Plant Cell Tiss Organ Cult (2010) 101:49–57
DOI 10.1007/s11240-009-9662-3

ORIGINAL PAPER

Agrobacterium-mediated transformation of mature *Prunus serotina* (black cherry) and regeneration of transgenic shoots

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Received: 13 August 2009 / Accepted: 10 December 2009 / Published online: 27 December 2009

Abstract A protocol for *Agrobacterium*-mediated transformation was developed for in vitro leaf explants of an elite, mature *Prunus serotina* tree. *Agrobacterium tumefaciens* strain EHA105 harboring an RNAi plasmid with the black cherry *AGAMOUS* (*AG*) gene was used. Bacteria were induced for 12 h with 200 μM acetosyringone for *vir* gene induction before leaf explant inoculation. Explants were co-cultured for 3 days, and then cultured on woody plant medium supplemented with 9.08 μM thidiazuron, 1.07 μM napthaleneacetic acid, 60 μM silver thiosulphate, 3% sucrose, plus 200 mg l^{-1} timentin in darkness for 3 weeks. Regenerating shoots were selected 27 days after initial co-culture, on Murashige and Skoog medium with 3% sucrose, 8.88 μM 6-benzylaminopurine, 0.49 μM indole-3-butyric acid, 0.29 μM gibberellic acid, 200 mg l^{-1} timentin, and 30 mg l^{-1} kanamycin for five subcultures. After 5–6 months of selection, transformation efficiencies were determined, based on polymerase chain reaction (PCR) analysis of individual putative transformed shoots relative to the initial number of leaf explants tested. The transformation efficiency was 1.2%. Southern blot analysis of three out of four PCR-positive shoots confirmed the presence of the neomycin phosphotransferase and *AG* genes. Transgenic shoots were rooted (37.5%), but some shoot tips and leaves deteriorated or died, making acclimatization of rooted transgenic plants difficult. This transformation, regeneration, and rooting protocol for developing transgenic black cherry will continue to be evaluated in future experiments, in order to optimize the system for several mature black cherry genotypes.

Keywords Agamous · Genetic engineering · Leaf explants · RNAi

Abbreviations

AS	Acetosyringone
BA	6-benzylaminopurine
GA_3	Gibberellic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	Napthaleneacetic acid
PCR	Polymerase chain reaction
STS	Silver thiosulphate
TDZ	Thidiazuron
nptII	Neomycin phosphotransferase
WPM	Woody plant medium

Introduction

Black cherry (*Prunus serotina* Ehrh.), also called wild black cherry and mountain black cherry, is native to the eastern US and parts of Canada. It is the only native species of the genus *Prunus* valued commercially for lumber production. The heartwood of black cherry varies from light to dark reddish brown and is valued for cabinets, furniture, architectural wood work, and veneer. There is a demand for high quality black cherry wood and increased interest in establishing plantations with improved black cherry genotypes.

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A reliable and efficient in vitro regeneration system to regenerate phenotypically normal plants is a prerequisite for genetic improvement of black cherry through transformation. There have been several reports of in vitro regeneration of black cherry using leaves. Hammatt and Grant (1998) were the first to report adventitious shoot regeneration from juvenile leaf explants of black cherry, however, a complete protocol including rooting and acclimatization of regenerated plantlets was not established. Espinosa et al. (2006) established a complete protocol for regeneration of black cherry plants from juvenile leaf explants. Recently, a regeneration system has been established for mature elite black cherry from in vitro leaf explants (Liu and Pijut 2008). There has only been one report of *Agrobacterium* infection of black cherry (Maynard and Fuernkranz 1989) using leaf pieces for regeneration and five *Agrobacterium* strains for gall formation tests. Therefore, developing a transformation system for mature black cherry will provide a promising approach to facilitate the transfer of valuable genes for the genetic improvement of black cherry.

