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Transformation of *Rhododendron* through microprojectile bombardment

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Abstract A method for producing transgenic *Rhododendron* plants via microprojectile bombardment was developed. Leaves from in vitro microshoots of *R. catawbiense* cv. Album Michx. were bombarded with the marker gene *uidA* (coding for β -glucuronidase, GUS) or *smGFP* (green fluorescent protein, GFP) in combination with *nptII* (neomycin phosphotransferase). Transgenic plants were regenerated under a biphasic selection strategy with initial selection on 50 mg/l kanamycin followed by stringent selection on 100 mg/l kanamycin. GFP expression was detected in leaf callus from transgenic plants. Histochemical GUS assays of transformed tissues indicated uniform expression throughout the transgenic plant, including leaves, stems, and roots. GUS expression was stable during all stages of plant development over 2 years, as demonstrated by characteristic blue staining of vegetative and floral buds. Molecular characterization of the transformants indicated that the transgenes were stably integrated into the *Rhododendron* genome through ten vegetative generations.

Keywords Woody ornamental · Biolistics · Transformation · Gene transfer

Abbreviations *2,4-D*: 2,4-Dichlorophenoxy acetic acid · *GFP*: Green fluorescent protein · β -*GUS*: β -Glucuronidase · *IBA*: Indole-3-butyric acid · *2iP*: 6-(γ - γ -Dimethylallylamino)purine · *nptII*: Neomycin phosphotransferase · *PCR*: Polymerase chain reaction · *uidA*: Gene encoding GUS · *WP*: Woody plant medium

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Introduction

Hybrid rhododendrons (*Rhododendron* L., Ericaceae Juss.) are some of the most popular flowering, broadleaf evergreens. Horticultural improvements in *Rhododendron* require long periods to produce flowering plants by traditional breeding methods. In addition, new trait development by conventional genetics is limited to existing germplasm. Genetic engineering approaches to horticultural improvement offer the possibility for introducing new traits using foreign DNA from diverse sources. Modifications of *Rhododendron* for disease and stress resistance, enhanced flower color, plant morphology, and growth habit are of major commercial importance and may be amenable to modern improvement methods based on gene transfer technology.

Previous research has explored the feasibility of incorporating selectable markers and reporter genes into *Rhododendron* via *Agrobacterium*-mediated transformation (Ueno et al. 1996; Pavingerová et al. 1997; Tripepi et al. 1999). Although transformed plants were recovered, regenerants showed various levels of chimerism (Ueno et al. 1996; Pavingerová et al. 1997). In addition, individual cultivars differed in their susceptibility to *Agrobacterium* (Tripepi et al. 1999), thereby making this system less than optimum. An alternative to *Agrobacterium*-mediated gene transfer is microprojectile bombardment. Biolistics has been used successfully for a number of woody species, including eucalyptus (Serrano et al. 1996), spruce (Tian et al. 2000) and grapevine (Kikkert et al. 1996). Using this method, Hsia and Korban (1998) demonstrated transient GUS expression in leaves and shoot tips of *Rhododendron* but failed to regenerate transgenic plants. Here we report the results of our study on biolistic transformation of *Rhododendron* leaf explants and the first successful recovery of transgenic *Rhododendron* using this method.

Materials and methods

Plant material

Rhododendron propagation and tissue culture were performed according to Brand and Kiyomoto (1997). Basal woody plant (WP) medium (Lloyd and McCown 1980) (pH 5.2 prior to autoclaving) containing 3% sucrose, 0.3% agar (Sigma, St. Louis, Mo.), and 0.1% Phytigel (Sigma) was used for all formulations. For shoot multiplication, WP was supplemented with 10 μ M 2iP (shoot multiplication medium, SMM). For shoot regeneration via organogenesis from excised leaves, WP medium was supplemented with 30 μ M 2iP and 1 μ M IBA (shoot induction medium, SIM).

