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Improvement of *Agrobacterium*-mediated transformation and rooting of black cherry

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Abstract An improved protocol for *Agrobacterium*-mediated transformation of an elite, mature black cherry genotype was developed. To increase transformation efficiency, vacuum infiltration, sonication, and a combination of the two treatments were applied during the cocultivation of leaf explants with *Agrobacterium tumefaciens* strain EHA105 harboring a *PsAGAMOUS* RNAi plasmid (pART27-PsAGRNAi). The effects of *Agrobacterium* culture density and cocultivation duration on transformation efficiency were examined using EHA105 harboring either pBI121-MDL4 or pBI121-PsTFL1. In addition, the effect of the binary vector on transformation efficiency was also studied. Fifteen-minute vacuum infiltration without sonication produced the highest transformation efficiency (21.7%) in experiments using pART27-PsAGRNAi. OD_{600} values of 1.0 and 1.5 resulted in a transformation efficiency of 5% when pBI121-PsTFL1 was used for transformation. Transformation efficiency of 5% was also obtained from 3-d cocultivation using construct pBI121-MDL4 whereas no shoots regenerated after 4-d cocultivation. The binary vectors used also impacted transformation efficiency. PCR and quantitative-PCR analyses were used to confirm the integration of transgenes and determine the copy number of the selectable marker gene, *neomycin phosphotransferase II*, in 18 putative transgenic lines. Rooting of transgenic black cherry shoots was achieved at a frequency of 30% using half-strength Murashige and Skoog medium supplemented with 2% sucrose, 5 μM naphthaleneacetic acid, 0.01 μM kinetin, and 0.793 mM phloroglucinol, and the resulting transgenic plants were successfully acclimatized.

Keywords Genetic transformation · *Prunus serotina* · Rooting · Sonication · Vacuum infiltration

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Introduction

Black cherry (*Prunus serotina* Ehrh.) is the only species in the genus *Prunus* that is of commercial importance as a timber species. Distributed throughout the northeastern United States, it is one of the most valuable hardwoods for high-end cabinetry, furniture, architectural millwork, paneling, and veneer (Marquis 1990; Cassens 2007). The demand from hardwood lumber mills for high-quality black cherry wood is high, and there is an increased interest in establishing professionally managed plantations of improved black cherry genotypes. However, the damage caused by cambial-mining insect pests such as the peach bark beetle (*Phloeotribus liminaris*), the lesser and greater peachtree borers (*Synanthedon pictipes* and *Synanthedon exitiosa*, respectively), and the agromyzid cambium miner (*Phytobia pruni*) triggers gummosis, a nonspecific defense response prevalent in members of the Rosaceae. The deposition of resinous gum can decrease the value of affected lumber by as much as 90% (Barnd and Ginzel 2008). To alleviate this problem, genetic engineering offers an alternative approach to traditional breeding in trees because it avoids the long juvenile period and enables transfer of specific traits into selected genotypes (Peña and Séguin 2001). This could be a potentially efficient approach to improve the resistance of black cherry to specific pests and eventually reduce the occurrence of gummosis.

An *Agrobacterium*-mediated transformation and regeneration system for black cherry was previously established (Liu and Pijut 2010), but the transformation efficiency obtained

was only 1.2%. Improvement of this system is therefore needed. Evaluation of different *Agrobacterium* strains and binary vectors, the level of acetosyringone (AS), *Agrobacterium* suspension concentration, and the durations of inoculation and cocultivation could lead to increased transformation rates (Amoah *et al.* 2001; Jin *et al.* 2005). In addition to these factors, sonication and vacuum infiltration have been widely used in a number of plant species to help increase cocultivation efficiency (Liu *et al.* 2005; Park *et al.* 2005; de Oliveira *et al.* 2009; Bakshi *et al.* 2011; Subramanyam *et al.* 2011), but have not been evaluated in *Prunus* species. Even though transgenic black cherry shoots form roots (Liu and Pijut 2010), most shoot tips and leaves died or deteriorated during the rooting process, making acclimatization of transgenic plants extremely difficult. Pérez-Clemente *et al.* (2004) also found that rooting was very difficult in transgenic shoots of peach (*Prunus persica* L).

The objective of this study was to improve the existing *Agrobacterium*-mediated transformation protocol for black cherry by testing sonication, vacuum infiltration, *Agrobacterium* concentration, cocultivation duration, and binary vector and to improve the rooting and acclimatization of transgenic plants, which would provide a good foundation for future genetic improvement of black cherry.

Materials and Methods

Plant material. Leaf explants for transformation and regeneration were excised from *in vitro* shoots of a mature elite black cherry genotype (BC3) as described previously (Liu and Pijut 2008, 2010). *In vitro* shoot cultures were maintained in Magenta™ GA-7 vessels on a modified Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) (M499; *Phyto*Technology Laboratories, Shawnee Mission, KS) with organics (100 mg L^{-1} myoinositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 0.1 mg L^{-1} thiamine HCl, and 2 mg L^{-1} glycine) supplemented with 3% (*w/v*) sucrose, 4.44 μM 6-benzyladenine (BA), 0.49 μM indole-3-butyric acid (IBA), 100 mg L^{-1} casein hydrolysate, 0.5 μM gibberellic acid (GA_3), and 0.7% (*w/v*) Bacto agar (No. 214030; Becton Dickinson, Franklin Lakes, NJ). 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 24 ±2°C under a 16-h photoperiod provided by cool-white fluorescent lamps (80–100 μmol m^{-2} s^{-1}) and were transferred to fresh medium every 3 wk.