Trees were once considered to be recalcitrant for genetic transformation. Progress has been made for several economically important *Prunus* species, using both immature and mature tissues. However, transformation and regeneration of transgenic plants of elite, mature genotypes or commercial cultivars is much more difficult than using tissue from juvenile sources with unknown characteristics. Ramesh et al. (2006) reported successful transformation and regeneration from leaf explants of the commercial almond cultivar, *Prunus dulcis* cv. Ne Plus Ultra. Transformation efficiencies were 5.6% for kanamycin selection [neomycin phosphotransferase (*nptII*); negative selection] and 6.8% for mannose selection [phosphomannose isomerase (*pmi*); positive selection]. Leaf explants of sour cherry (*Prunus cerasus* cv. 'Montmorency') and a dwarfing cherry rootstock cv. 'Gisela 6' (*P. cerasus* x *P. canescens*) have been transformed (Song and Sink 2006). Transformation results showed 3.1% of cv. 'Montmorency' and 3.3% of cv. 'Gisela 6' explants produced glucuronidase (GUS)- and polymerase chain reaction (PCR)-positive shoots. Transformation of plum (*Prunus domestica*) has been reported from leaf explants (Mikhailov and Dolgov 2007; Yancheva et al. 2002) and hypocotyl slices (Petri et al. 2008c). Transformation frequencies of 0.8% of the Belgian- 'Altesse simple' (Quetsche) clone and 2.7% for the Bulgarian local plum cultivar were reported by Yancheva et al. (2002). Mikhailov and Dolgov (2007) reported only 0.08% of explants on kanamycin selection and 1.3% of explants on hygromycin selection [hygromycin phosphotransferase (*hpt*)] of the cv. 'Startovaya' produced green fluorescent protein (GFP) and PCR-positive transgenic lines. Constructs containing a plum pox virus coat protein hairpin were used in successful transformation of the plum cv. 'Bluebyrd', and an average transformation efficiency of 25% of hypocotyl slices produced transgenic plants (Petri et al. 2008c). Transgenic apricot plants (*Prunus armeniaca*) cv. 'Helena' were regenerated from leaf explants with a transformation efficiency of 3.5–5.8% (Petri et al. 2008a, b). Similar transformation efficiencies of 3.6 ± 1% were reported for transgenic *Prunus persica* cv. 'Miraflores' (peach) (Pérez-Clemente et al. 2004). Leaf explants of chokecherry (*Prunus virginiana*) derived from a mature seed-propagated clone (NN) were regenerated with a transformation efficiency of 2.6% (Dai et al. 2007). Transformation efficiencies of 0.8 and 0.3% were reported for transgenic plants regenerated from hypocotyl segments of *Prunus salicina* (Japanese plum) varieties 'Angeleno' and 'Larry Anne', respectively (Urtubia et al. 2008).

In this study, we describe a transformation and regeneration methodology to produce transgenic black cherry with an RNAi construct with the C-function homeotic floral gene *AGAMOUS* (*AG*) from black cherry in order to produce reproductive sterility. Plants were regenerated from in vitro leaf tissues, established from an elite, mature black cherry tree. The integration of the transgenes was confirmed by Southern blot analysis.

Materials and methods

Plant material and tissue culture

Leaf explants from an elite mature genotype (#3) were obtained from in vitro shoot cultures as described by Liu and Pijut (2008). In vitro cultures of black cherry were maintained on Murashige and Skoog (MS) medium (1962) with 3% (v/v) sucrose, 8.88 μM 6-benzylaminopurine (BA), 0.49 μM indole-3-butyric acid (IBA), 0.29 μM gibberellic acid (GA_3), and 0.7% Difco-Bacto agar (Liu and Pijut 2008). The pH of the medium was adjusted to 5.7 before the addition of agar and autoclaved at 121°C for 20 min. Cultures were incubated at 25°C under a 16-h photoperiod provided by cool-white fluorescent lamps (100–140 μmol m^{-2} s^{-1}). Nodal segments were excised and transferred to fresh medium every 3 weeks for multiplication of shoots. Acetosyringone (AS) and all antibiotics such as kanamycin, timentin, spectinomycin, and rifampicin were filter-sterilized and added to media cooled to 50–60°C after autoclaving.

Adventitious shoot regeneration and rooting

An adventitious shoot regeneration and rooting protocol for black cherry (Liu and Pijut 2008) was employed with some modification for this study. Briefly, leaf explants were

cultured on woody plant medium (WPM) (Lloyd and McCown 1981) containing 9.08 μM thidiazuron (TDZ), 1.07 μM napthaleneacetic acid (NAA), and 60 μM silver thiosulphate (STS) in the dark for 3 weeks. These cultures were moved to light conditions (16-h photoperiod; 100–140 μmol m^{-2} s^{-1}) for 3 days, and then transferred to shoot elongation medium. Shoot elongation media consisted of MS medium with 3% (v/v) sucrose, 8.88 μM BA, 0.49 μM IBA, 0.29 μM GA_3, 200 mg l^{-1} timentin, and 30 mg l^{-1} kanamycin. Elongated shoots were rooted by dipping in 2.5 mM IBA for 3 min. Kanamycin (30 mg l^{-1}) was added to the media 27 days after co-culture. For all transformation experiments, freshly prepared leaf explants from in vitro-grown cultures were inoculated.

