfp, hrp, A1, and B1 are shown as *black rectangles*, and predicted products and sizes are shown as *double-headed arrows*; directions of transcription are shown as *arrows above the respective genes*

for 15–25 min. Co-cultivation was conducted at 25 °C for 3–5 days in the dark on callogenesis medium consisting of TE medium (Tang et al. 1998) supplemented with 10 mg/l 2,4-D, 4 mg/l BA, 4 mg/l kinetin, and 50 μ M acetosyringone, with the exception of experiments testing different concentrations of acetosyringone. Co-cultivated mature zygotic embryos were washed four to five times in sterile deionized water to reduce the *Agrobacterium* concentration, blotted on sterile filter paper to remove excess liquid, and transferred onto callogenesis medium supplemented with 500 mg/l Claforan (Hoechst-Roussel, Somerville, N.J., USA) (for *A. tumefaciens* GV3101) and 4.5 mg/l hygromycin to select stable transformants. Mature zygotic embryos were transferred to fresh selection medium at 3-week intervals. After three to four subcultures, plant regeneration was carried out according to a procedure previously described (Tang et al. 1998). The organogenesis medium consists of TE basal medium containing 0.5 mg/l of indolebutyric acid (IBA) and 2 mg/l BA.

Histological observation

For histological studies, transformed mature zygotic embryos and organogenic callus were fixed in formalin/acetic acid/ethyl alcohol (1:1:18, by vol.) for 24–48 h, dehydrated through a graded series of ethyl alcohol and tertiary butyl alcohol, and embedded in Tissue Prep2 paraffin (58–60 °C) (Fisher Scientific). Serial sections of 8 μ m in thickness were cut with a rotary microtome LEITZ1512 (Ernst Leitz, Midland, Ontario, Canada), and stained with 5% Fuchsin solution.

Histochemical assays and fluorometric analysis of GUS activity

Histochemical analysis of GUS expression was performed 24 h after co-cultivation with *A. tumefaciens*. Tissues were incubated in staining buffer (Jefferson et al. 1987) consisting of 100 mM sodium phosphate, 50 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid cyclohexylammonium salt, 0.1% β -mercaptoethanol, and 0.1% Triton X-100 (pH 7.2) at 37 °C for 16 h prior to observation. Stained plant materials were cleared with 70% ethanol for at least 24 h. A sample was scored as GUS positive if there was at least one discrete dark-blue region on the tissue. Fluorometric assays were performed using a TK100 fluorometer (Hofer, San Francisco,

Calif., USA). Total protein was extracted from plant tissue with extraction buffer (Jefferson et al. 1987) and was measured using the BCA protein assay Kit (Pierce, Rockford, Ill., USA) according to product instructions, with a SPECTRA max 250 Microplate Spectrophotometer. Fluorometry was conducted according to the protocol by Jefferson et al. (1987).

Polymerase chain reaction (PCR) analysis

Loblolly pine genomic DNA was extracted from 300–500 mg fresh tissue of control and putative transgenic plants, respectively, using a Genomic DNA Isolation Kit (Sigma) following the manufacturer's protocol. Primers used for amplification of insert DNA were the *aph(3')II* forward primer (nfp) 5'-acaacagacaatcggctgc-3' and reverse primer (nrp) 5'-aagaactgtcaagaaggcg-3', and the hygromycin phosphotransferase gene forward primer (hfp) 5'-ttcagctcgatgtaggagg-3' and reverse primer (hrp) 5'-agaagaagatgttggcgacc-3'. The primer set utilized for amplification across the junction between T-DNA and flanking plasmid DNA was 5'-cgttgaggatcaagccaca-3' (P3: outside right border) and 5'-aagaactgtcaagaaggcg-3' (nrp: inside right border). A total of 100–300 ng of genomic DNA was used as template in a 50- μ l PCR reaction mix containing 200 μ M each of dATP, dCTP, dGTP, dTTP, 35 pmol of each primer, 2.5 U Taq DNA polymerase (Promega), 1.5 mM MgCl₂, and 5 μ l 10 \times buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25 °C), 1% Triton X-100, 15 mM MgCl₂]. The reaction proceeded in a programmable MJ MiniCycler (MJ Research, Watertown, Mass., USA). The PCR conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 52 °C for 1 min, and 72 °C for 2 min. Cycling was followed with a final incubation of 72 °C for 10 min. PCR products were observed under UV after electrophoresis on a 1.0% agarose gel with 0.1% ethidium bromide. Molecular markers are λ DNA *HindIII* and 1-kb marker (Gibco-BRL).