Recently expanded shoots were cut from a mature *Rhododendron catawbiense* cv. Album Michx. plant using an ethanol-sterilized knife. Shoots were stripped of leaves, trimmed to 4 cm, washed in soapy water, and rinsed under running tap water for 5 min. They were then disinfested by agitation for 15 min in 10% commercial bleach (5.25% sodium hypochlorite) containing five drops of Tween 20 per 500 ml. Following three rinses in sterile distilled water, shoots were cut to 2 cm and placed on SMM medium in individual vessels.

During the establishment phase, explants were grown for the initial two subculture periods on 15 ml SMM in 25×150-mm culture tubes sealed with clear polypropylene caps. The cultures were thereafter maintained on 30 ml SMM in 200-ml glass jars sealed with B-caps (Magenta, Chicago, Ill.). Six explants, apical and multi-nodal segments (1–2 cm long), were transferred to each jar at subculture every 4 weeks. Leaves were excised from in vitro shoots and placed adaxial side up on SIM in petri dishes. All cultures were sealed with Handi-Wrap and incubated at 24 $^{\circ}$ ±2 $^{\circ}$ C under a 16/8-h (day/night) photoperiod with light provided by cool-white fluorescent tubes at an intensity of 40 μ mol m $^{-2}$ s $^{-1}$. Shoots regenerated from more than 98% of the leaf explants (22.2±1.3 shoots/leaf) in 6–8 weeks.

Plant gene expression vectors

Plasmid pFF19K contains the coding region of *nptII* for antibiotic selection, and pFF19G contains the *uidA* coding sequence to be used as a visible marker. Both are under the regulatory control of the CaMV 35S promoter, enhancer, and terminator (Fig. 1) (Timmermanns et al. 1990). Plasmid pCD3-326 contains the solubility-modified GFP coding sequence under the regulatory control of the CaMV 35S promoter and the *nos* terminator (Fig. 1) (Davis and Vierstra 1998).

Rhododendron transformation

One week prior to bombardment, leaves were excised from in vitro plantlets and incubated on SIM medium under the conditions described above. One hour prior to bombardment, 40 leaves were arranged in an open circle, adaxial side up, in the center of each petri dish containing SIM supplemented with 100 g/l sucrose, as an osmotic treatment. Explants were bombarded with pFF19K in combination with pCD3-326 or pFF19G using the Bio-Rad PDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, Calif.).

Plasmid DNA was precipitated onto 1- μ m gold particles as described by Kausch et al. (1995) with several modifications. Gold particles (60 mg) were resuspended in 1 ml 100% ethanol, and 35 μ l of this suspension (2.1 mg) was used to prepare five bombardments. The particles were pelleted by a brief centrifugation, the ethanol was removed, and the pellet was washed with 1 ml sterile water. The water was then removed and replaced by 25 μ l DNA (1 mg/ml) (equimolar concentrations of co-transforming plasmids). The following were added in the order mentioned, with mixing between each addition: 225 μ l sterile water, 250 μ l 2.5 M CaCl $_2$, and 50 μ l 0.1 M spermidine. The suspension was vortexed for 10 min at 4 $^{\circ}$ C. The DNA-coated particles were pelleted by

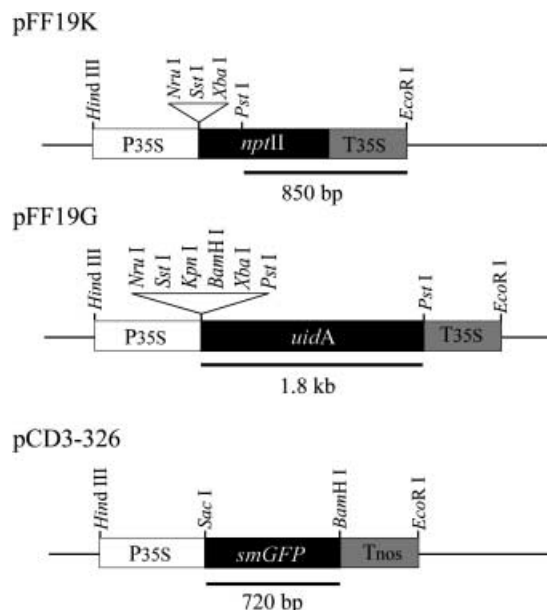


Fig. 1 Schematic representation of constructs used for *Rhododendron* transformation. Fragments of each construct used for probing Southern blots are underlined and the size of the fragment indicated