Binary vectors and Agrobacterium strain. Three binary vectors were used to test the efficiency of transformation (Fig. 1). pART27-PsAGRNAi, an RNAi plasmid targeting the black cherry *AGAMOUS* gene (*PsAG*) (Liu and Pijut 2010; Liu *et al.* 2010), was used to study the effects of vacuum infiltration and sonication (Fig. 1*c*). pBI121-MDL4, the binary vector pBI121 containing complementary DNA (cDNA) of the gene for (*R*)-(+)-mandelonitrile lyase isoform 4 (*MDL4*) from black cherry (Hu and Poulton 1999), was constructed and used to test the effect of cocultivation duration of leaf explants with *Agrobacterium* (Fig. 1*b*). To construct pBI121-MDL4, the rolC promoter–driven MDL4 with NOS terminator was first assembled in a pGEM-T Easy vector (Promega, Madison, WI), and the entire cassette was cut with *Hin*dIII and inserted into the same site of pBI121. pBI121-PsTFL1, the binary vector pBI121 containing cDNA of *TERMINAL FLOWER 1* (*TFL1*) from black cherry (*PsTFL1*; Wang and Pijut 2013) driven by the *CaMV 35S* promoter, was used to examine the impact of *Agrobacterium* concentration on transformation efficiency (Fig. 1*a*). To construct pBI121-PsTFL1, pBI121 was first digested with *Bam*HI and *Eco*RI to remove the β-glucuronidase gene (*GUS*) and NOS terminator, and an

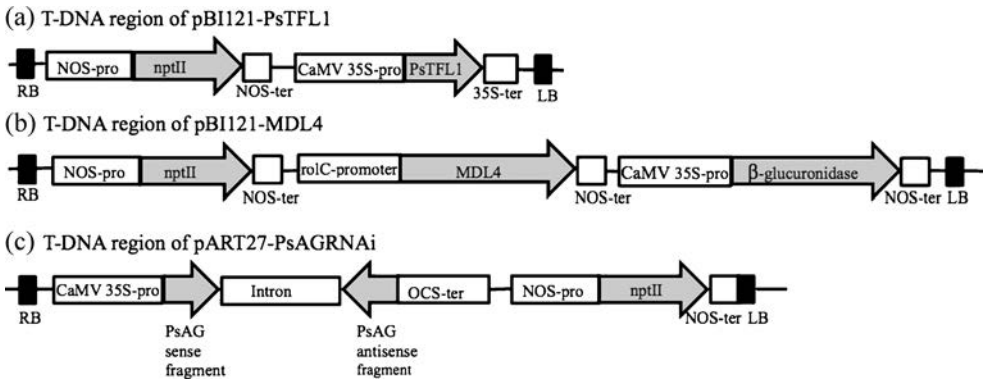

Figure 1. Schematic representation of the T-DNA region of the binary vectors used in transformation of black cherry. (*a*) T-DNA region of pBI121-PsTFL1. *NOS-pro* nopaline synthase promoter, *nptII* neomycin phosphotransferase II gene, *NOS-ter* nopaline synthase terminator, *CaMV 35S-pro* cauliflower mosaic virus 35S promoter, *PsTFL1 Prunus serotina TERMINAL FLOWER 1* gene, *35S-ter* cauliflower mosaic virus 35S terminator. (*b*) T-DNA region of pBI121-MDL4. *rolC-promoter* rolC promoter of *Agrobacterium rhizogenes* Ri plasmid, *MDL4* gene for (*R*)-(+)-mandelonitrile lyase isoform 4 from black cherry, *β-glucuronidase Escherichia coli* β-glucuronidase gene (GUS). (*c*) T-DNA region of pART27-PsAGRNAi. *PsAG Prunus serotina AGAMOUS* gene, *intron* partial PDK intron, *OCS-ter* OCS terminator.

enhanced green fluorescent protein (EGFP) fragment with 35S terminator was cut from the vector pd35E (Li *et al.* 2004) with the same restriction enzymes and ligated into pBI121. Then the EGFP fragment was replaced with *PsTFL1* by using *Xba*I, to yield pBI121-PsTFL1. Each construct was introduced into *Agrobacterium tumefaciens* strain EHA105 by heat shock (Hofgen and Willmitzer 1988) and then used for genetic transformation.