Southern blot

Genomic DNA was isolated from 5–8 g fresh tissue of control and putative transgenic plants according to the methods of Wagner et al. (1987). Buffers for Southern blot analysis, including TAE electrophoresis buffer, hybridization solution, and SSC solutions for final washes, were prepared according to Sambrook et al. (1989). Fifteen to thirty micrograms of DNA was digested overnight with the restriction enzymes *EcoRI* and/or *HindIII* (Boehringer Mannheim) at 37 °C, electrophoresed on a 1.0% agarose gel in TAE buffer and denatured with 0.5 N NaOH, then transferred to a nylon membrane using alkali transfer buffer. The DNA fixed on membranes was hybridized in hybridization solution at 65 °C with the probe (717-bp PCR fragment of the neomycin phosphotransferase gene or 596-bp PCR fragment of the hygromycin phosphotransferase gene), which was labeled with [³²P]dCTP (Ready to Go Labeling Beads (Pharmacia)), according to standard protocols (Sambrook et al. 1989). Membranes were washed twice in 2 \times SSC, 0.1% SDS at 65 °C for 5 min each, once in 0.5 \times SSC, 0.1% SDS at 62 °C for 15 min, and once in 0.1 \times SSC, 0.1% SDS at room temperature for 30 min, and exposed to Kodak X-Omat-AR films at –80 °C for 1–3 days.

Plant DNA/T-DNA junction analysis

The method to identify the junction sequences in putative transgenic plants was described by Zhou et al. (1997). One microgram of genomic DNA prepared by using a Genomic DNA Isolation Kit (Sigma) was digested by 10 units of *TaqI* restriction enzyme (NEBiolabs) in 50 μ l reaction volume at 65 °C overnight. Digested genomic DNA was extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). The upper aqueous phase was transferred to another 1.5-ml tube, DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold ethanol, and the pellet was dissolved in 20 μ l 10 mM Tris (pH 8.0), 1 mM EDTA. One microgram of pUC19 plasmid extracted from *Escherichia coli* strain JM109 was digested by 5 units of *AccI* restriction enzyme (NEBiolabs) at 37 °C overnight in 20 μ l reaction volume. Digested pUC19 was separated on a 1.0% agarose gel and the band representing pUC19 was purified with a QIAquick Gel Extraction Kit (QIAGEN). Purified genomic DNA (50 ng) and pUC19 plasmid DNA (150 ng) were ligated by 1 unit of T4 DNA ligase (Boehringer Mannheim) at 4 °C overnight in a 20- μ l reaction volume with 4 μ l of 5 \times ligase buffer [(1 \times ligase buffer is 50 mM Tris-HCl (pH 7.5 at 25 °C), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, and 25 μ g/ml bovine serum albumin)]. Five microliters of the ligation mixture was used for the first PCR in a 50- μ l mixture containing 200 μ M dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 0.1% Triton X-100, 1.5 mM MgCl₂, 2.5 units of *Taq* polymerase, and 200 ng each of primers A1 [5'-cagtaatagccgaatagcctctccacc-3' (from T-DNA)] and A2 [5'-tcgtatgtgtgtggaatcgtgagcgg-3' (from pUC19)]. The PCR conditions were an initial incubation at 94 °C for 5 min followed by 40 cycles of 94 °C for 45 s, 68 °C for 1 min (for primer annealing), 72 °C for 1 min. This was followed by 72 °C for 10 min and a 4 °C hold. After the PCR reaction, the products were purified using the QIAquick PCR Purification Kit. The second PCR reaction was carried out in 50 μ l reaction volume using primer B1 5'-cgaatagcctctccaccaag (from T-DNA), and primer B2 5'-aacagctatagacatg-3' (from pUC19), which are nested to the first primers in the first PCR amplification. Five-microliter aliquots of the first PCR product were used as template. The thermal cycling conditions were the same as those for the first PCR reaction, except that the annealing temperature was 54 °C. The second PCR products were separated on a 1.0% Agarose gel and purified with the QIAquick Gel Extraction Kit (QIAGEN). Purified PCR products were sequenced using Primer B1 (already given above) at the North Carolina State University sequencing facility using an ABI PRISM 377 DNA Sequencer (PE Applied Biosystem).

Results

Tissue culture and selection of stable transformants

Embryos of 24 open-pollinated families of loblolly pine were used as explants to conduct in vitro regeneration.

