A Simple Transformation System Using Adventitious Shoot Multiplication of Juneberry

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Abstract. A simple gene transfer method based on *Agrobacterium* -mediated transformation of adventitious multiplication of Juneberry (*Amelanchier laevis* L.) basal shoots is described. Evidence is presented for successful integration and expression of a transformed gene in greenhouse-grown transgenic plants. This method can transform woody perennials that are difficult to regenerate from leaf disks, protoplasts, or other tissue culture regimens.

Transformation of tree species was reported several years ago (Fillatti et al., 1987). Since then, introducing foreign genes into tree species has become more feasible for those trees that can be regenerated efficiently in vitro from leaf disks, protoplasts, or cell lines (Haissig, 1989). The purpose of this report is to describe a transformation system for Juneberry, which undergoes in vitro adventitious shoot multiplication from the bases of individual shoot explants that easily root in soil and develop into normal plants (Behrouz, 1981). We have used Juneberry as a model for other woody plant species that can proliferate adventitious shoots efficiently in vitro because of the plant's intrinsic value as a forest and ornamental species.

Juneberry shoot-tip explants (≈ 2 cm long) were harvested originally from a greenhouse-grown plant; surface-sterilized in 5.25% NaOCl; rinsed three times with autoclaved distilled water; and cultured in Magenta GA7 vessels (Magenta Corp., Chicago) containing a shoot multiplication medium (Behrouz, 1981). The multiplication medium consisted of Murashige and Skoog (MS) (1962) basal medium supplemented with 3% sucrose, myo-inositol at 100 mg·liter⁻¹, casein enzymatic hydrolysate at 200 mg·liter⁻¹, thiamine at 100 mg·liter⁻¹, and 2.5 μ M benzyladenine. This

medium is hereafter referred to as modified MS medium (MMSM).

About 100 adventitious shoots were regenerated from the basal cut portion of each single shoot cultured in MMSM within 6 to 10 weeks (Fig. 1). These in vitro-grown shoots were used further for the transformation studies described below.

A kill curve experiment was conducted to identify the level of kanamycin sulfate (KS) required for selecting putatively transgenic shoots in vitro. In this experiment, Juneberry shoot explants (2 cm long) were obtained from in vitro-cultured material. Explants were cul-

tured on MMSM supplemented with KS at 0, 4, 16, or 64 mg·liter⁻¹. Five jars, each containing 10 shoot explants, were used for each concentration. While KS at 4 mg·liter⁻¹ did not affect growth or shoot multiplication rate, those cultured in KS at 16 mg·liter⁻¹ reduced adventitious shoot production five-fold relative to controls. All of the shoots cultured in medium containing KS at 64 mg·liter⁻¹ showed evidence of bleaching and died within 2 weeks.

The plasmids pWB139 (Cheng et al., 1992) and pWB195 (Fig. 2) were constructed. These plasmids contain an in-frame translational fusion between the amino terminal 612 amino acids of a *Bacillus thuringiensis* var. *kurstaki* gene encoding the toxin I-ID73 fused to the neomycin phosphotransferase II (*npt II*) gene (rendering resistance to the aminoglycoside antibiotics KS and neomycin) from the *Escherichia coli* transposon Tn5. These plasmids are based on a modification of the plasmid pWB146 (Barnes, 1990). This plasmid's gene cassette is regulated by the cauliflower mosaic virus 35S (35S) promoter and tomato proteinase inhibitor I terminator (*Tom pi I*). Plasmid pWB139 also contains a nopaline synthase (*nos*) promoter upstream of the 35S promoter and a *nos* terminator downstream from the *Tom pi I* terminator. Plasmid pWB139 has only the right border at the 5' end, and pWB195 has a left border at the 5' end and a right border and over drive at the 3' end of the gene cassette. Unless specified, all reagents used for these experiments were from Sigma Chemical, St. Louis.

The disarmed *Agrobacterium tumefaciens* strain WAg11 (Barnes, 1990) containing plasmids pWB139 and pWB195 was used to transform Juneberry. To transform this species, the

Fig. 1. Regenerating adventitious shoots from a single control shoot cultured for 4 weeks on modified Murashige and Skoog (1962) medium supplemented with 2.5 μ M benzyladenine.