Agrobacterium-mediated transformation and regeneration of adventitious shoots. The basic procedure for transformation was conducted according to our previous method (Liu and Pijut 2010). The procedure consisted of a 12-h *vir* gene induction of *Agrobacterium* in induction medium containing 200 μM AS, a 60-min inoculation of explants in *Agrobacterium* suspension with gentle shaking, a 3-d cocultivation of leaf explants and *Agrobacterium*, 3 wk in the dark, 3 d under light on regeneration medium, and five subcultures for selection on shoot elongation medium containing 30 mg L^{-1} kanamycin at 24±2°C under a 16-h photoperiod (Liu and Pijut 2010). The effects of vacuum infiltration and sonication, concentration of *Agrobacterium* suspension, and duration of cocultivation were investigated independently in each factorial. The vacuum infiltration and sonication treatments were applied during the inoculation of leaf explants with *Agrobacterium* suspension. Vacuum infiltration at 63.5 cm Hg for 0, 5, 10, or 15 min combined with sonication for 0, 30, 60, or 90 s (Branson Ultrasonics, Danbury, CT) were tested. The effect of *Agrobacterium* concentration on transformation efficiency was evaluated by testing the bacterial solution at four optical density $(OD)_{600}$ values (0.5, 1.0, 1.5, or 1.8). To test the effect of cocultivation period, explants were cocultivated for either 3 or 4 d with *Agrobacterium* (OD_{600} of 1.0) on regeneration medium containing 100 μM AS. For each treatment, only one factor was examined while the rest of the factors were the same as in the existing protocol developed by Liu and Pijut (2010). Timentin (200 mg L^{-1}) was used to eliminate *Agrobacterium* overgrowth during the regeneration stage. There were three replications for each factorial, with 20 explants per replication. The kanamycin-resistant shoots were identified as transgenic shoots by PCR analysis. Transformation efficiency was calculated as the number of calli that formed transgenic shoots relative to the initial number of leaf explants per treatment.

PCR and copy number determination of transgenic plant lines. Genomic DNA of all regenerated shoots was isolated using the CTAB method (Murray and Thompson 1980), and PCR analysis was conducted for the marker gene *neomycin phosphotransferase II* (*nptII*) and the gene of interest in each construct. 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 fragment of *nptII*. Primers 5′-TGA AAA TAG TTC CGA GAG TCA AGC TTA GGC-3′ and 5′-GGC GTC TAG AAA GAA ACA ATT GAG AGG TAC-3′ were designed to amplify a 450-bp fragment of *PsAG*. Primers 5′-ATG GAG AAA TCA ACA ATG TCA GCT GTA GTA TTG GTG TTG AAC CTT TTG GTC CTT CAT CTT CAA TAT TCA GAG GTT CAC TCG CTT GCC AAT ACT TCT TCT GAG-3′ and 5′-TTA AAA AGC AAA GGA TAA TGC TGA CTT CAG GGA ATC CAT A-3′ amplified the 1,515-bp *MDL4* gene. Primers 5′-ATG TGA GTT AGC TCA CTC ATT AGG C-3′ and 5′-CTA GCG TCT TCT AGC TGC TGT TTC TCT CTG-3′ were used to amplify the 1,725-bp *CaMV 35S* promoter and *PsTFL1* cDNA. Each reaction contained the following reagents in 50 μL: 1 μL genomic DNA (20–100 ng), 1 μL 10 μM of each primer, 5 μL 10× PCR buffer (Clontech, Mountain View, CA), 1 μL dNTPs, 1 μL 50× Advantage® 2 Polymerase Mix (Clontech), and sterile water up to the final volume. The cycling conditions consisted of a denaturing step at 95°C for 3 min; 30 cycles at 95°C for 15 s, annealing for 40 s, and elongation for 30 s to 2 min at 68°C; followed by a final extension at 68°C for 3 min. The annealing temperature was determined based on the T_m value of the respective primers. After electrophoresis in 1% (*w/v*) agarose gels containing 0.5 μg mL^{-1} ethidium bromide, the PCR products were visualized under UV light.

The transgene copy number for *nptII* was determined by quantitative-PCR (qPCR). Black cherry mandelonitrile lyase isoform 2 (*MDL2*) was selected as a single-copy endogenous reference gene, confirmed by Hu and Poulton (1999) using Southern blot analysis. Genomic DNA from the leaves of transgenic and wild-type black cherry plant lines was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Primers 5′-TTG CTC CTG CCG AGA AAG TAT CCA-3′ and 5′-CGA TGT TTC GCT TGG TGG TCG AAT-3′ were designed to amplify a 100-bp fragment of *nptII*, and primers 5′-GTG AAA TCA ACA ATG TCA GCT ATA CTA GTA-3′ and 5′-GCA GAG GTA TTG GCA AGC GAT-3′ were used to amplify a 99-bp fragment of the *MDL2* reference gene. The qPCR was performed with the Stratagene Gene MX 3000 PM. Each reaction contained the following reagents in 20 μL: 2 μL DNA (approximately 100 ng), 1 μM of each primer, 10 μL Brilliant SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA), and sterile water up to the final volume. The cycling conditions consisted of DNA polymerase activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis from 55 to 95°C. Each sample was replicated three times, and relative standard curves of serial dilutions of genomic DNA for both target and reference genes were constructed. The transgene copy number in 18 independent lines was analyzed following the calculation reported by Weng *et al.* (2004).

Rooting and acclimatization of transgenic shoots. Robust transgenic shoots verified by PCR analysis were selected for

rooting trials and included lines of pART27-PsAGRNAi, pBI121-PsTFL1, and pBI121-MDL4. The rooting efficiency of transgenic shoots was tested on half-strength MS medium supplemented with 5 μM naphthaleneacetic acid (NAA); 0.01 or 0.1 μM kinetin; 0.793 mM, 1.586 mM, or 2.379 mM phloroglucinol (PG); 2% (*w/v*) sucrose; and 0.7% Bacto agar in Magenta™ GA-7 vessels. Neither kanamycin nor Timentin was added to the rooting medium. Shoots 2–3 cm in length were excised and transferred to various media treatments. Cultures were placed in the dark for 5 d before being exposed to a 16-h photoperiod. Each treatment was replicated three times with 10 shoots per replicate. The number of roots, root length, and percent rooting were recorded after 6 wk. After 6 wk, any unrooted shoots were transferred to fresh rooting medium of the same type for continuous root induction.