Table 2 Influence of genotypes on transformation frequency in loblolly pine

Strain	Genotype	Seed source	Concentration of bacteria (OD ₆₀₀)	% embryos expressing GUS
<i>A.t.</i> GV3101 ^a	7-2	IPC ^b	0.619	25
	WO3	IPC	0.954	98.8
	8-1020	TIP ^c	0.830	8.7
	8-1039	TIP	0.830	9.5
	8-1064	TIP	0.555	51.6
	8-1082	TIP	0.567	93.1
	11-1029	TIP	0.868	100

^a*A.t.* GV3101: *Agrobacterium tumefaciens* GV3101 (pPCV6NFHygGUSINT)

^bIPC: International Paper Company (Georgia); cones stored at 4 °C in paper bags

^cTIP: Tree Improvement Program, N.C. State (extracted from cones, dried, stored at 4 °C, in plastic bags)

Differences in regeneration frequency among the 24 families were observed (Table 1). Transient expression of the GUS reporter gene was then assayed in seven families representing a range of regeneration efficiencies (Table 2). Families WO3, 8-1082, and 11-1029 were selected for further transformation experiments. The minimal concentration of hygromycin for selection of transformed tissues was determined by culturing embryos of these three genotypes on callogenesis medium containing various concentrations of hygromycin (Fig. 2). Growth of mature zygotic embryos and callus formation were completely inhibited at 4.5 mg/l hygromycin in the fourth week of culture (Fig. 2). This concentration of hygromycin was used in the selection of transformed tissue. Embryos co-cultivated with *Agrobacterium* began to form calli 1–3 weeks after transfer onto callogenesis medium containing hygromycin. The frequency of transgenic calli increased during 3–8 weeks on fresh callogenesis medium supplemented with 500 mg/l Claforan and 4.5 mg/l hygromycin. The highest frequency of transgenic callus formation was obtained on the eighth week. The effect of different concentrations of acetosyringone in co-cultivation medium on transformation frequency was tested. Eight weeks after inoculation, the frequency of hygromycin-resistant callus formation was improved (Fig. 3). Concentrations of acetosyringone between 30 and 75 μ M resulted in the highest transformation frequencies.

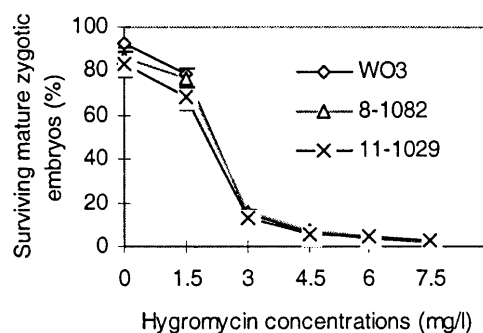


Fig. 2 Influence of hygromycin concentrations on the survival of mature zygotic embryos of three families of loblolly pine (*Pinus taeda*). Each treatment was replicated three times, and each replicate consisted of 30–90 mature zygotic embryos. Data are means \pm SD

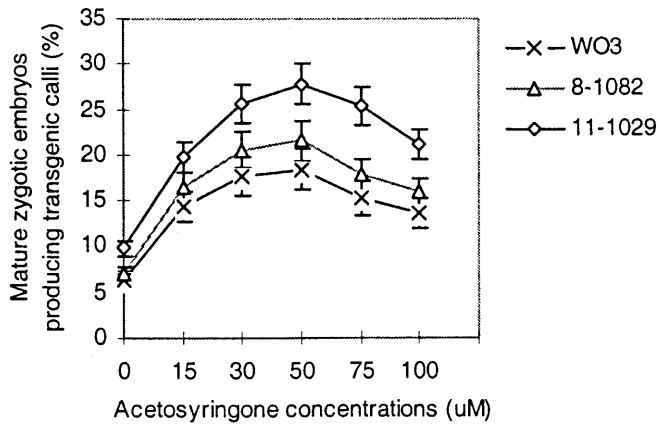


Fig. 3 Influence of acetosyringone concentrations on hygromycin-resistant callus formation of mature zygotic embryos of three families of loblolly pine. Each treatment was replicated three times, and each replicate consisted of 30–90 mature zygotic embryos. Data are means \pm SD

Three to five weeks after inoculation, callus was formed on cotyledons, hypocotyls, and radicles of mature zygotic embryos inoculated with bacteria. Proliferation of transgenic calli was achieved by subculturing these calli on fresh callogenesis medium with selected antibiotics.

Expression of the GUS gene in transformed tissues

The frequency of GUS expression among embryos and the number of blue spots per embryo varied between families (Table 3). The highest frequency of GUS-expressing embryos was obtained from family 11-1029 (100%). In a culture period of 5 weeks after infection, mature zygotic embryos of family 11-1029 had the highest mean number of blue spots per embryo (322 blue spots per embryo). Transient GUS expression was observed in cotyledons, hypocotyls, and in radicles of the mature zygotic embryos of genotypes WO3, 8-1082, and 11-1029. Transient GUS expression was observed mostly in cotyledons in other genotypes. Hygromycin-resistant callus with GUS expression was obtained from these genotypes tested. Activity of GUS enzyme was not detected in control embryos. A longer co-cultivation period (up to 2 weeks) and a longer sonication time (more

