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ORIGINAL PAPER

Agrobacterium-mediated transformation of black cherry for flowering control and insect resistance

Ying Wang · Paula M. Pijut

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Abstract Black cherry is one of the most valuable hardwood species for cabinetry, furniture, and veneer. The goal of this study was to develop transgenic black cherry plants with reproductive sterility and enhanced insect resistance. Black cherry *TERMINAL FLOWER 1* (*PsTFL1*) was overexpressed under the control of the CaMV 35S promoter in black cherry via *Agrobacterium*-mediated transformation, as a strategy for gene containment. *PsTFL1* is a homolog of *Arabidopsis TFL1* which is known to play a key role in regulating flowering time by counteracting with *FLOWERING LOCUS T* and repressing the transcription of the floral-related genes. The elevated expression level of *PsTFL1* was proven to be able to significantly delay flowering and cause abnormal floral structure which led to sterility in *Arabidopsis*. Therefore, the overexpression of *PsTFL1* was expected to induce the similar phenotype in black cherry to achieve reproductive sterility. To enhance insect resistance in black cherry, the black cherry endogenous genes encoding prunasin hydrolase isoform 3 (PH3) and mandelonitrile lyase isoform 4 (MDL4) were inserted into black cherry under the control of the phloem-specific promoter *rolC* or the CaMV 35S promoter. The two enzymes catalyze the hydrolysis of prunasin to mandelonitrile and the dissociation of mandelonitrile to hydrogen cyanide (HCN), respectively, and the overexpression of *PH3* or *MDL4* might accelerate the release of toxic HCN and lead to an effective protection from cambial-mining insects. Three independent transgenic lines of 35S::PsTFL1, three of rolC::MDL4-FLAG, two of rolC::PH3-FLAG, and eight of 35S::MDL4-FLAG were obtained. The integration of transgenes and the copy number of neomycin phosphotransferase were examined by polymerase chain reaction (PCR) and quantitative PCR (qPCR) analysis. The mRNA levels of *PsTFL1*, *MDL4*, and *PH3* were examined by real-time qPCR and were compared to the wild-type. The expression level of *PsTFL1* in the three 35S::PsTFL1 lines were 3.7–5.8-times higher than that of the wild-type. However, the mRNA level of *MDL4* in the 35S::MDL4-FLAG lines and rolC::MDL4-FLAG lines, and the mRNA level of *PH3* in the rolC::PH3-FLAG lines showed no significant change indicating that transgene silencing was induced. Western blot analysis was carried out using anti-FLAG antibody to detect the FLAG-tagged PH3 in the transgenic line that had slightly increased mRNA level of PH3, but no signal was detected.

Keywords Cyanogenesis · Flowering control · Gene silencing · Genetic transformation · Insect resistance · *Prunus serotina*

Introduction

Black cherry (*Prunus serotina* Ehrh.) is the only member in the genus *Prunus* that is of commercial importance as a timber species for cabinetry, furniture, and veneer (Marquis 1990). The damage caused by cambial-mining insects, such as the peach bark beetle (*Phloeotribus liminaris*), and the lesser and greater peachtree borers (*Synanthedon pictipes* and *S. exitiosa*, respectively), triggers black cherry to exude gum at the site of injury which dramatically

Y. Wang
Department of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center (HTIRC), Purdue University, 715 West State St., West Lafayette, IN 47907, USA

P. M. Pijut (✉)
Northern Research Station, HTIRC, USDA Forest Service, 715 West State St., West Lafayette, IN 47907, USA
e-mail: ppijut@purdue.edu; ppijut@fs.fed.us

decreases yields of high-quality black cherry lumber for veneer (Barnd and Ginzel 2008). In addition to traditional breeding, genetic engineering offers an alternative approach to improve the resistance of black cherry to pests with the advantages of avoiding the long juvenile periods and enabling the transfer of traits into selected genotypes (Peña and Séguin 2001).