Transgenic plants with a shoot and root system were rinsed in distilled water to remove residual agar and transferred to 10 cm×9 cm plastic pots containing a moist, autoclaved potting mix (Fafard Growing Mix, Agawam, MA). Rooted plantlets were maintained in closed 3.8-L zipper-lock plastic bags to maintain a high relative humidity in the culture room at 24±2°C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}), with four holes punched below the zipper lock for gas exchange. Plantlets were watered as needed and gradually acclimatized (by opening the bags) over a period of 2 wk. Plantlets that showed slow growth were stored at 4°C in the dark for 4 mo and then exposed to a 16-h photoperiod (80 μmol m^{-2} s^{-1}) at 24±2°C.

Statistical analysis. Data were analyzed with an analysis of variance (ANOVA) using the ANOVA procedure in SAS, version 9.2 (SAS Institute 1999). Tukey's test was applied to distinguish significant differences between treatments at α=0.05.

Results and Discussion

The conditions for *Agrobacterium*-mediated gene transfer need to be optimized for each plant species because of differences in the types of explant tissues used and in the response to transformation and regeneration. In this study, we evaluated such factors as the conditions of inoculation and cocultivation, *Agrobacterium* inoculum density, and plasmid vector in order to improve transformation of black cherry.

Effects of vacuum infiltration and sonication. Combinations of 0, 30, 60, or 90 s sonication and 0, 5, 10, or 15 min vacuum infiltration were tested in our study. Among the 16 treatments, 60 and 90 s sonication increased the transformation rate (1.33 calli producing shoots/20 explants=6.7%) over 30 s sonication (0%), but when combined with vacuum infiltration, the rate decreased. Vacuum infiltration for 15 min without sonication resulted in the highest transformation efficiency (4.33 calli producing shoots/20 explants=21.7%) and the highest number of transgenic shoots regenerated per explant (Table 1). When either sonication or vacuum infiltration was applied alone, longer durations were found to produce higher

Table 1. Effect of sonication and vacuum infiltration on transformation efficiency of black cherry

Sonication (s)	Vacuum infiltration (min)	No. of leaf explants infected	No. of calli producing shoots	No. of transgenic shoots
–	5	20	0.67±0.67b	0.67±0.67b
–	10	20	1±1.00b	3.33±3.33ab
–	15	20	4.33±0.88a	14.67±4.06a
30	–	20	0	0
60	–	20	1.33±0.33ab	1.67±0.67b
90	–	20	1.33±0.88ab	5±4.51ab
30	5	20	0	0
30	10	20	0	0
30	15	20	0.33±0.33b	0.33±0.33b
60	5	20	0.67±0.33b	3.67±2.03ab
60	10	20	0	0
60	15	20	0	0
90	5	20	0	0
90	10	20	0	0
90	15	20	0.67±0.67b	1±1.00b

All treatments in this experiment used pART27-PsAGRNAi. Each treatment was replicated three times. Mean values ± standard error followed by the same *letter* were not significantly different by Tukey's multiple comparison test (p=0.05)

Table 2. Effect of *Agrobacterium* concentration and cocultivation duration on transformation efficiency of black cherry

Treatments	No. of leaf explants infected	No. of callus producing shoots	No. of transgenic shoots
Agrobacterium concentration (OD_{600})[z]			
0.5	20	0.33±0.33ab	0.33±0.33a
1.0	20	1±0.00a	1.33±0.33a
1.5	20	1±0.00a	2.67±1.53a
1.8	20	0.33±0.33ab	2±2.00a
Cocultivation duration (d)[y]			
3	20	1±0.00a	8.67±3.84a
4	20	0b	0a

Each treatment was replicated three times. Mean value ± standard error followed by the same *letter* were not significantly different by Tukey's multiple comparison test (p=0.05)

[z] Tests of *Agrobacterium* concentration used pBI121-PsTFL1 and 3-d cocultivation

[y] Tests of cocultivation duration used pBI121-MDL4 at OD_{600}=1.0

Table 3. Effect of binary vector on transformation efficiency

Binary vector	No. of leaf explants infected	*Agrobacterium* inoculum OD_{600}	Sonication (s)	Vacuum infiltration (min)	Cocultivation duration (d)	Transformation efficiency (%)
pART27-PsAGRNAi	20	1.0	0	15	3	21.7a
pBI121-PsTFL1	20	0.5	0	15	3	1.65bc
	20	1.0	0	15	3	5.00b
	20	1.5	0	15	3	5.00b
	20	1.8	0	15	3	1.65bc
pBI121-MDL4	20	1.0	0	15	3	5.00b
	20	1.0	0	15	4	0.00d

Each treatment was replicated three times. Values followed by the same *letter* were not significantly different by Tukey's multiple comparison test (p=0.05)

transformation efficiencies than shorter durations. Vacuum infiltration has long been used to increase transformation efficiency in many plant species and is known to improve penetration of *Agrobacterium* cells into plant tissue layers (Subramanyam *et al.* 2011), whereas sonication creates small channels in the tissue and facilitates entry of *Agrobacterium* cells into internal plant tissues (Trick and Finer 1997). In addition, the microwounding created by sonication may release phenolic compounds that stimulate the virulence of *Agrobacterium* (de Oliveira *et al.* 2009). However, no pattern was found in transformation efficiency when sonication and vacuum infiltration were applied together.