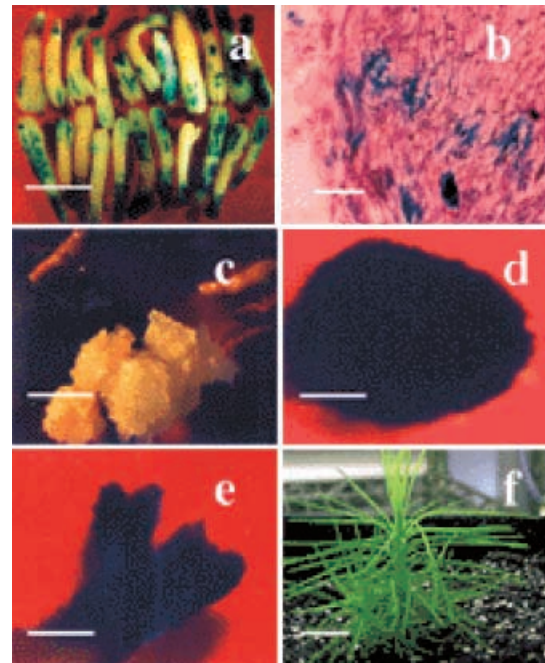


Fig. 4a–f *Agrobacterium tumefaciens*-mediated transformation and transgenic plant regeneration in loblolly pine. **a** GUS expression in embryos treated with acetosyringone, which was added to callus-induction medium, and either sonicated (*upper row*) or not sonicated (*lower row*). **b** GUS-expressing cells in callus derived from cotyledons. **c** Hygromycin-resistant calli derived from cotyledons, hypocotyl, and radicle. **d** GUS-expressing hygromycin-resistant calli derived from cotyledons. **e** GUS-expressing and hygromycin-resistant adventitious shoots. **f** Putative transgenic regenerated plantlet established in soil. Bars = 0.1 mm (**b**), 0.5 cm (**a**, **c–e**), 1 cm (**f**)

than 60 s) resulted in more embryos showing positive GUS expression, but also led to a considerable decrease in embryo survival. GUS expression was observed in adventitious buds regenerated from callus derived from co-cultivated mature zygotic embryos co-cultivated with *Agrobacterium* for genotypes WO3, 8-1082, and 11-1029. Figure 4 shows representative photographs of different stages in the process.

To improve the transformation efficiency, acetosyringone (virulence inducer) was added to media during the period of co-cultivation. The frequency of GUS-expressing embryos and hygromycin-resistant callus

Table 3 Influences of acetosyringone and sonication on transformation frequency of loblolly pine

Genotypes	Number of blue GUS spots per embryo			
	Control (no sonication, no acetosyringone)	Acetosyringone (50 μM)	Sonication (30 s)	Acetosyringone (50 μM) + sonication (30 s)
WO3 ^a	17 \pm 4c	82 \pm 5b	65 \pm 6b	281 \pm 17a
8-1082	32 \pm 5c	91 \pm 8b	89 \pm 10b	302 \pm 19a
11-1029	55 \pm 6c	112 \pm 9b	108 \pm 15b	321 \pm 21a

^aEmbryos were transformed with *A. tumefaciens* GV3101 (pPCV6NFHygGUSINT); and the number of blue GUS spots per embryo was obtained by counting 10–15 embryos randomly selected from among those expressing GUS. Data are shown as means \pm SD. Values followed by different letters are significantly different ($\alpha=0.05$) by ANOVA

formation was improved. Addition of acetosyringone to co-cultivation medium is more efficient for improving transformation efficiency than addition to the bacterial growth medium (data not shown). Sonication of mature zygotic embryos increased the frequency of GUS-expressing embryos and the number of blue spots per embryo. For most of the genotypes tested, acetosyringone and sonication synergistically enhanced *A. tumefaciens*-mediated transformation frequency in loblolly pine (Table 3). Histological observation of GUS-expressing embryos and calli showed that transformed cells may be on the surface or/and inside of cotyledons or the cambium of hypocotyls (data not shown), and on the surface or/and inside of calli derived from cotyledons, hypocotyls, and radicles of transformed embryos.

Differentiation of transgenic shoots and plant regeneration

Nine to twelve weeks after hygromycin-resistant callus was transferred to organogenesis medium, adventitious buds were formed on the callus surface. The frequency of adventitious bud formation on organogenesis medium containing BA and IBA in the 12th week of culture ranged from 8 to 20% (Fig. 5). Both rooting of adventitious buds and acclimatization of regenerated plantlets were carried out according to a procedure previously described (Tang et al. 1998). Rooting frequencies of 4–17% were observed and the growth and phenotype of regenerated transformed plantlets appeared similar to the non-transformed controls. Twenty-seven regenerated plantlets from three families of loblolly pine were transferred from culture flasks into a perlite/peatmoss/vermiculite (1:1:1, by vol.) soil mixture, and nineteen acclimatized plantlets were successfully established in soil. Fluorometric assay of GUS activity in transgenic tissues and plantlets of different families of loblolly pine showed that the highest levels of GUS activity were obtained from needles of transgenic plantlets of three genotypes (Fig. 6).