Agrobacterium strain and binary vector

A RNAi plasmid containing the black cherry *AG* gene (GenBank accession number EU938540.1) was used in this study. Plasmid PsAGRNAi (Fig. 1) which contains sense and antisense *AG* from black cherry was developed for this study (Liu et al. 2009). The plasmid was transformed into *A. tumefaciens* strain EHA105 by the freeze–thaw method (Höfgen and Willmitzer 1988). The RNAi cassette from pHannibal was ligated to pART27 which contains the *nptII* gene, under the control of the CaMV 35S promoter and NOS terminator. *Agrobacterium* strain EHA105 was kindly provided by Dr. Stanton Gelvin (Purdue University). EHA105: PsAGRNAi was streaked and incubated on solid Luria-Bertani media (LB) supplemented with 200 mg l^{-1} spectinomycin and 20 mg l^{-1} rifampicin at 28°C for about 48 h to obtain single colonies.

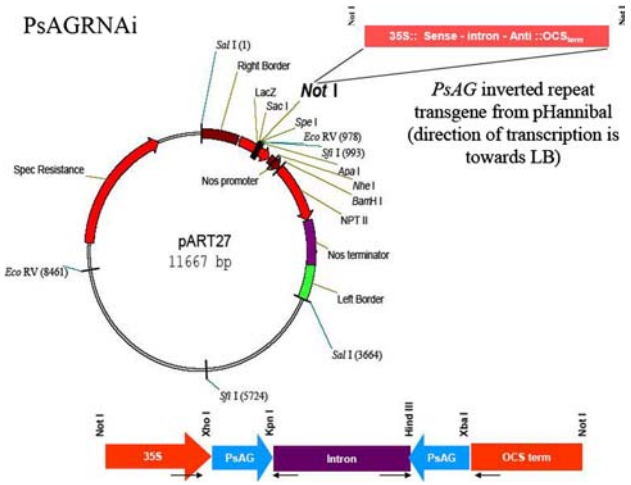

Fig. 1 Map of construct PsAGRNAi (*PsAG* gene RNAi silencing construct). RNAi cassette was shown in red flanking by *Not* I site. Primer positions were shown by black arrows. Primers 35S and PDK were used to confirm sense fragment, which amplifies partial 35S promoter, *AG* sense, and partial PDK intron. Primers PDK and OCS term were used to confirm the antisense fragment

Transformation

A single colony was inoculated into 10 ml of liquid LB medium with 200 mg l^{-1} spectinomycin and incubated at 28°C for 24 h with constant agitation (175 rpm). An additional 40 ml LB was then added and the culture was grown overnight at the same culture conditions to an OD_{600} = 0.8–1.0. The cells were then centrifuged at 3,000 rpm for 20 min and the pellet was diluted to OD_{600} = 0.2 in an induction medium (Gelvin 2006) supplemented with 200 μM AS at pH 5.2, and cultured 6–24 h at 25°C on an orbital shaker (100 rpm) to induce the *vir* genes. This step was reported to help the transfer of T-DNA, which was enhanced by low pH and phenolic compounds such as AS (Gelvin 2006; Vernade et al. 1988). The bacterial suspension was again centrifuged and the pellet re-suspended in liquid WPM consisting of WPM salts, 9.08 μM TDZ, 1.07 μM NAA, 2% (w/v) sucrose, and 100 μM AS, pH 5.6. The first two apical, fully expanded leaves from proliferating in vitro shoots were placed in sterile water, and several wounds were made on the midrib of each leaf (three to five wounds per explant). Leaves were then inoculated for 60 min in the bacterial suspension with gentle shaking (50 rpm). Leaf explants were then blotted dry on sterile filter paper and cultured in the dark on WPM with 9.08 μM TDZ, 1.07 μM NAA, and 100 μM AS. After 72 h co-cultivation, leaf explants were washed three times (5 min each) in liquid co-cultivation medium plus 500 mg l^{-1} timentin, rinsed twice in the same medium without timentin, and then blotted dry on sterile filter paper. The explants were then transferred to regeneration medium (WPM with 9.08 μM TDZ, 1.07 μM NAA, 200 mg l^{-1} timentin, and 60 μM STS) with the abaxial side up, and cultured in the dark at 25°C for 3 weeks, before being transferred to the 16-h light for 3 days. Explants were then transferred to shoot elongation medium with 200 mg l^{-1} timentin and 30 mg l^{-1} kanamycin for five sub-cultures. After selection, leaves from regenerated putative transgenic shoots were harvested for PCR analysis. Transgenic shoots were transferred to shoot elongation medium containing 20 mg l^{-1} kanamycin to elongate and multiply shoots. Sixty-five explants were used for each transformation experiment; and the experiment was replicated five times. To inhibit *Agrobacterium* overgrowth, cultures were transferred to fresh medium with timentin and kanamycin every 2–3 weeks.