Effects of Agrobacterium concentration and cocultivation duration. The *Agrobacterium* concentration used for inoculation could influence transformation efficiency. Although an OD_{600} of 1.0 or 1.5 produced a higher transformation efficiency than an OD_{600} of 0.5 or 1.8 (Table 2), the differences were not significant. This result was consistent with that reported by Amoah *et al.* (2001), who discovered that an OD_{600} in the range of 1.0–1.5 was optimal, and further increases in cell density led to reduction in transformation efficiency of wheat (*Triticum aestivum* L.). In contrast, increased transient gene expression levels were observed in *Arabidopsis* seedlings following infiltration with *Agrobacterium* at an OD_{600} of 2 (Marion *et al.* 2008). De Clercq *et al.* (2002) tested an OD_{600} of 1.6–2.4 and found it had a detrimental effect on the explant survival of tepary bean (*Phaseolus acutifolius*).

Cocultivation duration has also been evaluated for optimization of transformation. Duration usually ranges from 2 to 4 d, and a longer period of cocultivation was reported to have a positive impact, but the effect of increasing coculture time varies among species and may lead to *Agrobacterium* overgrowth (Xing *et al.* 2007; Dutt and Grosser 2009; Yang *et al.* 2010). Our results showed that a 3-d cocultivation produced 5% transformation efficiency (1 out of 20 explants) when the construct pBI121-MDL4 was used, whereas no shoots regenerated after 4 d of cocultivation (Table 2). When inoculation intensity was beyond a threshold value, plant cell viability could be decreased (Amoah *et al.* 2001). Wu *et al.* (2003) found that a longer cocultivation period could reduce the capacity of wheat immature embryos to form callus and regenerate. In summary, *Agrobacterium* density did not substantially affect transformation efficiency of black cherry.

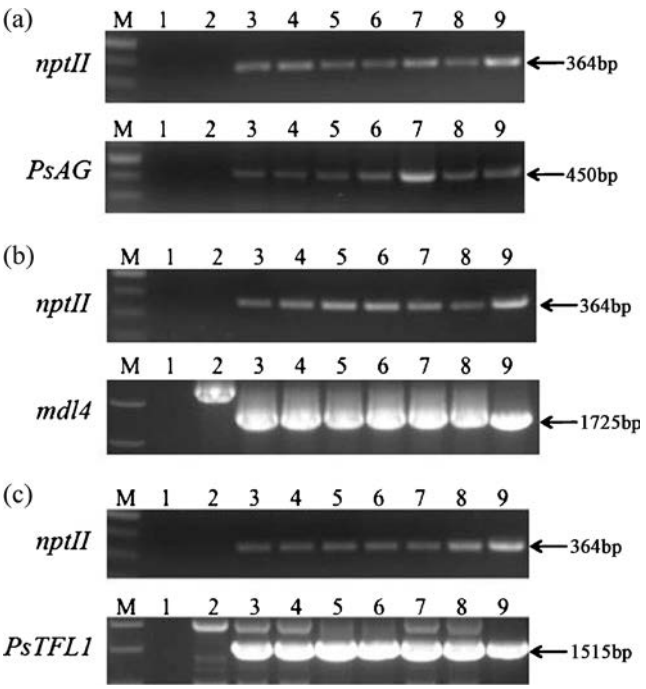

Figure 2. PCR analysis of transgenes in the genome of transgenic black cherry plants. (*a*) A 364-bp *nptII* fragment and a 450-bp *PsAG* gene fragment amplified from genomic DNA of six transgenic plants transformed with pART27-PsAGRNAi. (*b*) A 364-bp *nptII* fragment and a 1,725-bp *MDL4* cDNA amplified from genomic DNA of six transgenic plants transformed with pBI121-MDL4. (*c*) A 364-bp *nptII* fragment and a 1,515-bp CaMV 35S promoter and *PsTFL1* amplified from genomic DNA of six transgenic plants transformed with pBI121-PsTFL1. *Lane M* 100-bp or 1-kb DNA ladder, *lane 1* no-template control, *lane 2* wild-type, *lanes 3–8* six independent transgenic lines, *lane 9* the transformation vector.

Effects of different plasmid vectors. Three different constructs (pART27-PsAGRNAi, pBI121-MDL4, and pBI121-PsTFL1; Fig. 1) were used for black cherry transformation. Under identical transformation conditions (*Agrobacterium* suspension at OD_{600} of 1.0, no sonication, 15-min vacuum infiltration, and 3 d cocultivation), a higher transformation efficiency was observed with the use of pART27-PsAGRNAi (21.7%) than with pBI121-MDL4 or pBI121-PsTFL1 (5% each), indicating that the vector may have a strong impact on transformation (Table 3). Under these conditions, the transformation efficiencies of pBI121-PsTFL1 and pBI121-MDL4 were both 5%, suggesting that the binary vector itself, rather than the genes incorporated, played an important role in transformation efficiency. Differences in T-DNA delivery efficiency between different vectors were also reported by Amoah *et al.* (2001) in their study of wheat transformation, in which the size and organization of the T-DNA region in each vector were thought to contribute to the differences in T-DNA mobility and delivery. The copy numbers of both pART27 and pBI121 in *Agrobacterium* are low, and both vectors are larger than 10 kb, although pART27 is smaller than pBI121 (Gleave 1992; Chen *et al.* 2003). The higher efficiency of the pART27-based vector relative to those based on pBI121 might result from differences in the T-DNA regions or backbones.