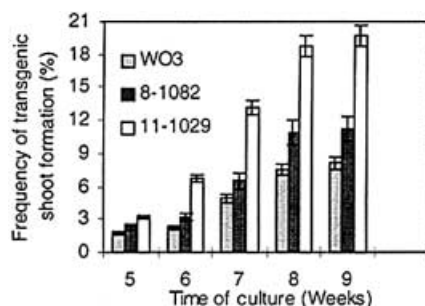


Fig. 5 Differences between families of loblolly pine in the regeneration frequency (%) of transgenic adventitious buds. Each treatment was replicated 4 times, and each replicate consisted of 30–150 callus tissues. Data are means \pm SD

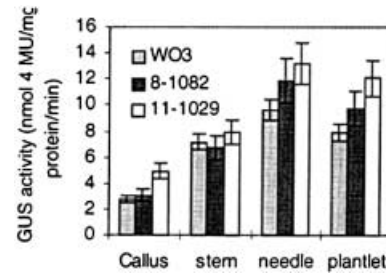


Fig. 6 Fluorometric assay of GUS activity in transgenic tissues and regenerated plantlets of different families of loblolly pine. Each treatment was replicated three times, and each replicate consisted of 300–500 mg of tissue. 4 MU 4-Methylumbelliferone. Data are means \pm SD

PCR analysis of the transformants

PCR analysis was carried out as a rapid identification for the insert DNA in hygromycin-resistant calli from three families (WO3, 8-1082, and 11-1029) of loblolly pine. The expected 717-bp band (for *aph(3')II*) and/or 596-bp band (for *hpt*) were amplified in the hygromycin-resistant GUS-expressing calli. No 717-bp band (for *aph(3')II*) and/or 596-bp band (for *hpt*) were amplified

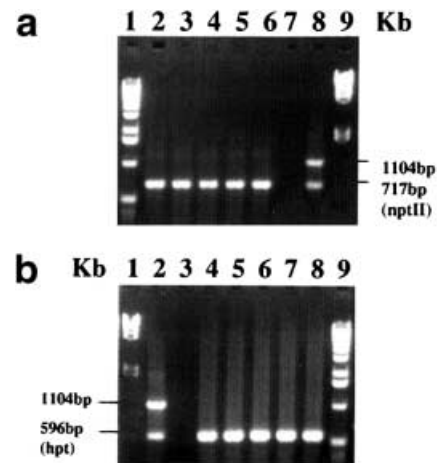


Fig. 7a, b PCR analysis of DNA isolated from putative transgenic plantlets of loblolly pine. **a** Detection of insert *aph(3')II* (*nptII*) gene from plasmid pPCV6NFHygGUSINT. Lane 1 1-kb molecular markers (Gibco-BRL); lanes 2–6 transgenic regenerated plants from genotypes WO3 (lane 2), 8-1082 (lanes 3, 4), and 11-1029 (lanes 5, 6), respectively; lane 7 non-transgenic regenerated plantlet control; lane 8 pPCV6NFHygGUSINT plasmid control (1,104-bp band containing a 300-bp DNA fragment outside right border of T-DNA); lane 9 λ DNA *HindIII* molecular markers (Gibco-BRL). **b** Detection of insert *hpt* gene from plasmid pPCV6NFHygGUSINT. Lane 1 λ DNA *HindIII* molecular markers (Gibco-BRL); lanes 2 pPCV6NFHygGUSINT plasmid control (1,104-bp band containing a 300-bp DNA fragment outside right border of T-DNA); lane 3 non-transgenic regenerated plantlet control; lanes 4–8 transgenic regenerated plants from genotypes WO3 (lane 4), 8-1082 (lanes 5, 6), and 11-1029 (lanes 7, 8), respectively; lane 9 1-kb molecular markers (Gibco-BRL). The absence of the 1,104-bp band amplified by primers P3 and nrp from templates obtained from transgenic plantlets suggests that little if any residual *Agrobacterium* remains in the plantlets, and that the T-DNA is integrated into the pine genome

in the non-transformed calli. Putative transgenic plantlets derived from hygromycin-resistant calli from three families of loblolly pine were also checked by PCR. All of the hygromycin-resistant plantlets tested showed the 717-bp band and/or 596-bp band (Fig. 7a, b). These PCR results confirm that the regenerated plantlets from hygromycin-resistant calli contain the transgene derived from the pPCV6NFHygGUSINT plasmid. Southern hybridization of genomic DNA was carried out to confirm that the T-DNA was integrated into the plant genome.