Rooting of transgenic shoots

Shoots were rooted using an IBA dip method described previously by Liu and Pijut (2008). Shoots (2–3 cm in length) were excised and the basal end dipped in 2.5 mM IBA for 3 min, then placed in Magenta GA-7 vessels containing half-strength MS medium supplemented with

2% (v/v) sucrose, 0.7% Difco-Bacto agar, and 200 mg l^{-1} timentin. Cultures were placed in the dark for 4 days prior to transfer to a 16-h photoperiod. Percent rooting was calculated 3 weeks after root induction treatment.

PCR analysis

DNA was extracted from 100 mg of in vitro grown leaves from putative transgenic shoots using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). To screen black cherry transformants, the primers *PsAG-Hind* III 5′-TGA AAA TAG TTC CGA GAG TCA AGC TTA GGC -3′ and *PsAG-Xba* I 5′- GGC GTC TAG AAA GAA ACA ATT GAG AGG TAC -3′ were used to amplify a 450 bp product of the black cherry *AG* gene. Primers 5′-AAT ATC ACG GGT AGC CAA CG -3′ and 5′- TGC TCC TGC CGA GAA AGT AT-3′ were used to amplify a 364 bp *nptII* gene fragment. Reactions were performed in a 25 µl volume, containing 2.5 µl 10× PCR buffer (Invitrogen, Carlsbad, CA), 4 mM $MgCl_2$, 0.2 mM each of dNTP, 1 mM each of primer, 1 U of Go Taq DNA polymerase (Promega), and 300–400 ng DNA. Reactions were subjected to an initial cycle of 3 min at 94°C and 35 cycles of 40 s at 94°C, 40 s at 55°C (for *nptII*) or 58°C (for black cherry *AG*), 1 min at 72°C, followed by a final extension at 72°C for 7 min. Amplified DNA was detected under UV light, after electrophoresis of the amplification reaction mixture for each sample in 1% (w/v) agarose gels and staining with ethidium bromide. PCR products were purified with QIAquick Gel Extraction Kit (Qiagen) for sequencing PCR. Sequencing PCR products were purified by the Performa Dye Terminator Removal System (Edge Biosystems, Gaithersburg, MD) and sequenced at the Purdue Genomic Center (West Lafayette, IN).

Southern blot analysis

Genomic DNA of black cherry was isolated from PCR-positive leaves. To confirm the insertion of *nptII* gene, a total of 10 µg genomic DNA was digested with restriction enzyme *Bam*HI at 37°C in a water bath overnight. The digested DNA fragments were separated on 0.8% agarose gels. For Southern blot analysis to confirm the insertion of the black cherry *AG* gene, two restriction enzymes *Xba* I and *Xho* I were used to digest genomic DNA. After electrophoresis, the agarose gel was soaked in 0.25 M HCl for 10 min followed in the denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min, and then the DNA fragments from the gel were transferred to a nylon membrane. The transferred DNA was immobilized by UV irradiation (Stratagene UV crosslinker, 120 mJ). The template used as probes were PCR amplification products: 364 bp *nptII* gene and 450 bp black cherry *AG* gene. A total of 30 ng DNA in 45 µl TE buffer was labeled by 5 µl [α-^{32}P] dCTP Random Prime Labeling System (Rediprime II; GE Healthcare Bio-Sciences Corp.). Pre-hybridization was performed for at least 1 h in the hybridization solution (Sigma, St. Louis, MO, Cat # H703) at 65°C with gentle shaking; after adding the denatured probe, the hybridization was carried out overnight under the same conditions. After hybridization, the membranes were washed twice (30 min each time in the same conditions mentioned above) with a solution containing 2× SSC and 0.1% SDS, then once in the solution of 0.5× SSC and 0.1% SDS, 0.1% PPi at 62°C for 20 min. The washed membrane was wrapped in plastic wrap and exposed to a radioactive phosphorus film (autoradiography) at room temperature overnight.

Experimental design

Transformation experiments were conducted in a completely randomized design with ten or more Petri dishes per transformation. Each dish contained at least six leaves. Experiments were replicated five times.