Confirmation of transgene integration and analysis of copy number. A total of 18 independent transgenic black cherry lines, including six lines each of pART27-PsAGRNAi, pBI121-PsTFL1, and pBI121-MDL4, were propagated for further examination. PCR and qPCR analysis were conducted to confirm the presence of the transgenes. The *nptII* and target genes were amplified from the genomic DNA of each transgenic line (Fig. 2). The copy number of *nptII* in each of the 18 transgenic lines was determined by qPCR (Table 4), with the calculation based on standard curves of both the target and reference genes (Fig. 3). Among the 18 independent lines, four contained a single copy of *nptII* per tetraploid genome and nine lines had 4–12 copies of *nptII* per tetraploid genome. The other five lines had transgene copy number of less than one, which suggested that they might be chimeras. The formation of chimeras after *Agrobacterium*-mediated transformation has been reported (Charity *et al.* 2002; de Oliveira *et al.* 2009). In these studies, PCR analysis detected the presence of the transgene, but Southern blot analysis showed negative results.

Single-copy transformants are desirable because of their more stable and predictable expression. Although compared to particle bombardment, *Agrobacterium*-mediated transformation is generally thought to produce transformants with low transgene copy number, the integration of multiple transgene copies using *Agrobacterium*-mediated transformation has also been reported. Abou-Alaiwi *et al.* (2012) observed three or more inserts of the transgene in transgenic *Centaurea montana* (mountain bluet). Zhang *et al.* (2010) also reported high transgene copy numbers in transgenic *Cattleya* orchid obtained using *Agrobacterium*-mediated transformation. In the present study, it was not clear whether the copy number

Table 4. C_T value of the reference gene *MDL2* and the target gene *nptII* for each transgenic black cherry line and the estimated copy number of *nptII* for each line

Sample	C_T (MDL2)	C_T (nptII)	4′ (X_0/R_0)	Estimated *nptII* copy no.[z]
AGRNAi-1	19.52±0.08	29.31±0.36	0.011±0.37	Chimera
AGRNAi-2	18.80±0.10	22.73±0.11	0.86±0.15	1
AGRNAi-3	17.60±0.11	21.68±0.11	0.82±0.16	1
AGRNAi-4	19.60±0.20	19.86±0.04	12.23±0.04	12
AGRNAi-5	18.89±0.14	20.58±0.10	4.43±0.17	4
AGRNAi-6	18.78±0.22	21.17±0.19	2.66±0.29	3
MDL4-1	18.70±0.08	28.80±0.20	0.0093±0.22	Chimera
MDL4-2	18.74±0.27	26.64±0.13	0.046±0.30	Chimera
MDL4-3	19.02±0.03	24.85±0.19	0.21±0.19	Chimera
MDL4-4	17.25±0.14	19.06±0.20	4.44±0.24	4
MDL4-5	18.02±0.09	19.42±0.12	5.78±0.15	6
MDL4-6	19.14±0.20	30.26±0.38	0.0043±0.43	Chimera
PsTFL1-1	18.45±0.27	22.28±0.23	0.94±0.35	1
PsTFL1-2	19.64±0.08	20.31±0.17	8.98±0.19	9
PsTFL1-3	20.37±0.03	21.00±0.13	8.88±0.13	9
PsTFL1-4	18.51±0.11	22.26±0.12	0.99±0.16	1
PsTFL1-5	17.84±0.11	20.81±0.15	1.83±0.19	2
PsTFL1-6	19.01±0.07	26.54±0.11	0.060±0.13	Chimera

Values equal mean ± standard deviation. Each real-time PCR reaction was replicated three times