centrifugation at 500 rpm for 5 min, washed with 100% ethanol, and resuspended in a final volume of 55 μ l of 100% ethanol (anhydrous). The suspension was sonicated for 10 s immediately before dispensing. Ten-microliter aliquots of the DNA suspension was placed onto each macrocarrier and allowed to air dry. The bombardment parameters were: rupture discs, 7,584 kPa (1,100 psi) (Bio-Rad); rupture-disc-to-macrocarrier gap distance, 10 mm; macrocarrier fly distance, 10 mm; stopping screen-to-target distance, 5 cm; partial vacuum, 94.82 kPa (28 inches Hg).

Selection of transformants

After particle bombardment, leaves were transferred to SIM and allowed to recover 48 h in the absence of selection. After this recovery period, tissues were transferred to SIM containing a moderate level (50 mg/l) of kanamycin. After 4 weeks on this medium, tissues were transferred to medium containing 100 mg/l kanamycin, and incubated as described previously. Three shoots (BTR-1, BTR-2, BTR-3) regenerated from bombarded leaves after 4 months and were transferred to SMM containing 100 mg/l kanamycin.

Rooting, acclimatization, and plant growth

Apical and multi-nodal pieces (1–2 cm long) were used to perpetuate the cultures as described previously. Transgenic shoots (3 cm long) were rooted in Redi-earth Plug and Seedling Mix (The Scotts Co, Marysville, Ohio) in covered, clear plastic trays, and the humidity was gradually decreased to ambient conditions following an 8-week rooting period. Rooted plants were transferred to Metro-mix 360 (The Scotts Co) in 98-cell plug trays and kept under mist and 50% shade for 1 week, then transferred to the greenhouse. Plants were grown under a 16/8-h (day/night) photoperiod with light provided by high-intensity discharge lamps and fertilized with 200 ppm N (Peters 20-20-20 General Purpose, The Scotts Co) once every 2 weeks. After 2 months, plants were transferred to 4-inch plastic pots with Metro-mix 510 (The Scotts Co) and grown under the same regime for 6 months. The plants were transplanted to 1-gallon pots (300S Nursery Supplies, Fairless Hills, Penn.) in a soil-less mix consisting of 2:1:1 composted

bark:sand:peat and grown outdoors in a 50% shade-covered hoop-house. They were over-wintered in an unheated, white plastic-covered hoop-house.

Nucleic acid isolation and molecular analysis

PCR analysis of primary transformants was completed with DNA extracted (Knapp and Chandlee 1996) from approximately 100 mg of tissue from shoots and/or callus that proliferated in the presence of kanamycin after 4 months of selection and from unbombarded controls of the same age. As plant tissues became available through the subculture of shoots, PCR analysis was repeated twice with DNA extracted from three to four leaves.

PCR analysis was completed using primers specific for internal regions of the gene coding sequences of (1) *nptII*: 5'-GAA-CAAGATGGATTGCAC-3' and 5'-GAAGAACTCGTCAAGAA-GG-3', which yields an 800-bp amplification; (2) *uidA*: 5'-CGT-CCTGTAGAAACCCCAAC-3' and 5'-CCAATCCAGTCCATTATGC-3', which yields a 950-bp amplification product; (3) GFP: 5'-GTGAAGGTGATGCAACATACG-3' and 5'-GCAGCTGTTC-CAAACTCAAG-3', which yields a 583-bp product. Twenty-five-microliter reactions were performed in 1× *Taq* polymerase buffer without MgCl₂ (Promega, Madison, Wis.), 2.5 mM MgCl₂, 0.2 mM each dNTP, 0.5 μM each primer, 0.5 U *Taq* polymerase (Promega). PCR amplification parameters included an initial 5-min denaturation at 95°C followed by 30 cycles of 45 s at 95°C, 45 s at 58°C, 60 s at 72°C, and a final 5-min extension at 72°C in a PTC-100 programmable thermal controller with a heated lid (MJ Research, Watertown, Mass.). PCR products were separated on a 1.0% agarose gel and visualized with ethidium bromide (Sambrook et al. 1989).