Southern blot analysis

Trangenic plantlets from independent transformation events of all three families (WO3, 8-1082, and 11-1029) were analyzed by Southern hybridization. Two restriction enzymes were used together in digestion of genomic DNA; *EcoRI* recognizes sites within the T-DNA (Fig. 1), while *HindIII* has no recognition site within the T-DNA but will cut at the flanking *HindIII* site in genomic DNA. This digestion should yield a different size fragment for each insertion site of T-DNA into pine genomic DNA. No bands were detected in non-transformed plants, whereas bands were observed in transgenic plants. These results confirm the presence of foreign genes integrated into the *Pinus taeda* genome. The Southern blots for transformed plants showed single bands representing junctions between T-DNA and adjacent plant DNA (Fig. 8).

Plant DNA/T-DNA junction analysis

Anchored PCR was utilized to isolate right-border insertion fragments from transgenic plants derived from families WO3, 8-1082, and 11-1029. The first PCR amplification normally does not produce a visible band on agarose gels stained with ethidium bromide because the

Fig. 9 Plant DNA/T-DNA junction analysis. (T) The right border T-DNA region from pPCV6NFHygGUSINT, indicating the right border (*double underlined*) and break sites (indicated by *arrow-heads*) in the T-DNA are shown. (A)–(C) Sequence from each anchored PCR fragment including the same region from T-DNA (*underlined*). (A) Family WO3, (B) family 8-1082, (C) family 11-1029. The junction fragments from different parts of the same plant have the same sequence

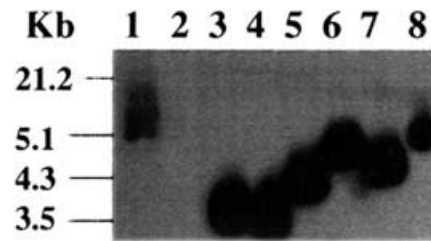
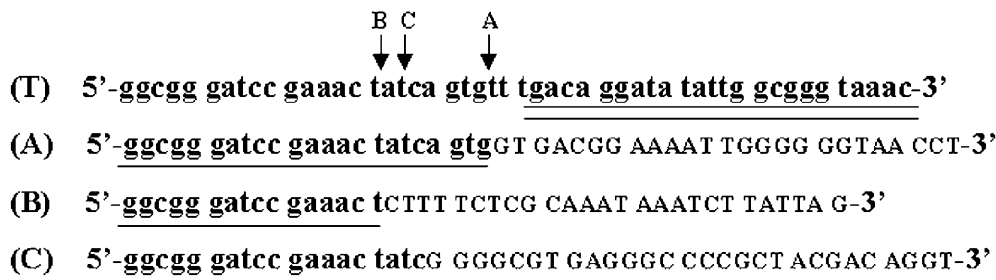


Fig. 8 DNA was digested overnight with the restriction enzymes *EcoRI* and *HindIII* (Boehringer Mannheim) and was hybridized (at 65 °C) with the *aph(3')II* probe (717-bp PCR product). Lane 1 Plasmid DNA of pPCV6NFHygGUSINT (5 µg); lane 2 DNA from a non-transformed plant; lanes 3–8 DNA from transgenic plants of genotypes WO3 (lanes 3, 4), 8-1082 (lanes 5, 6), and 11-1029 (lanes 7, 8), respectively (30 µg each)

copy number of the fragment that can be amplified in the ligation mixture is too low. Nested PCR primers are used and the second PCR usually produces visible bands that can be used for sequencing. Sequencing of second PCR products from these putative transgenic plants showed that all these transgenic plants have plant DNA/T-DNA insertion fragments (Fig. 9), which is consistent with the southern blot data (single-copy insertion) above. Our results also demonstrated that the same DNA/T-DNA junction sequence was obtained from different parts (roots, stems, and needles) of a transgenic plant.

Discussion

The data on *A. tumefaciens*-mediated transformation of different genotypes of loblolly pine presented here show a useful procedure for the transformation and regeneration of transgenic loblolly pine plantlets. This is the first report on transgenic plantlet regeneration via *A. tumefaciens*-mediated transformation in loblolly pine. Regeneration varies between open-pollinated families of loblolly pine, but several families show high regeneration potential. The efficiency of the transformation events was dependent on the families and on the organs and tissues infected. High transformation rate and GUS expression were observed in families WO3, 8-1082 and 11-1029, and lower transformation rates were observed in other families. Among different tissues, high GUS expression was observed in cotyledons, hypocotyls, and radicles of genotypes WO3, 8-1082 and 11-1029, and in cotyledons of other genotypes. These results are different

from those of Tzfira et al. (1996), who found that *Pinus halepensis* embryos transformed with *A. rhizogenes* showed GUS expression primarily in radicles. We attribute this difference to a more rapid proliferation of callus in our system.