Results

Transgenic shoots regenerated from leaf explants

Leaf explants, transformed with *A. tumefaciens* (EHA105) containing the construct PsAGRNAi, doubled in size and produced callus after 2–3 weeks culture in darkness (Fig. 2b). Callus appeared mainly on the wounded edges and midrib, and became reddish (anthocyanin production) when cultures were transferred to the light. Adventitious shoot buds began to develop 2 weeks after transfer to elongation media and shoots were visible around 3 weeks (Fig. 2c). Leaf explants individually sub-cultured to shoot elongation medium every 2–3 weeks continued growth and development. No shoot buds were regenerated when explants were placed on medium with kanamycin immediately after co-cultivation (data not shown). However, using a 27-day selection, 5–10 shoots survived after selection. Kanamycin killed non-transgenic shoots which first became reddish or purple, then appeared bleached showing increased susceptibility to kanamycin (Fig. 2e, f), and eventually died after five sub-cultures on kanamycin medium. Transgenic shoots developed a main stem with leaves and elongated after selection (Fig. 2d, g), whereas false positive transgenic shoot buds formed as a cluster of leaves without a main stem (Fig. 2e). Regenerated shoot buds showed low vigor, stunted growth, browning of the tissue, and eventually ceased growth in subsequent passages on medium with kanamycin. Among the three treatments for the 27-day selection, 12 h induction with AS

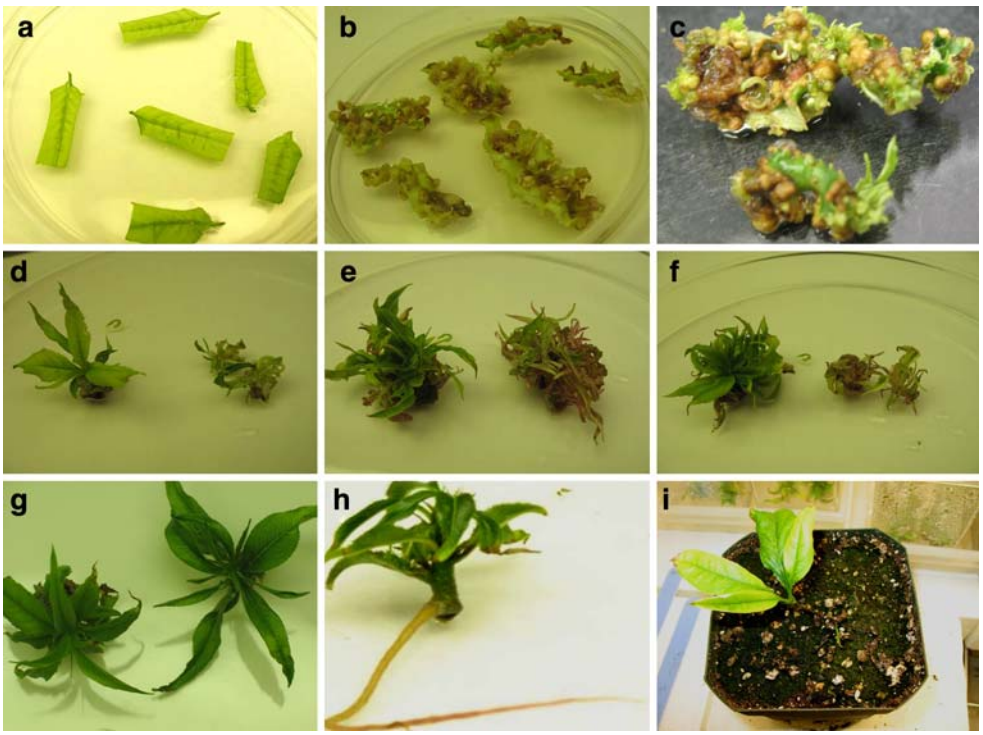

Fig. 2 Transformation and regeneration of black cherry leaf explants. **a** Leaf explants after inoculation, **b** Callus formed 3 weeks after transformation, **c** Putative transgenic shoots regenerated from 27-day selection, **d** Shoots after one sub-culture selection on kanamycin medium; transgenic shoot (*left*), non-transgenic shoot (*right*), **e** Shoots after two sub-cultures on 30 mg l^{-1} kanamycin medium; transgenic shoot (*left*), non-transgenic shoot (*right*), **f** Shoots after three sub-cultures on kanamycin medium; transgenic shoot (*left*), non-transgenic shoot (*right*), **g** Elongated transgenic shoots, **h** Rooted transgenic shoots, **i** Rooted transgenic shoot 4 weeks after acclimatization

produced the best results: 40% of kanamycin-resistant shoots were PCR-positive shoots (Table 1). Longer induction time (24 h) decreased the transformation efficiency and no transgenic shoots were recovered. It was possible that overgrowth of *Agrobacterium* was not at the exponential growth stage and the infection ability was decreased. *Agrobacterium* overgrowth was observed. Many leaf explants became brown and died after 3 days co-culture, and it was difficult to eliminate the bacterial growth. *Agrobacterium* grew even after several washings with 500 mg l^{-1} timentin. Overgrowth of bacteria decreased the regeneration efficiency; therefore, the chance for transformed cells to regenerate also decreased.