Genomic DNA (10 μg) from BTR-1 and BTR-3 plantlets regenerated under the selection conditions described above was digested with *PstI/EcoRI* and with *HindIII* (Promega) for probing with *nptII*. BTR-1 was digested with *HindIII* and *BamHI/SacI* for probing with GFP. BTR-3 was digested with *PstI/EcoRI* and *BamHI/HindIII* for probing with *uidA*. All digested DNAs were electrophoresed on 1% agarose gels and visualized with ethidium bromide. The DNAs were blotted to Hybond-N+ positively-charged nylon membranes (Amersham-Pharmacia Biotech, Piscataway, N.J.) with 10× SSC (0.15 M sodium citrate, 1.5 M sodium chloride) according to the manufacturer's recommended procedure. DNA was fixed to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, Calif.). The 850-bp *PstI/EcoRI* fragment of pFF19K, the 720-bp *BamHI/SacI* fragment of pCD3-326, and the 1.8-kb *PstI* fragment of pFF19G were labeled according to the manufacturer's instructions (Amersham ECL). Blots were prehybridized, hybridized, and washed according to the manufacturer's recommendations. Signal generation and detection were performed as described by Amersham. Blots were placed in development folders (Gibco BRL, Gaithersburg, Md.) and exposed to Kodak X-Omat AR film for 4–12 h.

Histochemical GUS staining

BTR-3 shoots, flower buds, vegetative buds, and roots were stained for *uidA* expression by immersion in GUS staining solution containing 0.1 M sodium phosphate buffer, pH 7.0, 0.1% Triton-X100, and 0.5 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl glucuronic acid dissolved in N,N'-dimethylformamide) followed by vacuum infiltration at 94.82 kPa for 5 min for microshoots and for up to 30 min for vegetative and floral buds (modified from Jefferson 1989). After infiltration, all samples were incubated at 37°C overnight. Chlorophyll was removed from samples by incubation in 70% ethanol for 24 h.

Visualization of GFP

Leaves from BTR-1 cultures were excised from putatively transformed shoots and induced to form callus on WP medium supple-

mented with 10 μM 2iP and 10 μM 2,4-D (callus induction medium, CIM). Plates were incubated at 25°C in the dark for 4 weeks. GFP was visualized by illuminating calli with a hand-held long-wave ultraviolet lamp (Spectronics, Westbury, N.Y.) with an excitation wavelength of 365 nm. Fluorescing calli were photographed with Kodak Ektachrome 100 slide film.