Among the many different methods to confer pest resistance, utilizing cyanogenesis in black cherry is a novel strategy. Cyanogenesis is considered to be an ancient plant defense system that exists in more than 2,500 plant species (Møller 2010). Upon tissue disruption caused by chewing insects, mix of the cyanogenic glucosides and the compartmentalized enzymes catalyzing their degradation leads to liberation of toxic hydrogen cyanide (HCN). The first effort to use this natural defense mechanism through metabolic engineering was conducted by Tattersall et al. (2001) who transferred the entire biosynthetic pathway of the cyanogenic glucoside, dhurrin, from *Sorghum bicolor* to *Arabidopsis*, and successfully conferred resistance to the flea beetle (*Phyllotreta nemorum*). Black cherry is also one of the cyanogenic plant species. The cyanogenic glucoside in the leaves and cambium tissue of black cherry is prunasin, and the two enzymes involved in cyanogenesis are prunasin hydrolase (PH) and mandelonitrile lyase (MDL). Prunasin is first degraded to mandelonitrile in the presence of PH. Mandelonitrile then dissociates enzymatically into HCN and benzaldehyde in the presence of MDL. However, the effective defense not only depends on cyanide potential, but on the rapid release of HCN as well (Selmar et al. 1989). The latter may be achieved by overexpressing the enzymes, β-glucosidase and hydroxynitrile lyase (HNL), involved in cyanogenesis. Siritunga and Sayre (2004) overexpressed HNL in cassava (*Manihot esculenta*), and proved that the elevated level of HNL accelerated cyanogenesis. Therefore, it is promising to overexpress the enzymes in black cherry for rapid cyanogenesis, and successful protection against insects. The two enzymes involved in cyanogenesis in leaves of black cherry are PH and MDL. Both of these have multiple isoforms, and have been characterized (Hu and Poulton 1999; Zhou et al. 2002).

Furthermore, gene containment must be achieved since transgene flow is an environmental concern, and will likely be required by regulatory agencies regarding planting of transgenic trees. Various strategies for transgene containment have been developed, including chloroplast transformation for maternal inheritance, tissue-specific gene excision, approaches that target reproductive structures or seed formation and germination, and RNA interference of genes involved in floral initiation and development. A large number of genes are found to be involved in the flowering process, and with their function fully understood, more and more could be targeted for intervention. *TERMINAL FLOWER1* (*TFL1*) has been widely studied in a number of plant species, and is known to delay flowering time and suppress the transition from juvenile- to adult-stage through the interaction with the bZIP transcription factor *FD* and transcriptional repression of *FD*-dependent floral meristem identity genes (Hanano and Goto 2011). It has an antagonistic activity with *FLOWERING LOCUS T* (*FT*), and its level is also regulated by downstream genes in the flowering pathway, such as *APETALA1* (*AP1*). *TFL1* homologous genes have been cloned and characterized from several tree species, such as apple (*Malus* × *domestica*), Japanese pear (*Pyrus pyrifolia*) (Esumi et al. 2005), *Populus* (Igasaki et al. 2008; Mohamed et al. 2010), Japanese apricot (*Prunus mume* Sieb. et Zucc.) (Esumi et al. 2010), and black cherry (*P. serotina* Ehrh.) (Wang and Pijut 2013). To our knowledge, this is the first study to use *TFL1* for flowering control in *P. serotina*.

Here we describe the development of transgenic black cherry plantlets overexpressing the *PsTFL1* gene through *Agrobacterium*-mediated transformation for reproductive sterility. We also report the first attempt to overexpress the endogenous genes encoding PH or MDL in black cherry for insect resistance.

Materials and methods

Plant materials

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^{TM} GA-7 vessels on a modified Murashige and Skoog (MS) basal medium (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, USA). 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 weeks.