Integration of transgenes

PCR verified the transformation of black cherry with the construct PsAGRNAi, showing a 450 bp fragment of black cherry *AG* gene (Fig. 3). Since the *AG* fragment within the PsAGRNAi construct was from cDNA, we believe the PCR product was amplified from the transgene instead of the endogenous *AG* gene which has three introns and the size of the fragment amplified from the wild type should be about 1.8 kb. The *nptII* gene was amplified for four independent transgenic lines (Fig. 4), with a 364 bp *nptII* gene fragment. Sequencing results of the PCR products confirmed that the products were amplified from the *AG* and

Table 1 Summary of transformation experiments with *Prunus serotina* (black cherry)

No. leaf explants	No. days selection after co-cultivation	*Vir* gene induction time (h)	Explant inoculation time (min)	No. days co-culture	AS in co-culture media	Total shoots after kanamycin selection	PCR – positive shoots
325	27	6	40	3	+	5	1[a]
325	27	12	60	3	+	10	4
110[b]	27	24	60	3	+	2	0

Abbreviations: acetosyringone (AS); polymerase chain reaction (PCR)

[a] Plant did not survive after confirmation

[b] This method led to *Agrobacterium* overgrowth, no formation of callus on leaf explants after transformation, and leaf explants eventually died

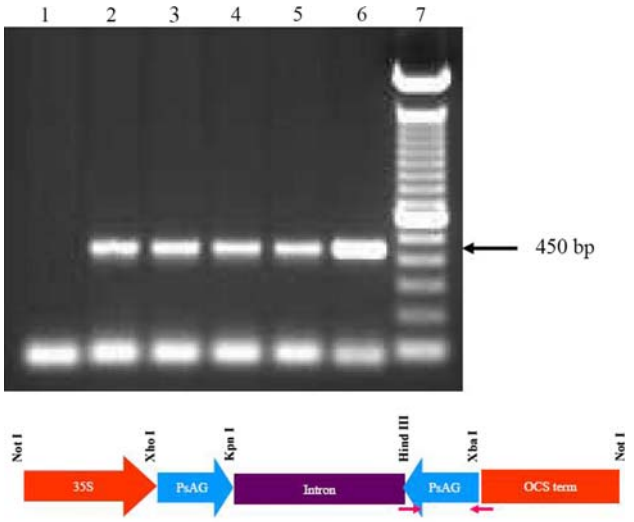

Fig. 3 RNAi expression cassette (*below*) and PCR image for transgenic black cherry with the construct PsAGRNAi. A 450 bp black cherry *AG* gene fragment was amplified. Primer positions were indicated by *arrows*. *Lane 1*, nontransformed shoot, *Lanes 2–5*, transgenic BC3-t1, -t2, -t3, -t4, *Lane 6*, plasmid PsAGRNAi, and *Lane 7*, 100 bp DNA ladder

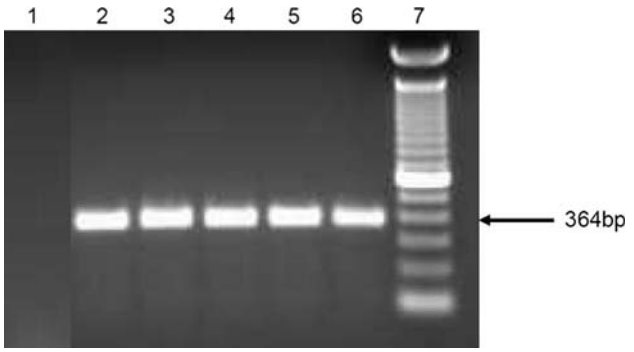

Fig. 4 PCR detection of *nptII* gene in transgenic black cherry. A 364 *nptII* gene fragment was amplified from transgenic plants. *Lane 1*, non-transformed shoot, *Lanes 2–5*, transgenic BC3-t1, -t2, -t3, -t4, *Lane 6*, 2 µl PCR product from plasmid PsAGRNAi, *Lane 7*, 100 bp DNA ladder

nptII genes, respectively, with a 100% nucleotide match (data not shown).

To confirm the presence and integration of the transgene into the transformants, Southern blot analysis was performed. To confirm *nptII* gene, two lines were analyzed. In each of the two transformants, the *nptII* probe hybridized to the DNA fragments from *Bam* HI digested genomic DNA (Fig. 5). The positive control was PCR product of *nptII* gene (364 bp). Examination of *nptII* hybridization pattern indicated that one to two copies of the transgene existed in the transgenic lines. The genomic DNA from the control