Results and discussion

Transformation, selection and regeneration of transgenic plants

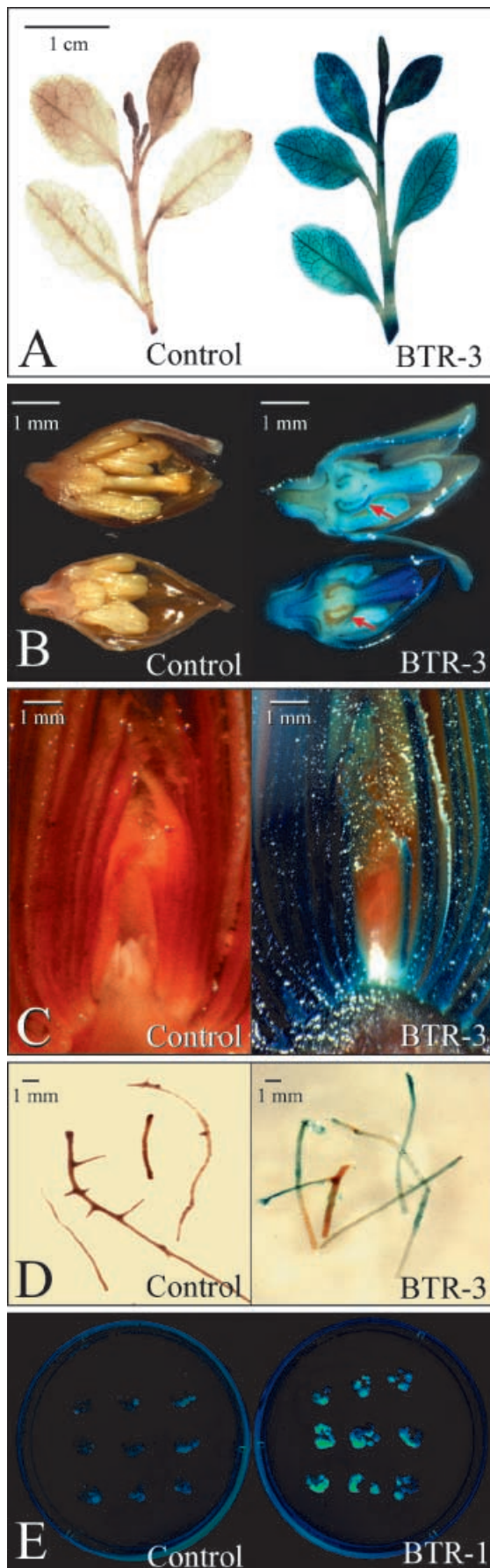
Three independent transformation events were recovered from a total of 1,500 bombarded leaves of *Rhododendron catawbiense* cv. Album. Three hundred leaves were bombarded with pFF19K in combination with pCD3-326 (BTR-1), 600 leaves were bombarded with pFF19K alone (BTR-2), and 600 leaves were bombarded with pFF19K in combination with pFF19G (BTR-3). One shoot from each of BTR-1 and BTR-2 and one green callus from BTR-3 developed from the cut edge of leaves after 4 months on selective medium. Transformation efficiency for this system (0.2%) was low compared to that (5%) reported by Ueno et al. (1996) when leaves and nodes were transformed with *Agrobacterium*. However, Hsia and Korban (1998) reported only one blue spot on a single leaf when rhododendron leaves were bombarded with the GUS gene, suggesting that the penetration of rhododendron leaf cells by gold particles is difficult, that wounding caused by the process results in a high incidence of cell death, or that rhododendron leaf tissues degrade exogenous DNA before transient expression or incorporation into the genome occurs.

All putatively transformed tissues transferred to SIM containing 100 mg/l kanamycin proliferated and formed multiple shoots. Apical and multi-nodal segments were subcultured, as described previously, on SMM medium containing 100 mg/l kanamycin to propagate putative transformants. During this culturing period BTR-2 grew slowly, and several segments died. After 3 months, explants were subcultured to SMM lacking kanamycin.

A high percentage of the microcuttings rooted (>95%) within 8 weeks when grown in humidity chambers in a seedling potting mix. Rooted plantlets were readily acclimated to greenhouse conditions with few losses. Transgenic and control (non-transformed) plants had similar morphology and growth rates (data not shown).

GUS and GFP analysis

Leaves, stems, vegetative buds, floral buds, and roots from BTR-3 produced the characteristic blue color in all tissues when stained for GUS expression (Fig. 2A–D), indicating that the CaMV 35S promoter drives expression ubiquitously in *Rhododendron* and that the plants were fully transformed rather than chimeric. Although some regions appeared to be lightly stained or unstained,



these regions were more likely the result of insufficient penetration by the staining solution. Rhododendron tissues are particularly waxy and often lignified, thereby making saturation with the GUS staining solution difficult (personal observation). The flower buds shown in Fig. 2B illustrate this phenomenon. The upper bud was completely stained light blue with dark-blue staining of the ovaries, whereas the lower bud showed stronger staining throughout the flower organs, but completely lacked blue color in the ovaries.

Due to high levels of autofluorescence, GFP was difficult to detect in the roots, shoots, or leaves when whole BTR-1 plants were illuminated. However, when calli generated from leaves were illuminated with a hand-held UV lamp at 365 nm, GFP fluorescence was clearly visible (Fig. 2E).