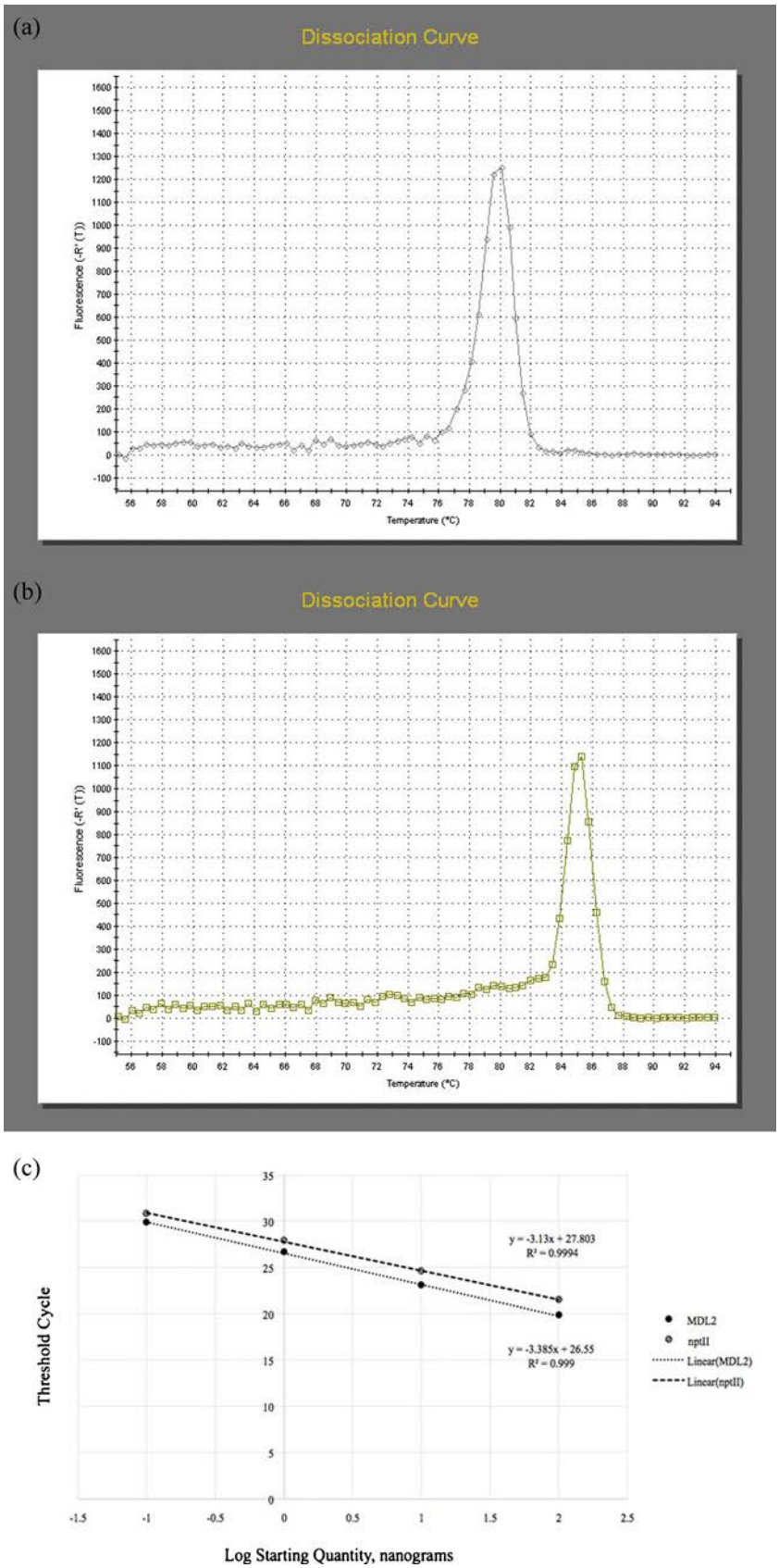

Figure 3. qPCR dissociation curves and relative standard curves. (*a*) Dissociation curve of the reference gene, *MDL2*. (*b*) Dissociation curve of the target gene, *nptII*. (*c*) Relative standard curves of *MDL2* and *nptII* obtained by plotting the threshold cycle (C_T) value versus the log of each initial concentration of genomic DNA.

Table 5. *In vitro* root formation of transgenic black cherry microshoots after 6 wk on rooting medium

NAA + kinetin (μM)[z]	Rooting (%)	Mean no. of roots/shoot	Mean root length (cm)
5+0.01	30.0±5.8a	3.1±1.1a	4.6±0.9a
5+0.1	10.0±0.0b	4.0±1.0a	4.4±1.5a

Values equal mean ± standard error for 10 shoots per treatment. Means in each *column* followed by the same *letter* were not significantly different according to Tukey's multiple comparison test (p=0.05). Data were collected after 6 wk. *NAA* naphthaleneacetic acid

[z] Both media contained 0.793 mM phloroglucinol

of the transgene was related to factors such as inoculation intensity and infection time.