Binary vectors and *Agrobacterium* strain

The binary vector pBI121 containing cDNA of *PsTFL1* under the control of the CaMV 35S promoter was used for

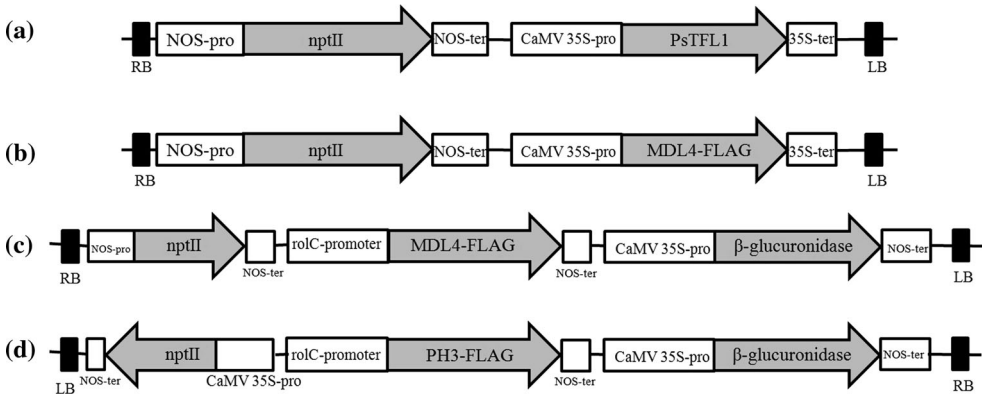

Fig. 1 Schematic representation of the T-DNA region of the four binary vectors. **a** pBI121 (35S-PsTFL1), **b** pBI121 (35S-MDL4-FLAG), **c** pBI121 (rolC-MDL4-FLAG), and **d** pCAMBIA2301 (rolC-PH3-FLAG). *NOS-pro* Nopaline synthase promoter, *nptII* neomycin phosphotransferase gene, *NOS-ter* nopaline synthase terminator, *CaMV 35S-pro* cauliflower mosaic virus 35S transcript promoter, *PsTFL1 Prunus serotina Terminal Flower 1* gene, *35S-ter* cauliflower mosaic virus 35S terminator, *MDL4-FLAG* FLAG tagged *mandelonitrile lyase* isoform 4, *PH3-FLAG* FLAG tagged *prunasin hydrolase* isoform 3

flowering control in black cherry (Wang and Pijut 2013) (Fig. 1a). The binary vector pBI121 containing cDNA of (R)-(+)-mandelonitrile lyase isoform 4 from black cherry (Hu and Poulton 1999) under the control of the CaMV 35S promoter, and the *rolC* promoter of *Agrobacterium rhizogenes* were constructed, respectively, for *MDL4* overexpression (Fig. 1b, c). The binary vector pCAMBIA2301 containing cDNA of prunasin hydrolase isoform 3 driven by the *rolC* promoter was used to overexpress *PH3* in black cherry (Zhou et al. 2002) (Fig. 1d). The *rolC* promoter region and cDNA fragment of *PsTFL1*, *MDL4*, and *PH3* were obtained by polymerase chain reaction (PCR) based on the available sequence information (DQ160187; Wang and Pijut, unpublished; AF043187; AF221526). The FLAG-tag (DYKDDDDK) was added to the C terminus of MDL4 and PH3 by PCR using a 3′ primer containing the FLAG sequence before the stop codon. 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