Molecular analysis

All three putative transformants were positive for the presence of *nptII* when DNA was analyzed by PCR (Fig. 3A), although the band from BTR-2 was faint. Additionally, BTR-1 was PCR-positive for GFP and BTR-3 was PCR-positive for *uidA* (Figs. 3B, 4C, respectively). In PCR reactions using DNA from subsequent extractions, no *nptII* amplification product was detected in BTR-2, but the 800-bp fragment was clearly visible in both BTR-1 and BTR-3. To test for chimerism, we transferred leaves from subcultured shoots of all three putative transformants back to SIM containing 100 mg/l kanamycin for a secondary screen. Only leaves from BTR-1 and BTR-3 were able to produce adventitious shoots on this medium. The lack of a PCR amplification product, the inability to form adventitious shoots on kanamycin, and its slow growth all indicate that the BTR-2 transformant was chimeric. The transformed tissues were either sacrificed for DNA extractions or were outgrown by non-transformed cells when transferred to medium lacking kanamycin, resulting in the loss of the transformant. Chimerism of transgenic rhododendrons generated through *Agrobacterium*-mediated methods has been reported by Pavingerová et al. (1997) and is likely the result of de novo meristem formation from multiple cells rather than from single cells. The screening of leaves from putatively transformed shoots in a second round of selection will therefore be an important tool for recovering fully transformed plants.

Southern analysis was performed on DNA from transgenic lines BTR-1 and BTR-3. For analysis of the selectable marker *nptII*, DNA was digested with *Pst*I and *Eco*RI to cut out a fragment containing the coding region and the CaMV 35S terminator and with *Hind*III to determine the number of inserts integrated into the plant ge-

Fig. 2A–E Expression of marker genes in transgenic rhododendron. **A–D** GUS expression in transformant BTR-3: **A** shoots, **B** flower buds (arrows indicate ovaries), **C** vegetative buds, **D** roots. **E** GFP visualization of calli from transformant BTR-1