Acetosyringone is a phenolic compound that is released by the wounded plant cells and is a virulence inducer similar to syringaldehyde (Stachel et al. 1985). It plays an important role in the natural infection of plants by *A. tumefaciens* because it activates the virulence genes of the Ti plasmid and initiates the transfer of the T-DNA region into plant cells. A positive effect of phenolic compounds on *A. tumefaciens*-mediated transformation has been demonstrated in many plant species, such as *Arabidopsis thaliana* (Sheikholeslam and Weeks 1987), *Nicotiana tabacum* (Godwin et al. 1991), *Cucumis sativus* (Sarmiento et al. 1992), and *Malus pumila* (James et al. 1993). An increase in transformation efficiency upon addition of acetosyringone was also reported in hybrid larch (Levee et al. 1997) and Norway spruce (Wenck et al. 1999). The addition of 30–75 μ M acetosyringone during the co-cultivation step increased the induction frequency of hygromycin-resistant calli and GUS-expressing tissues. Sonication, in most cases, increased the frequency of GUS-expressing embryos and the number of blue spots per embryo. This was useful to optimize the conditions for transformation by *A. tumefaciens*, and led to the establishment of stable transformation in loblolly pine. Southern blot results show that these are transformants in which the GUS gene has typically integrated at one site in the plant genome (this is confirmed by the plant DNA/T-DNA junction analysis). This is different from the results of transformation of *Pinus radiata* via particle bombardment, where the integration of tandem or multimer copies in transformed plants was also observed (Walter et al. 1998). The same DNA/T-DNA junction sequence was obtained from different source tissues of a specific transgenic plant in this investigation. This demonstrates that one specific transgenic line originated from only one single cell.

Other transformation methods reported for generation of transgenic pine plantlets include *Agrobacterium* transformation of embryogenic cultures (Levee et al. 1999) and biolistic transformation of embryogenic cultures (Walter et al. 1998). The method reported here uses mature zygotic embryos as explants, while most embryogenic cultures of pine must be initiated from immature embryos, which typically are collected during a brief period after fertilization occurs in early summer. Mature seeds are available year-round and can be stored at 4 °C, unlike embryogenic cultures which frequently lose embryogenic potential upon long-term culture and must be preserved in cryogenic storage to allow continued availability. The method described in this report is likely to be very useful for smaller laboratories that do not have the resources to initiate, maintain and preserve embryogenic cultures of loblolly pine. The advantage of embryogenic cultures, of course, is in the much larger numbers of plantlets that can be obtained from a single

culture. Thousands or perhaps millions of mature somatic embryos could be produced from a single transformation event, while the organogenesis method used here will produce at most 100–200 plantlets. Further multiplication of those plantlets could be achieved using rooted cuttings, however, and the relative merits of these alternative systems will in the final analysis depend on the specific objectives of the laboratory conducting the work. The transformation and regeneration method we described should prove useful for experimental analysis of gene function in transgenic loblolly pine.

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References

- Birch RG (1997) Plant transformation: Problems and strategies for practical application. *Annu Rev Plant Physiol Plant Mol Biol* 48:297–326
- Charest PJ, Michel MF (1991) Basis of plant genetic engineering and its potential application to tree species. Petawawa National Forestry Institute, Forestry Canada, Information Report PI-X-104:48
- Charest PJ, Devantier Y, Lachance D (1996) Stable genetic transformation of *Picea mariana* (black spruce) via microprojectile bombardment. *In Vitro Cell Dev Biol* 32:91–99
- Ellis DD, McCabe DE, Mcinnis S, Ramachandran R, Russell DR, Wallace KM, Martinell BJ, Roberts DR, Raffa KF, McCown BH (1993) Stable transformation of *Picea glauca* by particle acceleration. *Biotechnology* 11:84–89
- Godwin I, Gordon T, Ford-lloyd B, Newbury HJ (1991) The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Rep* 9:671–675
- Goldfarb B, Strauss SH, Howe GT, Zaerr JB (1991) Transient gene expression of microprojectile-introduced DNA in Douglas fir cotyledons. *Plant Cell Rep* 10:517–523
- Hansen G, Chilton MD (1996) “Agrolistic” transformation of plant cells: Integration of T-strands generated in planta. *Proc Natl Acad Sci USA* 93:14978–14983
- Huang Y, Diner AM, Karnosky DF (1991) *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix decidua*. *In Vitro Cell Dev Biol* 27:201–207
- Humara JM, Lopez M, Ordas RJ (1999) *Agrobacterium rhizogenes*-mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of *uidA* gene transfer. *Plant Cell Rep* 19:51–58
- James DJ, Uratsu S, Cheng J, Negri P, Viss P, Dandekar AM (1993) Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. *Plant Cell Rep* 12:559–563
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6:3901–3907
- Klimaszewska K, Devantier Y, Lachance D, Lelu MA, Charest PJ (1997) *Larix laricina* (tamarack): somatic embryogenesis and genetic transformation. *Can J For Res* 27:538–550
- Levee V, Lelu MA, Jouanin L, Cornu D, Pilate G (1997) *Agrobacterium tumefaciens*-mediated transformation of hybrid larch