Agrobacterium was inoculated in liquid Luria–Bertani (LB) medium to OD_{600} of 0.8–1.0 and then cultured in an induction medium (Gelvin 2006) containing 200 μM acetosyringone (AS) for 16–24 h. Thirty to 60 leaf explants were wounded transversely along the midrib (3–5 cuts per explant) and exposed to 25–50 mL of *Agrobacterium* suspension at OD_{600} of 1.0. Fifteen-min vacuum infiltration (62.5 cm Hg) was applied followed by 1 h gentle shaking (50 rpm). Leaf explants were then blotted dry and cultured on woody plant medium (WPM) (Lloyd and McCown 1981) with 9.08 μM thidiazuron (TDZ), 1.07 μM naphthaleneacetic acid (NAA), and 100 μM AS in the dark for 3 days. After removing the *Agrobacterium* by rinsing in the same liquid medium containing timentin, explants were cultured on WPM containing 9.08 μM TDZ, 1.07 μM NAA, 200 mg L^{-1} timentin, and 60 μM silver thiosulphate (STS) in the dark for 3 weeks. Explants were then exposed to the 16 h photoperiod for 3 days and transferred to shoot elongation medium [MS medium with 3 % (w/v) sucrose, 8.88 μM BA, 0.49 μM IBA, 0.29 μM GA_3, 30 mg L^{-1} kanamycin, and 200 mg L^{-1} timentin] for at least five subcultures (Liu and Pijut 2010; Wang and Pijut 2014). Regenerated shoots were then harvested for further analysis.

PCR analysis and copy number determination of transgenic plant lines

After transformation and regeneration, genomic DNA from leaves of independent putative transgenic shoots was isolated using the DNeasy Plant Mini Kit (Qiagen, USA) and PCR analysis was conducted to confirm the integration of the selectable marker gene neomycin phosphotransferase (*nptII*) and the genes of interest, including *PsTFL1*, *MDL4*, and *PH3*, respectively. Each reaction contained the following reagents in 50 μL:1 μL DNA (approximately 20–50 ng), 1 μL 10 μM of each primer (Table 1), 5 μL 10× PCR buffer (Clontech, USA), 1 μL dNTP, 1 μL 50× Advantage 2 Polymerase Mix (Advantage, USA), and sterile water was added to make the final volume. The cycling conditions consisted of a denaturating step at 95 °C for 3 min, 30 cycles at 95 °C for 15 s, annealing for 40 s, elongation for 30 s to 2 min at 68 °C, and followed by a final extension at 68 °C for 3 min. The annealing

Table 1 Primers used in this study

Name	Primer sequence (5′–3′)	Purpose
nptII-F	AAT ATC ACG GGT AGC CAA CG	PCR analysis
nptII-R	TGC TCC TGC CGA GAA AGT AT	
PsTFL1-F	ATG TGA GTT AGC TCA CTC ATT AGG C	PCR analysis
PsTFL1-R	CTA GCG TCT TCT AGC TGC TGT TTC TCT CTG	
MDL4-F	AAG GTA CCC GGG 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	PCR analysis
MDL4-R	AAG CGG CCG CTT ACT TGT CGT CAT CGT CTT TGT AGT CAA AAG CAA AGG ATA ATG CTG ACT TCA GGG AAT CCA TA	
PH3-F	AAC CGC GGA TGG CAA TGC AGT TAG GCT CTT TGT GTG CGA TGC TTC TTA TTG GCT TTG CAT TGA G	PCR analysis
PH3-R	AAC TGC AGT CAC TTG TCG TCG TCG TCC TTG TAA TCC ATA ATT TCA TAC CCA GCT TTG GTG TCC CTA GCA TTA TCG TCC ACA CGT AC	
nptIIq-F	TTG CTC CTG CCG AGA AAG TAT CCA	Copy number detection
nptIIq-R	CGA TGT TTC GCT TGG TGG TCG AAT	
MDL2q-F	GTG AAA TCA ACA ATG TCA GCT ATA CTA GTA	Copy number detection
MDL2q-R	GCA GAG GTA TTG GCA AGC GAT	
PsActin-F	CCC TGG ACT ATG AGC AAG AAC	Reference gene for expression analysis
PsActin-R	CAA TGA GTG ATG GTT GGA AGA G	
PsTFL1q-F	GCCTCTGGTTGTTGGGAGAG	Expression analysis of *PsTFL1*
PsTFL1q-R	GATAGAGCTCATATCCATTGCAGAC	
MDL4q-F	CTC TAC ATT CCC TGC CAC AC	Expression analysis of *MDL4*
MDL4q-R	GAA GTA TGA ATA GCC TCC CCT G	
PH3q-F	TCC ACC CTC TCA TTG TCC AGT	Expression analysis of *PH3*
PH3q-R	CGT TTG CAG CAC CTT CTA CC	

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.