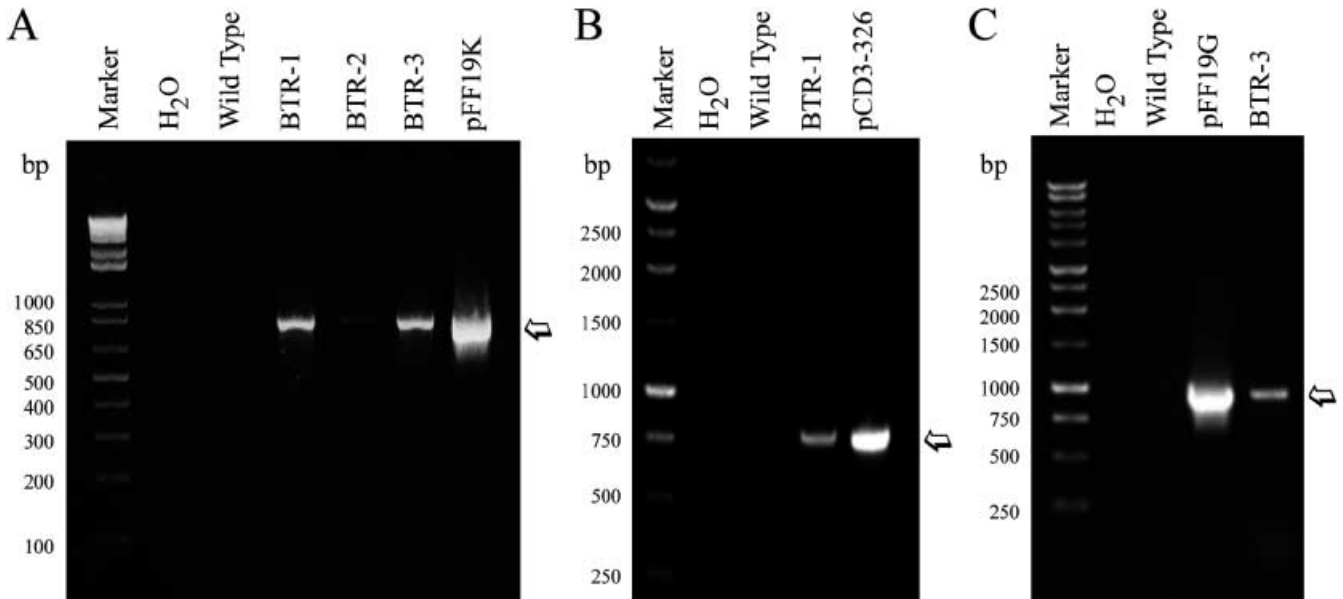


Fig. 3A–C PCR analysis for the presence of transgenes in primary transformants. **A** PCR amplification of *nptII*. Lanes: Marker 1-kb Ladder Plus (Gibco BRL, Gaithersburg, Md.), H_2O negative control reaction containing no DNA, *Wild Type* non-transformed *Rhododendron* DNA, *BTR-1* putative transformant co-bombarded with pFF19K and pCD3-326, *BTR-2* putative transformant bombarded with pFF19K and pFF19G, *BTR-3* putative transformant co-bombarded with pFF19K and pFF19G, pFF19K 1-ng positive control plasmid

DNA. The *arrow* indicates the expected 800-bp PCR amplification product. **B** PCR amplification of GFP. Lanes: Marker, H_2O , *Wild Type* and *BTR-1* as in **A**; pCD3-326 1-ng positive control plasmid DNA. The *arrow* indicates the expected 583-bp PCR amplification product. **C** PCR amplification of *uidA*. Lanes: Marker 1-kb DNA ladder (Promega, Madison, Wis.), H_2O and *Wild Type* as in **A**, pFF19G 1-ng positive control plasmid DNA, *BTR-3* as in **A**. The *arrow* indicates the expected 950-bp PCR amplification product

