Agrobacterium-Mediated Transformation of Chokecherry (*Prunus virginiana* L.)

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Abstract. Chokecherry (*Prunus virginiana* L.) was transformed using *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pBI121 carrying the neomycin phosphotransferase gene (*nptII*) and β -glucuronidase (GUS) gene (*uidA*). Plants were regenerated from the *Agrobacterium*-infected leaf tissues through organogenesis on woody plant medium (WPM) supplemented with MS (Murashige and Skoog) vitamins, 10 μ M 6-benzyladenine (BA), and 250 mg·L⁻¹ cefotaxime plus 500 mg·L⁻¹ carbenicillin plus 15 mg·L⁻¹ kanamycin (CCK₁₅). Transformation was verified with polymerase chain reaction (PCR) and Southern blot analysis. Four of 150 (2.67%) initial explants produced GUS- and PCR-positive shoots. Southern blot analysis confirmed that the transgenes were integrated into the chokecherry genome. Transgenic in vitro shoots were rooted in half-strength MS medium containing 10 μ M naphthalene acetic acid. Rooted plants were transferred to potting mix and grown in the greenhouse. This research shows a potential for future improvement of chokecherry and other *Prunus* species. Chemical names used: 6-benzyladenine (BA), naphthalene acetic acid (NAA), acetosyringone (AS), 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium (X-Glu), cefotaxime, carbenicillin, kanamycin.

Chokecherry (*Prunus virginiana* L.) is a small tree or large shrub widely distributed across the northern Great Plains in the United States and Canada. Native to North America, chokecherry is well adapted to a variety of severe conditions such as alkaline soils and harsh Winters and is a valuable food resource and shelter for wildlife. Chokecherry is one of the native species (pincherry, cranberry, blueberry, and so on) used in small fruit production for beverages, fresh fruit, dried fruit products, and wine. It is also used as an ornamental plant because of the beautiful white flowers in Spring and colorful leaves and fruits in Fall.

The development of the native fruit industry in the northern Great Plains is largely impeded by lack of high-quality and high-yield cultivars. Chokecherry suffers several diseases, including black knot and X-disease (incited by a cell-wall-less prokaryotic phytoplasma) (Guo et al., 1996). The damage by these diseases is severe. No effective methods are available to control these diseases.

These diseases and infected trees can only be removed. Therefore, utilization of disease-resistant plants is the best method to manage these diseases. Conventional approaches for chokecherry breeding is generally difficult and time-consuming because of its high heterozygosity, polyploidy, and long juvenile period. Thus, genetic engineering offers a useful tool to complement the conventional breeding method for chokecherry improvement.

Transgenic plants have been obtained in many woody species (Poupin and Arce-Johnson, 2005). In the genus *Prunus*, transformation of several species have been documented, including peach (Hammerschlag and Smigocki, 1998; Pérez-Clemente et al., 2004; Scorza et al., 1995a), plum (Scorza et al., 1995a, 1995b; Yancheva et al., 2002), almond (Ainsley et al., 2002; Miguel and Oliveira, 1999), apricot (Petri et al., 2004), and cherry (Dolgov et al., 1999; Song and Sink, 2006). However, most of these studies used immature tissues (immature embryos)

or leaves from a juvenile plant and very few studies recovered whole transgenic plants. To our knowledge, only Dolgov et al. (1999) and Song and Sink (2006) reported that transgenic plants were regenerated from leaf tissues of mature cherry species and no research has been reported on chokecherry transformation.

This study was carried out to develop a gene transformation protocol for future gene transfer of chokecherry. The method of genetic transformation of chokecherry might be also useful for genetic engineering of other *Prunus* species.

Materials and Methods

Plant materials. In vitro cultures of chokecherry clone NN were initiated by Zhang et al. (2000) using shoot tips from a mature seed-propagated chokecherry plant grown at the USDA Plant Materials Center in Bismarck, N.D. In vitro shoots were maintained in Murashige and Skoog (1962) medium (MS) supplemented with 2.5 μ M 6-benzyladenine (BA), 3% sucrose, and solidified with 0.7% agar (Difco Co., Detroit, Mich., #0140-01-0). The pH was adjusted to 5.7–5.8 before autoclaving. In vitro shoots were subcultured every 4 weeks to fresh media in 100-mL baby food jars containing 25 mL medium each and cultured at 25 °C under cool-white light at 36 μ mol·m⁻²·s⁻¹ with a 16- to 8-h photoperiod. All other experiments were performed under these conditions unless otherwise noted.