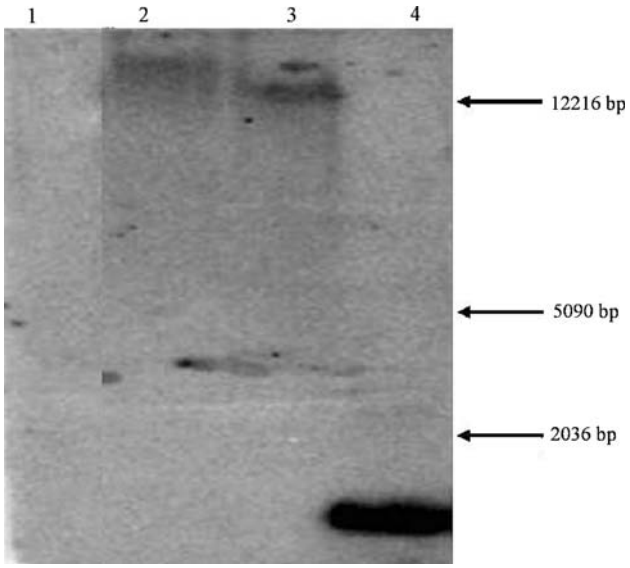

Fig. 5 Southern blot analysis of transgenic black cherry using the *nptII* gene as the probe. Plant DNA samples (10 µg) were digested with *Bam* HI. *Lane 1*, DNA from non-transformed leaves, *Lanes 2–3*, transgenic *lines* (BC3-t1,-t2), *Lane 4*, positive control, 100 pg PCR product of 364 bp fragment of *nptII* gene

non-transformed shoot did not show hybridization bands. For Southern blot to confirm the black cherry *AG* gene, three transgenic lines were analyzed. Double restriction enzymes (*Xba* I and *Xho* I) should cut out a 1.6 kb fragment of the transgene (*AG* sense, 810 bp intron and *AG* antisense). The transgene was released by double restriction enzyme digestion and two independent lines showed positive hybridization bands of the transgene (Fig. 6). One putative independent line showed no band or one faint band, which may have resulted from insufficient DNA used. DNA from the control non-transformed shoot did not show any hybridization bands.

Rooting of shoots

Three weeks after root induction with IBA, roots developed with a mean length of 1.7 cm and an average of one to three roots per shoot (Fig. 2h). The rooting percentage of clonal copies of the four transgenic lines was 37.5%. One rooted transgenic plantlet was acclimatized in soil, new leaves appeared after 4 weeks, and this plantlet survived for 2 months in the culture room (Fig. 2i). Although transgenic shoots rooted, most shoot tips and leaves died or deteriorated making acclimatization extremely difficult. This may be because the high concentration of IBA (2.5 mM) disturbed the endogenous hormone balance which caused the production of ethylene, and ethylene leads to senescence. The rooting protocol for transgenic shoots needs to be further investigated.

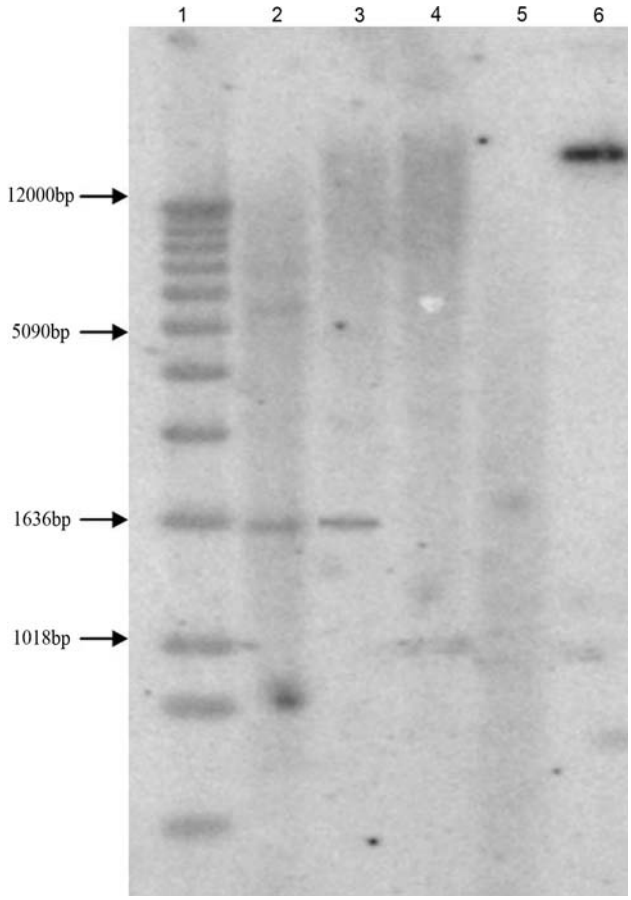

Fig. 6 Southern blot analysis using the black cherry *AG* gene as the probe. Plant DNA samples were double digested with *Xba* I and *Xho* I. *Lane 1*, 1 kb DNA ladder, *Lanes 2–3*, transgenic lines BC3-t1, -t2, *Lane 4*, putative transgenic line BC3-t3, *Lane 5*, non-transformed line, *Lane 6*, positive control, 100 pg plasmid PsAGRNAi