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pWB 139

pWB 195

Fig. 2. Restriction maps of pWB195 and pWB139. Scale: 300 nucleotides/cm (except the vector pRK 252).

shoot multiplication area (0.5-cm-long basal cut ends) of shoot explants was co-cultivated overnight at $24 \pm 2^\circ\text{C}$ in sterile, borosilicate, 12×75 -mm culture tubes (VWR Scientific, South Plainfield, N.J.) containing a 1:1 dilution of an overnight culture of *A. tumefaciens* carrying pWB139 or pWB195 and MMSM supplemented with KS at $20 \text{ mg}\cdot\text{liter}^{-1}$ and tetracycline at $10 \text{ mg}\cdot\text{liter}^{-1}$.

After co-cultivation, explants were rinsed twice in MS medium containing Augmentin 250 (Beecham, Bristol, Tenn.) at $250 \text{ mg}\cdot\text{liter}^{-1}$, then cultured on solidified phytoagar (Gibco/BRL Life Technologies, Gaithersburg, Md.) at $6.5 \text{ mg}\cdot\text{liter}^{-1}$, MMSM containing Augmentin at $250 \text{ mg}\cdot\text{liter}^{-1}$, and KS at $16 \text{ mg}\cdot\text{liter}^{-1}$. The effective activity of KS may have been reduced somewhat by the presence of Augmentin during the selection culture period. Cultures were maintained for 3 to 4 months at $24 \pm 2^\circ\text{C}$ under a 16-h photoperiod of $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The regenerated shoots were transferred monthly onto fresh medium containing Augmentin and KS. After 3 to 4 months, KS-resistant and control (nontransformed) shoots were transferred for rooting to 7.5-cm-diameter (250-ml) clay pots containing a medium of 1 peat : 1 perlite (v/v). The pots containing shoots were enclosed in plastic bags for 2 to 3 weeks to maintain high humidity. Small holes (2 to 3 mm in diameter) were made in the bags every 2 to 3 days to acclimate the plantlets to the lower relative humidity in the greenhouse. Plantlets also were acclimated to greenhouse conditions by increasing irradiance during 3 to 4 weeks of rooting. Rooted shoots (plantlets) were transferred to the greenhouse. All plantlets rooted when 2- to 3-cm-long shoots with more than four well-expanded leaves were used.

DNA was extracted from young leaves of putatively transformed and control plants following the method of Rogers and Bendich (1985). Bam HI (a site at the start codon of the *Bt* gene)-digested genomic DNA was electrophoresed on 1% agarose gels, then transferred to a Nytran nylon membrane (Schleicher and Schuell, Keene, N.H.) following procedures of Southern (1975). A 2.6-kb Bam HI/Hind III fragment from pWB139 containing *Btk/npt II* codons was used as a probe. Filters containing restricted DNA were incubated with a ^{32}P -

labeled probe [^{32}P -deoxycytidine 5'-triphosphate, 800 Ci/mmol ($1 \text{ Ci} = 37 \text{ GBq}$); New England Nuclear Research Products, Boston] prepared using random hexamers (Pharmacia LKB Biotechnology, Piscataway, N.J.) following procedures of Feinberg and Vogelstein (1983). Filters were washed using standard

methods (Sambrook et al., 1989) and subjected to autoradiography using Kodak X-Omat AR-5 film (Eastman Kodak, Rochester, N.Y.). The restriction enzymes and the Klenow fragment of DNA polymerase I were from New England Biolabs, Beverly, Mass.