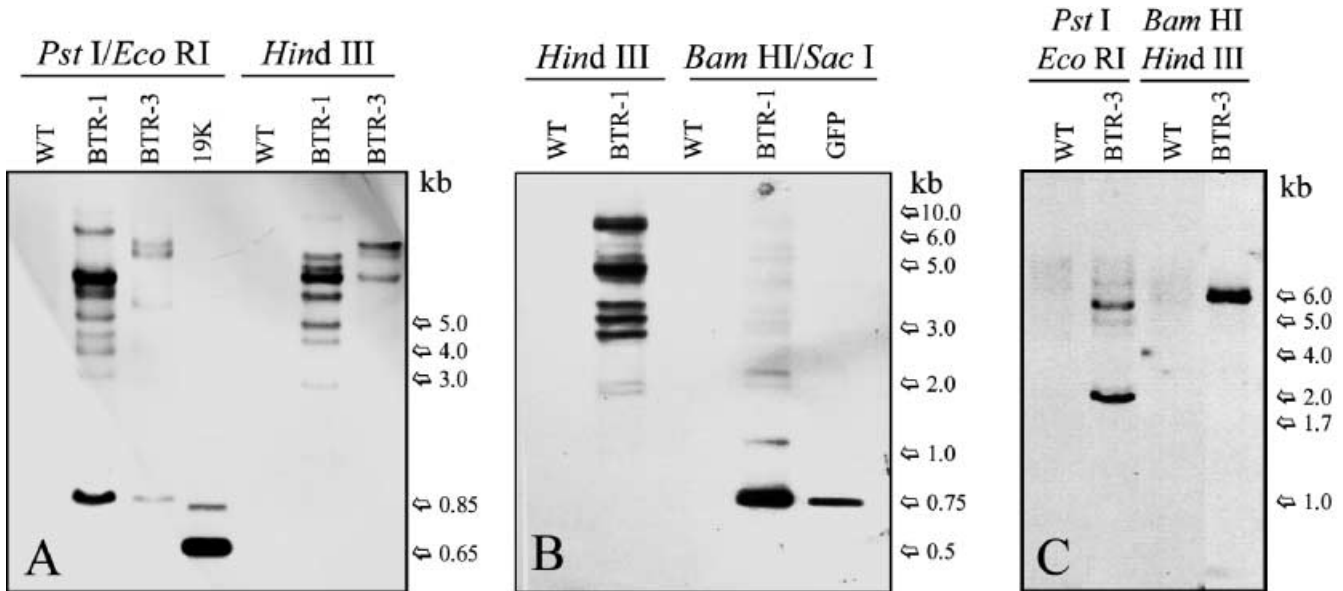


Fig. 4A–C Southern analysis of transformed rhododendrons. **A** DNA blot hybridized with the 850-bp *nptII*/CaMV terminator fragment of pFF19K. Lanes: *WT* Non-transformed rhododendron, *BTR-1* transformant co-bombarded with pFF19K and pCD3-326, *BTR-3* transformant co-bombarded with pFF19K and pFF19G, *19K* 20 pg of plasmid pFF19K positive control. **B** DNA blot hybridized with the 743-bp GFP coding sequence of pCD3-326. Lanes: *WT* and *TR-1* as in **A**; *GFP* 10 pg of plasmid pCD3-326 positive control. **C** DNA blot hybridized with 1.8-kb GUS coding sequence of pFF19G. Lanes: *WT* and *BTR-3* as in **A**. DNAs were digested with the enzymes indicated above lanes. *Arrows* indicate molecular weight size markers

nome (Fig. 4A). Both *BTR-1* and *BTR-3* contained multiple copies of *nptII*. *BTR-1* had a minimum of seven copies of the transgene, whereas *BTR-3* contained three copies of *nptII*, as observed in the lanes digested with *HindIII*. The expected 0.85-kb fragment was clearly visible in the *PstI/EcoRI* digest of *BTR-1* but only faintly visible in *BTR-3*, indicating either rearrangements of the transgenes or incomplete digestion of the DNA. *BTR-1* was further analyzed for the presence of GFP (Fig. 4B). DNA was digested with *HindIII* to determine number of

inserts and with *Bam*HI and *Sac*I to drop out the coding region. Ten copies of GFP were incorporated into the genome of this transgenic plant. A clean 0.75-kb fragment was observed when DNA was digested with *Bam*HI and *Sac*I, indicating rearrangements were absent within the coding regions of the transgenes. A Southern blot from DNA of BTR-3 probed with the *Pst*I fragment of pFF19G revealed the presence of three copies of the GUS transgene (Fig. 4C). The 1.8-kb fragment containing the coding region of *uidA* was clearly visible in the *Pst*I/*Eco*RI-digested DNA. Multiple bands in the high-molecular-weight range of the *Bam*HI/*Hind*III digest indicated incorporation into the plant genome. No hybridization signals were detected in total DNA from non-transformed control plants when probed with *nptII*, GFP, or *uidA* (lanes WT).

Interestingly, no gene silencing was observed in either transgenic line even though multiple copies of both transgenes were present and the CaMV 35S promoter drove expression of all transgenes, factors that regularly lead to silencing (reviewed by Stam et al. 1997). Leaves from propagated shoots of both BTR-3 and BTR-1 have been periodically tested for kanamycin resistance over 20 months and ten vegetative generations without a change in response to the antibiotic. Additionally, shoots, leaves, and roots from all ten vegetative generations of BTR-3 consistently show high levels of GUS expression when stained with X-Gluc. After more than 2 years, dormant vegetative and flower buds also showed strong GUS expression. Although Van Blokland et al. (1994) observed gene silencing in primary transformants of petunia, silencing was not observed until the T₃ generation in transgenic rice plants expressing a chitinase gene (Chareonpornwattana et al. 1999). The effects of multiple transgenes will be assessed in transgenic rhododendron T₁ progeny as seed becomes available. Transgenic plants are currently undergoing field trials to evaluate growth and morphology compared to non-transformed controls.

This research is the first report of the recovery of transgenic *Rhododendron* generated via microprojectile bombardment. Although the transformation efficiency (0.2%) was less than that reported (5%) by Ueno et al. (1996) using an *Agrobacterium*-mediated transformation system, we believe that the biolistics approach has the potential to lead to high-efficiency transformation without the complication of host specificity that is unavoidable with the *Agrobacterium*-mediated method. To this end, we are currently optimizing parameters that increase the frequency of particle penetration, minimize damage to target tissues, enhance adventitious shoot formation from leaf lamina, reduce transgene copy number, and limit the incidence of both escapes and chimeras.

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