- (*Larix kaempferi* (*L. decidua*) and transgenic plant regeneration. *Plant Cell Rep* 16:680–685
- Levee V, Garin E, Klimaszewska K, Seguin A (1999) Stable genetic transformation of white pine (*Pinus strobus* L.) after cocultivation of embryogenic tissues with *Agrobacterium tumefaciens*. *Mol Breed* 5:429–440
- Loopstra CA, Stomp AM, Sederoff RR (1990) *Agrobacterium*-mediated DNA transfer in sugar pine. *Plant Mol Biol* 15:1–9
- Mathur J, Szabados L, Schaefer S, Grunenberg B, Lossow A, Jonas-Straube E, Schell J, Koncz C, Koncz-kalman Z (1998) Gene identification with sequenced T-DNA tags generated by transformation of *Arabidopsis* cell suspension. *Plant J* 13:707–716
- Meyer P (2000) Transcriptional transgene silencing and chromatin component. *Plant Mol Biol* 43:221–234
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York
- Sarmiento GG, Alpert K, Tang FA, Punja ZK (1992) Factors influencing *Agrobacterium tumefaciens*-mediated transformation and expression of kanamycin resistance in pickling cucumber. *Plant Cell Tiss Org Cult* 31:185–193
- Sederoff R, Stomp AM, Chilton WS, Moore LW (1986) Gene transfer into loblolly pine by *Agrobacterium tumefaciens*. *Biotechnology* 4:647–649
- Sheikholeslam SN, Weeks DP (1987) Acetosyringone promotes high frequency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. *Plant Mol Biol* 8:291–298
- Stachel SE, Messens E, Montague MV, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318:624–629
- Stomp AM, Loopstra C, Chilton WS, Sederoff RR, Moore LW (1990) Extended host range of *Agrobacterium tumefaciens* in the genus *Pinus*. *Plant Physiol* 92:1226–1236
- Stomp AM, Weissinger A, Sederoff RR (1991) Transient expression from microprojectile-mediated DNA transfer in *Pinus taeda*. *Plant Cell Rep* 10:187–190
- Tang W, Ouyang F, Guo ZC (1998) Plant regeneration through organogenesis from callus induced from mature zygotic embryos of loblolly pine. *Plant Cell Rep* 17:557–560
- Tian L, Seguin A, Charest PJ (1997) Expression of the green fluorescent protein gene in conifer tissues. *Plant Cell Rep* 16:267–271
- Trick HN, Finer JJ (1997) SAAT: sonication-assisted *Agrobacterium*-mediated transformation. *Transgenic Res* 6:329–336
- Tzfira T, Yarnitzky O, Vainstein A, Altman A (1996) *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepensis* Mill. *Plant Cell Rep* 16:26–31
- Wagner DB, Furnier GR, Saghai-Maroo MA, Williams SM, Dancik BW, Allard RW (1987) Chloroplast DNA polymorphisms in lodgepole and jack pine and their hybrids. *Proc Natl Acad Sci USA* 84:2097–2100
- Walter C, Smith DR, Connett MB, Grace L, White DWR (1994) A biolistic approach for the transfer and expression of a *gusA* reporter gene in embryogenic cultures of *Pinus radiata*. *Plant Cell Rep* 14:69–74
- Walter C, Grace LJ, Wagner A, White DWR, Walden AR, Donaldson SS, Hinton H, Gardner RC, Smith DR (1998) Stable transformation and regeneration of transgenic plants of *Pinus radiata* D. Don. *Plant Cell Rep* 17:460–469
- Walter C, Grace L, Donaldson SS, Moody J, Gemmill JE, van der Maos S, Kvaalen H, Lonneborg A (1999) An efficient Biolistic transformation protocol for *Picea abies* embryogenic tissue and regeneration of transgenic plants. *Can J For Res* 29:1539–1546
- Wenck AR, Quinn M, Whetten RW, Pullman G, Sederoff R (1999) High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). *Plant Mol Biol* 39:407–416
- Zhou YX, Newton RJ, Gould JH (1997) A simple method for identifying plant/T-DNA junction sequences resulting from *Agrobacterium*-mediated DNA transformation. *Plant Mol Biol Rep* 15:246–254