In a typical experiment, up to 100 adventitious shoots were regenerated from control (nontransformed) and co-cultivated explants within 6 to 8 weeks. When putatively transformed shoot explants were selected in KS at $16 \text{ mg}\cdot\text{liter}^{-1}$ containing MMSM, 15 to 20 adventitious shoots were regenerated from each cultured shoot. Of 38 randomly selected Juneberry adventitious shoots regenerated after co-cultivation with *Agrobacterium* carrying pWB139, pWB195, or both and selected in KS at $16 \text{ mg}\cdot\text{liter}^{-1}$ containing MMSM, nine showed positive hybridization signals on southern blots (Fig. 3). DNA was digested with Bam HI. Any integrated DNA cut with Bam HI will cut at this site and in the plant genome 3' to the point of integration. Any piece that hybridizes with a *Btk/npt II* probe should be $>4 \text{ kb}$ for pWB139 and $>3.3 \text{ kb}$ for pWB195. Since pWB139 has no left-border t-DNA sequences,

Fig. 3. Autoradiogram of Southern blot of Bam HI-digested total DNA from Juneberry plants co-cultivated with *Agrobacterium* containing pWB139 (lanes 1-4), pWB139 and pWB195 (lanes 5-8), or pWB195 (lane 9). Lane C contains DNA from a nontransformed plant and lane P from the plasmid pWB195 DNA digested with Bam HI and HindIII. Molecular weights are given in kilobase pairs.

Fig. 4. Western blot of protein extracted from four independent transformants probed with anti-*npt II* antibody: positive control 29 kDa *npt II* protein (lane 1), four independently transformed plants (lanes 3-6), and a negative control plant (lane 9). Lanes 2 and 8 were left empty. Numbers on the right refer to protein marker sizes in kiloDaltons.

a characteristic that would denote the 3' integration site, predicting the size of the inserted DNA is difficult. Bands represent fragments extending from the internal Bam HI site to a distal site in the plant genome where plasmid integration has been terminated. Lanes 1 to 4 represent DNA from plants transformed with pWB139, lanes 5 to 8 represent DNA from plants transformed using both pWB139 and pWB195, and lane 9 represents DNA from a plant transformed with pWB195 only. Most lanes representing pWB195-transformed plants have only high-molecular-weight DNA bands of varying lengths, all >3.3 kb, as predicted by the presence of the left-border sequences. The size heterogeneity of DNA bands giving a positive hybridization signal indicates that no shoots were contaminated further with *Agrobacterium*.

To detect expression of the *npt II* gene in plants transformed by co-cultivation with *Agrobacterium* containing pWB139 and pWB195, protein immunodetection assays were performed on SDS PAGE western blots of plant extracts (Burnette, 1981; Laemmli, 1970; Towbin et al., 1979). Western blots were probed using a rabbit anti-*npt II* antibody (5'-3' Inc., West Chester, Pa.), to which 1% normal goat serum (Gibco BRL Life Technologies) was added while the primary anti-

body was incubated with the membrane to reduce the background. Purified *npt II* protein, used as positive control, was also from 5'-3' Inc. Other reagents for alkaline phosphatase-based immunodetection of bound goat, anti-rabbit secondary antibody were from Promega Biotech, Madison, Wis. Nitrocellulose membranes were from Schleicher and Schuell. Leaf extracts of four independently transformed plants showed a positive *npt II* signal on western blots (Fig. 4, lanes 3-6), whereas the nontransformed control did not react positively with the anti-*npt II* antibody. These four plants correspond to transgenic plants used in lanes 5 to 8 of Fig. 3. Although the gene cassettes in the transforming plasmids are an in-frame translational fusion of *Btk/nptII* coding sequences, the sizes of positive bands on the western blot are similar to those for the control *npt II* protein. This result suggests the fusion protein is cleaved to remove the *npt II* sequences by plant cytoplasmic proteases. A slightly smaller band is also seen and may represent a proteolytic product.

The system reported herein was used to demonstrate the possibility of transforming tree species that cannot be regenerated from leaf disks, but that efficiently produce adventitious shoots in vitro. An insect bioassay was not performed, since Juneberry is resistant to

most lepidopteran insects [e.g., tobacco hornworm (*Manduca sexta* L.)] used in *Bt* transformation studies.

Although producing adventitious shoots does not eliminate the possibility of somaclonal variation (Larkin and Scowcroft, 1981), all plants regenerated in vitro were morphologically alike. Although samples for molecular analysis were taken from different parts of each transgenic plant, more experiments are required to demonstrate if these transgenic plants are chimerically transformed.

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