Rooting and acclimatization of transgenic shoots. Although successful rooting and acclimatization of transgenic black cherry shoots has previously been achieved, the frequency was very low and this step in the process needed to be improved. Root induction of transgenic black cherry shoots was initially tested using various protocols (Padilla *et al.* 2003; Espinosa *et al.* 2006; Du and Pijut 2008; Liu and Pijut 2010). Further, we examined combinations of IBA at 2.5, 4.9, 7.4, or 9.8 μM and NAA at 2.7, 5.4, or 8.1 μM. However, the shoots only responded to the rooting medium containing 5 μM NAA and 0.01 μM kinetin, while the rooting rate obtained from the rest of the protocols was either zero or very low (data not shown). Because the combination of NAA and kinetin was the best combination of plant growth regulators for inducing roots on transgenic black cherry shoots among all the protocols tested, a preliminary experiment was designed to test the rooting percentage on medium containing 2.5, 5, or 7.5 μM NAA and 0.01 μM kinetin; 5 μM NAA was confirmed to be the optimal level (data not shown). Padilla *et al.* (2003) used 5 μM NAA and 0.01 μM kinetin in the culture medium for rooting of transgenic plum shoots (*Prunus domestica* L.); under these conditions, they obtained 90% rooting and successfully acclimatized the resulting plantlets. Petri *et al.* (2008) obtained rooting of transgenic plum on medium containing 5 μM NAA and 0.1 μM kinetin. Zimmerman (1984) reported that 4–7 d of dark treatment applied at the beginning of the rooting stage was effective in stimulating rooting of 'Delicious' apple (*Malus domestica* Borkh.). Druart *et al.* (1982) observed changes in endogenous phenolic levels and peroxidase activity induced by darkness. The phenolics might serve as antioxidants to protect auxins from oxidation during the root formation phase (Fett-Neto *et al.* 2001). Caboni *et al.* (1997) studied the *in vitro* rooting ability of almond (*Prunus dulcis* Mill.), a species closely related to black cherry. Microcuttings of seven genotypes of almond were cultured on MS medium containing 5 μM IBA and maintained in the dark for 5 d before being transferred to light. Their data showed that the total phenol content increased and peroxidase activity decreased during the first day of the root induction process in the easiest-to-root genotype, while this trend was not observed in the most difficult-to-root genotype. Their findings also suggested that the increased level of phenol compounds inhibited peroxidase activity, which led to a significantly higher free indole-3-acetic acid (IAA) endogenous level in the basal part of microcuttings of the easiest-to-root genotype than in the difficult-to-root genotype. Because the free IAA endogenous levels were believed to have a positive effect on root formation, these results showed an indirect relationship between phenol content and rooting ability.

Phloroglucinol is a novel substance reported to stimulate root formation in plant tissue culture that may act as an auxin synergist or auxin protector (Teixeira da Silva *et al.* 2013). Phloroglucinol has been used to enhance rooting of several *Prunus* species, including wild cherry (*Prunus avium* L.; Hammatt and Grant 1997), European plum (*P. domestica*; Petri and Scorza 2010), and Chinese plum (*Prunus salicina*; Zou 2010). In the present study, the rooting rate of transgenic black cherry was higher in medium supplemented with PG (data not shown) than without PG. After 6 wk, 30% of transgenic shoots developed roots in half-strength MS medium supplemented with 2% (*w/v*) sucrose, 5 μM NAA, 0.01 μM kinetin, and 0.793 mM PG with 5 d of dark treatment before being exposed to light, while there was only 10% rooting in medium containing 5 μM NAA, 0.1 μM kinetin,

Figure 4. Rooting and acclimatization of transgenic black cherry shoots. (*a*) Roots developed from the basal end of an *in vitro* transgenic shoot after 6 wk on rooting medium containing 5 μM NAA, 0.01 μM kinetin, and 0.793 mM PG. (*b*) Plantlets obtained after acclimatization. (*c*) Healthy elongating transgenic black cherry plant after 4 mo of cold treatment.

and 0.793 mM PG after the same duration of dark treatment (Table 5; Fig. 4*a*). In addition, after the initial 6-wk rooting period, when unrooted shoots were transferred to fresh rooting medium, many of these started to root. After an additional 2 wk, the total rooting percentage reached 50% on rooting medium with 5 μM NAA, 0.01 μM kinetin, and 0.793 mM PG, while 43.3% of shoots rooted on the medium with 5 μM NAA, 0.1 μM kinetin, and 0.793 mM PG. This suggested that rooting of transgenic shoots could take as long as 2 mo, and rooting frequency might depend on time in addition to auxin, cytokinin, and PG concentrations. The two levels of kinetin used here did not cause significant differences in rooting percentage at 8 wk. However, transgenic black cherry shoots rooted more quickly on rooting medium with 0.01 μM kinetin than on medium with 0.1 μM kinetin (Table 5). Among three levels of PG (0.793 mM, 1.586 mM, and 2.379 mM), 0.793 mM gave the highest rooting percentage (data not shown). It was also noticed that certain independent transgenic lines were easier to root than others. For example, the transgenic line MDL4-5 was able to root in the medium containing 5 μM NAA and either 0.01 or 0.1 μM kinetin, while the other MDL4 lines had no root formation, which indicated that root development might also be affected by the position where transgenes integrate into the genome or by the selection of a somaclonal variant that exhibited low root formation. After acclimatization, most of the transgenic plantlets tended to show very slow growth and development (Fig. 4*b*). Plantlets stored at 4°C in the dark for 4 mo and then exposed to a 16-h photoperiod (80 μmol m^{-2} s^{-1}) at 24±2°C continued to grow and develop normally (Fig. 4*c*).

In conclusion, several factors involved in *Agrobacterium*-mediated transformation of black cherry were evaluated. A procedure using *Agrobacterium* inoculum at an OD_{600} of 1.0 or 1.5 combined with a 15-min vacuum infiltration during inoculation and a 3-d cocultivation was found to be the most efficient for black cherry transformation. Compared to our previous protocol, the transformation efficiency was increased from 1.2% to as high as 21.7%, depending on the binary vector. PCR and qPCR analyses confirmed the integration of transgenes and measured the copy number of the *nptII* gene in the genome. An improved rooting protocol was also developed for transgenic black cherry shoots. The rooting efficiency was increased from 37.5% using the original rooting protocol developed by Liu and Pijut (2010) to 50% obtained after 2 mo of root induction using the current rooting method. Therefore, this study enhanced the effectiveness of transformation, regeneration, and rooting of transgenic black cherry for further genetic engineering.

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