Plant transformation. *Agrobacterium* strain EHA105 (Hood et al., 1993), carrying pBI121 (Clontech, Palo Alto, Calif.) containing the *nptII* gene encoding for neomycin phosphotransferase and the *uidA* coding for β -glucuronidase (GUS) (Fig. 1), was grown overnight in LB (Luria-Bertani) medium with 100 mg·L⁻¹ kanamycin at 28 °C in a shaker at 150 rpm. Cells were collected by centrifugation at 6000 rpm for 15 min, resuspended to 1.0 O.D. at Abs⁶⁰⁰ in fresh LB medium supplemented with 20 μ M acetosyringone (AS) without kanamycin, and incubated at 28 °C in a shaker at 150 rpm for 2 h. One-month-old in vitro leaves were cut through the main vein once and submerged in a bacterial culture solution for 30 min at 28 °C. Leaf explants ($\approx 0.5 \times 0.5$ cm) were then removed from the culture, blotted on sterilized paper towels, and transferred to a woody plant medium (Lloyd and McCown,

Fig. 1. Schematic representation of the T-DNA portion of pBI121 plasmid (Clontech, Palo Alto, Calif.). The vector was introduced into the disarmed *Agrobacterium tumefaciens* EHA 105. RB and LB: T-DNA right and left borders; Nos-pro: neomycin phosphotransferase promoter; Nos-ter: neomycin phosphotransferase terminator; *nptII*: neomycin phosphotransferase gene; *uidA*: β -glucuronidase gene; 35S-Pro: CaMV 35S promoter from cauliflower mosaic virus.

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1980) supplemented with MS vitamins, 10 μM BA, 0.7% agar, and 200 μM acetosyringone in Petri dishes (100 mm $\times$ 15 mm, 25 mL medium) for cocultivation in the dark for 72 h at room temperature. Each Petri dish contained 25 leaf explants and replicated three times (Petri dishes). The experiment was concurrently repeated twice. After cocultivation, a total of 150 leaf explants were washed three times with sterile deionized and distilled water (ddH_2O) and once with sterile ddH_2O plus 250 $\text{mg}\cdot\text{L}^{-1}$ cefotaxime and 500 $\text{mg}\cdot\text{L}^{-1}$ carbenicillin, and then 10 explants per Petri dish (100 mm $\times$ 15 mm) containing 25 mL regeneration medium [WPM supplemented with MS vitamins, 10 μM BA, 0.7% agar, and 250 $\text{mg}\cdot\text{L}^{-1}$ cefotaxime plus 500 $\text{mg}\cdot\text{L}^{-1}$ carbenicillin plus 15 $\text{mg}\cdot\text{L}^{-1}$ kanamycin (CCK_{15})] (antibiotics were added into the autoclaved medium when the medium temperature cooled down to $\approx 50^\circ\text{C}$) for regeneration under shoot culture conditions previously mentioned. Calluses developed from leaf tissues after the first month of culture in CCK_{15} medium. Calluses were then detached from original leaf explants and transferred to fresh regeneration medium for shoot regeneration. Shoots regenerated from CCK_{15} -containing medium were proliferated in MS supplemented with 2.5 μM BA and CCK_{15} . Proliferated shoots were rooted based on the method of Dai et al. (2004). Rooted plants were transferred to Sunshine Mix #1 (Fisons Western Corp., Vancouver, Canada) and grown in the greenhouse.

Histochemical β -glucuronidase assay. Leaves from these regenerated shoots were subjected to GUS screening as described by Jefferson (1987). In brief, in vitro young leaves were submerged in a GUS staining solution containing 200 μL dH_2O and 200 μL X-Glu solution (2 $\text{mg}\cdot\text{mL}^{-1}$; Gold Biotechnology, Inc., St. Louis, Mo.). Submerged leaves were incubated at 37°C overnight and then bleached with 70% to 100% ethanol gradually. GUS staining was observed under the microscope and photographed.