Discussion

An efficient gene delivery system is very critical for genetic modification of *Prunus serotina*. *Agrobacterium* strain EHA105 has been reported effective for transformation of other woody species such as apple (De Bondt et al. 1996; Ko et al. 2000), citrus (Peña et al. 1995), pear (Mourgues et al. 1996), blueberry (Cao et al. 1998), pomegranate (Terakami et al. 2007), and plum (Urtubia et al. 2008). This *Agrobacterium* strain also proved effective for black cherry transformation.

Black cherry was sensitive to kanamycin. No shoot regeneration from leaf explants for this elite mature genotype occurred when kanamycin was used at 15 mg l^{-1} or greater (Liu and Pijut 2008). In this study, when kanamycin at 15 or 30 mg l^{-1} was used for early selection, no transgenic shoots were produced (data not shown). Using a late selection strategy, transgenic shoots could survive on a medium with 30 mg l^{-1} kanamycin. Using a 27-day selection strategy enabled transformed cells to regenerate, while non-transformed shoot buds were killed. This procedure gave rise to 40% of surviving shoots being true transgenic shoots, and this result was similar to previous studies. Almond (*P. dulcis*) was similarly sensitive and 15 mg l^{-1} kanamycin was used for selection of almond transformants (Miguel and Oliveira 1999). Delayed selection (incorporating antibiotics into the culture medium, such as kanamycin, several days after the start of regeneration) was a successful strategy for transformation of apple (Yao et al. 1995; Yepes and Aldwinckle 1994) and apricot (Machado et al. 1992). Ramesh et al. (2006) used a 70-day delayed selection strategy for transformation of *P. dulcis* to address the adverse effect of kanamycin which inhibited organogenesis. Similarly, in apricot, 20 days of delayed selection was found to greatly improve transformation and selection efficiency (Petri et al. 2006). In this study, it was found that kanamycin also inhibited shoot elongation. After PCR confirmation, kanamycin was decreased to 20 mg l^{-1}, allowing transgenic black cherry shoots to elongate and multiply. A total of four independent transgenic lines were obtained. Southern blot analysis of three PCR-positive shoots showed the integration of the T-DNA into the black cherry genome.

Vir gene induction with AS was widely used to improve transformation of recalcitrant tree species. Transgenic apple shoots were obtained by *Agrobacterium* virulence induction for 5 h, and then co-cultivation for 3 days (Dandekar et al. 2006; James et al. 1993; Maximova et al. 1998). Transgenic poplar was achieved by 4 h induction (Ma et al. 2004). In this study, an induction period from 6 to 24 h was tested. It was found that 12 h induction was suitable to enhance the transfer of the T-DNA and produce transgenic shoots. Because the entire process (from transforming leaf explants to confirming transgenic shoots) was lengthy (6–8 months), and many factors were involved, it was difficult to determine which one factor (selection, induction, inoculation, co-culture, AS in the media, etc.) contributed the most to the transformation efficiency. These factors, along with others, will need to be evaluated more closely in future experiments.

Although *Agrobacterium*-mediated transformation has become the preferred method for plant genetic engineering, many economically important plant species, and elite cultivars of particular species are still highly recalcitrant. Attempts to increase the efficiency of plant transformation by identifying or manipulating more highly virulent *Agrobacterium* strains (e.g., Pazour and Das 1990) or by improving bacterial culture conditions (Jin et al. 1990) have been reported. *Agrobacterium*-mediated transformation is a multi-factor, complex interaction process. To achieve maximal *vir* gene expression for T-DNA transfer, further investigations are needed for many factors to achieve efficient black cherry transformation.

The rooting method for transgenic *Prunus serotina* needs to be improved. Transgenic shoots of some woody species are recalcitrant to rooting. Ramesh et al. (2006) reported 38% rooting rate for transgenic almonds. Padilla et al. (2003) were able to obtain 49 to 91% rooting rate for transgenic plum shoots by the addition of 5 μM NAA to the rooting medium. However, in another plum transformation system (Petri et al. 2008c), some plantlets died after acclimatization. In our study, several in vitro surviving transgenic rooted shoots usually only had one root (Fig. 2h).

This transformation, regeneration, and rooting protocol for developing transgenic black cherry will continue to be evaluated in future experiments, in order to optimize the system for several mature black cherry genotypes.

Acknowledgments The authors gratefully acknowledge Drs. W. David Dai and Guo-Qing Song for their constructive review and suggestions for the improvement of this manuscript.

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