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 μM 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 μM 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 were: 6-benzyladenine (BA), naphthalene acetic acid (NAA), acetosyringone (AS), 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium (X-Glu), cefotaxime, carbenicillin, and 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 (Petrí 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 μM 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 μmol·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 μM 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 (0.5 × 0.5 cm) were then removed from the culture, blotted on sterilized paper towels, and transferred to a woody plant medium (Lloyd and McCown, 1980). In vitro shoots were transferred to potted mix and grown in the greenhouse. Rooted plants were transferred to field conditions in the North Dakota State University Teaching and Research Center, Fargo, N.D., in Fall 2005.

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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: nopaline synthase promoter; Nos-ter: nopaline synthase terminator; nptII: neomycin phosphotransferase gene; uidA: β-glucuronidase gene; 3SS-Pro: CaMV 35S promoter from cauliflower mosaic virus.
containing 200 m et al. (1994). Polymerase chain reactions of cherry plants based on the method of Lodhi. DNA was extracted from young leaves of in leaves were submerged in a GUS staining solution (2 mg/mL) for cocultivation in the dark for 72 h and developed per the manufacturer’s instructions. 

**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’-CGACGG CATGGCGCATGGTTGACGGACG-3’ and nptII forward: 5’-GCTCTGATGGTAGTCTAGTGCAAGGCGG-3’. Northern blot analysis. Approximately 25 to 35 μg of genomic DNA was digested in 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 DNA from 25 to 40 ng of pH121 plasmid DNA was used as a positive control. The blot was probed with randomly primed 32P-labeled nptII PCR product (4 μL dH2O, 1 μL 6-mer oligo primers, 1.5 μL 5 mm dNTPs, 1.5 μL 10 μL Klenow buffer, 1 μL Klenow polymerase, and 5 μL dCTP 32P). Blot was prehybridized at 65 °C for 6 h in the hybridization solution (1% bovine serum albumin (BSA) Fraction V (Sigma), 0.5 μM NaH2PO4 (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 μM ethylenediamin 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× SSC (200 μM sodium chloride and 200 μM sodium citrate) for 35 min followed by 0.5× SSC + 0.1% SDS, and 0.1× 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 carrying pBI121 produced callus on CCK15 media, whereas no callus developed without cocultivation. Callus was detached from original leaf explants and transferred to the same regeneration medium containing CCK15. 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 medium to detect remaining Agrobacterium. All cultures remained free of bacteria after being subcultured in CCK15 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 nptII gene was being expressed in the leaf tissue. 

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chokecherry in this study was 2.7% (four of 150), which is higher than most transformation 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 mg 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 mg L⁻¹ kanamycin. To increase efficiency of selection, we exposed leaf explants to selective medium (containing 20 mg 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 infection 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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