Polymerase chain reactions. Genomic DNA was extracted from young leaves of in vitro transformed and nontransformed chokecherry plants based on the method of Lodhi et al. (1994). Polymerase chain reactions (PCRs) were carried out in 25 μL volume containing 200 μM dNTPs, 1 μM each of oligonucleotide primer, 2.5 units DNA *Taq* Polymerase (Promega, Madison, Wis.), and 25 ng DNA. The reaction conditions were: one cycle at 94°C for 5 min, 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 30 s and then one cycle at 72°C for 7 min. Amplified DNA fragments (10 μL of reaction) were electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide. The primers used for screening transgenes were: *nptII* reverse: 5'-GCAGG CATGCCATGGGTCACGACGA-3' and *nptII* forward: 5'-GCCCTGAATGAACTG CAGGACGAGGC-3' and *uidA* reverse: 5'-CCCGCAATAACATACGGCGTG-3' and *uidA* forward: 5'-CCTGTAGAAACCC

Fig. 2. A transformed plant was recovered (A); β -glucuronidase staining of a young leaf from a nontransformed (B) and a transformed (C) chokecherry. The blue (see the online version in color at www.ashs.org) color in C is the result of active beta-glucuronidase activity.

CAACCCGTG-3', which produced 410 bp and 365 bp products, respectively.

Southern blot analysis. Approximately 25 to 35 μg of genomic DNA was digested in a 50- μL reaction with 1 μL *EcoRI* + 1 μL *HindIII* restriction enzymes at 37°C for 2.5 h, electrophoresed on a 0.8% TAE (Tris-acetate EDTA) agarose gel, and blotted to a positively charged Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, U.K.). Similarly, restricted DNA from untransformed clone NN was used as a negative control, whereas 25 to 40 ng of pBI121 plasmid DNA was used as a positive control. The blot was probed with randomly primed ^{32}P -labeled *uidA* PCR product (4 μL dH_2O , 1 μL 6-mer oligo primers, 1.5 μL 5 mM dNTPs, 1.5 μL 10 $\times$ Klenow buffer, 1 μL Klenow polymerase, and 5 μL dCTP ^{32}P). Blot was prehybridized at 65°C for 6 h in the hybridization solution [1% bovine serum albumin (BSA) Fraction V (Sigma), 0.5 M NaH_2PO_4 (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 mM ethylenediamine tetraacetic acid (EDTA)]. DNA probe was added directly to the blot in the prehybridization mixture, hybridized at 65°C for 16 h, and then washed with $2 \times \text{SSC}$ (200 μM sodium chloride and 200 μM sodium citrate) for 35 min followed by $0.5 \times \text{SSC} + 0.1\%$ SDS, and $0.1 \times \text{SSC} + 0.1\%$ SDS for 10 min each at 65°C on a shaker. The blot was exposed to x-ray film (Kodak, N.Y.) at -80°C for 72 h and developed per the manufacturer's instructions.

Results and Discussion

Transformation of chokecherry. After 4 to 6 weeks of culture, leaf tissue cocultivated with *Agrobacterium* EHA105 carrying pBI121 produced callus on CCK_{15} media, whereas no callus developed without cocultivation. Callus was detached from original leaf explants and transferred to the same regeneration medium containing CCK_{15} . New shoots were regenerated from callus tissue after 4-week culture. From 150 initial explants, nine callus lines produced shoots.

β -glucuronidase staining. To prevent false-positive GUS staining and PCR from contamination of remaining *Agrobacterium*, in vitro shoots from nine regenerated lines were grown in antibiotic-free MS media to detect remaining *Agrobacterium*. All cultures remained free of bacteria after being subcultured in CCK_{15} medium two to three times,

indicating that all bacteria had been killed by antibiotics (cefotaxime and carbenicillin) during subculturing. GUS staining identified four regenerated lines that stained GUS-positive in leaves (Fig. 2), indicating that the *uidA* gene was being expressed in the leaf tissue.

Polymerase chain reactions. Four GUS-positive regenerated lines were proliferated to have enough leaf tissue for genomic DNA extraction and subjected to PCR verification. The expected fragments of *uidA* (356 bp) and *nptII* (410 bp) genes were successfully amplified from all transformants using the specific primers (Fig. 3).

Southern blot analysis. Genomic DNA was digested with *HindIII* and *EcoRI*. Gel electrophoresis showed that DNA was well digested and separated. The double-digested DNA samples were hybridized with the ^{32}P -labeled fragment of the 356-bp probe prepared from the PCR product of the *uidA* gene. The result showed that all four regenerated lines exhibited one distinctive restriction fragment (Fig. 4), confirming that the *uidA* gene was integrated into chokecherry genome.

In this study, transformation of four regenerated lines (T1-2, T1-4, T1-5, and T1-6) of 150 initial leaf explants has been confirmed. The transformation frequency of

Fig. 3. Polymerase chain reaction amplification of *uidA* and *nptII* genes in transformed plants. Lanes 1-4 and 8-11 are transformed lines T1-6, T1-5, T1-4, and T1-2 amplified with primers specific for *nptII* and *uidA*, respectively. Lanes 6 and 13 are negative controls of untransformed clone NN. Lanes 7 and 14 are 1-kb DNA ladders. Lanes 5 and 12 are positive controls of pBI121.

Fig. 4. Confirmation of transgene (*uidA*) in transformed chokecherry. Lane L: 1-kb DNA ladder; Lanes NT, P, and 1 to 4 are double-digested DNA with *EcoRI* and *HindIII* from nontransgenic clone NN, plasmid pBI121, and transgenic lines: T1-6, T1-5, T1-4, and T1-2, showing a predicted band of ≈ 3.0 kb.

chokecherry in this study was 2.7% (four of 150), which is higher than most transformations for *Prunus* species using mature materials (Petri and Burgos, 2005). Only one recent study reported a slightly higher transformation frequency obtained from cherry transformation (Song and Sink, 2006). Many factors such as genotype, *Agrobacterium* strain, conditions of infection, and cocultivation affect transformation frequency. The chokecherry clone used in this study is very amenable to regeneration from leaf tissue (Dai et al., 2004). Thus, higher transformation efficiency may be achieved if some improvements can be made during the *Agrobacterium* infection and transformant selection. For example, preconditioning explants, application of a vacuum technique during the infection process, and using different *Agrobacterium* strains will increase the transfer DNA delivery efficiency. In this transformation system, kanamycin was used as a selection agent to identify transgenic cell lines. Preliminary experiments showed that the tolerance of chokecherry leaf tissue to kanamycin appeared to vary greatly with the developmental stage of in vitro plants (data not shown). Regeneration of chokecherry from leaf tissue was sensitive to kanamycin concentration. High concentration of kanamycin ($>20 \text{ mg}\cdot\text{L}^{-1}$) completely inhibited regeneration from leaf tissue. However, the tolerance to kanamycin increased dramatically after in vitro shoots formed. In vitro shoots did not exhibit any damage after 8 to 12 weeks at 45 to $80 \text{ mg}\cdot\text{L}^{-1}$ kanamycin. To increase efficiency of selection, we exposed leaf explants to selective medium (containing $20 \text{ mg}\cdot\text{L}^{-1}$ kanamycin) immediately after cocultivation with *Agrobacterium*. No shoots were regenerated and explants were dying in the first 4 weeks, whereas control leaf explants (in the medium without kanamycin) produced many shoots within 4 weeks. Decreasing kanamycin concentration only several units resulted in escapes forming many nontransformed shoots. It may be helpful to culture infected explants in nonselective (no kanamycin) medium before transfer to selective (with kanamycin) medium. This may allow infected explants to recover from in-

fection and acclimate to new culture conditions, allowing the plant cells to initiate regeneration more easily.

To date, most transgenic plants of *Prunus* species have been initiated with embryogenic tissues (immature embryos) or leaves from a juvenile plant. This limits application of gene transformation for improving vegetatively propagated species, especially for elite cultivar improvement because of the segregation of seed-propagated plants. In this study, chokecherry transformation was achieved using leaf tissue from a mature tree. Therefore, this method can be used to improve single or several traits without changing any other genetic makeup, providing a useful tool in development of new cultivars of this and other species.

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