Transgene copy number of *nptII* was determined by quantitative PCR (qPCR) (Weng et al. 2004). Primers nptIIq-F and R were designed to amplify a 100 bp fragment of *nptII* from genomic DNA. *Mandelonitrile lyase* isoform 2 (*MDL2*) was selected as a single-copy endogenous reference gene confirmed by Hu and Poulton (1999) using Southern blot analysis, and was amplified with the primers MDL2q-F and R. The qPCR was performed with the Stratagene Mx3000P. Each reaction contained the following reagents in 20 μL:2 μL DNA (approximately 50 ng), 2 μL 10 μM of each primer, 10 μL Brilliant SYBR Green QPCR Master Mix (Agilent Technologies, USA), and sterile water was added to make final volume. The cycling conditions consisted of DNA polymerase activation at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and followed by a melting curve analysis from 55 to 95 °C. Each sample was replicated three times and the relative standard curve of the serial dilution of genomic DNA for both target and reference genes were constructed. Gene copy number of *nptII* in the 16 independent lines was analyzed following the calculation reported by Weng et al. (2004).

Expression analysis of target genes in transgenic black cherry

The relative expression of *PsTFL1*, *MDL4*, and *PH3* in the transgenic black cherry lines 35S::PsTFL1, 35S::MDL4-FLAG, rolC::MDL4-FLAG, and rolC::PH3-FLAG, respectively, was investigated by real-time qPCR (RT-qPCR). Total RNA was extracted from the leaves of the wild-type and transgenic shoots using the RNeasy Plant Mini Kit (Qiagen). Genomic DNA was removed by using the DNA-free DNase Treatment and Removal Kit (Ambion, USA), and the first-strand cDNA was synthesized with AccuScript High-Fidelity cDNA Synthesis Kit (Agilent Technologies). *Actin* in black cherry was used as a reference gene to normalize the data. Each reaction contained the following reagents in 20 μL:5 μL cDNA, 2 μL 10 μM of each primer, 10 μL Brilliant SYBR Green QPCR Master Mix (Agilent Technologies), and sterile water was added to make the final

volume. The cycling conditions consisted of DNA polymerase activation at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 1 min, and followed by a melting curve analysis from 55 to 95 °C and was performed with the Strategene Mx3000P. Each sample was replicated three times. The relative expression levels were calculated using the delta–delta–Ct method with efficiency correction (Pfaffl 2001).

Protein isolation and western blot analysis

Approximately 100 mg leaf and stem tissues of the wild-type, rolC::PH3-FLAG-1, and rolC::PH3-FLAG-2 were harvested for protein extraction with the Plant Total Protein Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). Total proteins were separated on a 12.5 % SDS-PAGE gel and examined by immunoblotting using anti-FLAG antibody (Sigma).

Rooting and acclimatization of transgenic shoots

The three transgenic lines that showed increased expression of *PsTFL1* were micropropagated on the same selection medium, and 2–3 cm long shoots were excised and rooted on half-strength MS medium supplemented with 5 μM NAA, 0.01 μM kinetin, 0.793 μM phloroglucinol, 2 % (v/v) sucrose, and 0.7 % Bacto agar with 5 days in the dark chamber before exposure to a 16 h photoperiod. The roots of transgenic shoots were rinsed in distilled water to remove residual agar and plantlets were transferred to 10 cm × 9 cm plastic pots containing a moist, autoclaved potting mix (Fafard Growing Mix, Agawam, MA, USA). Rooted plantlets were maintained in closed 3.8 L zip-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 zip-lock for gas exchange. Plantlets were watered as needed and gradually acclimatized (opening of the bags) over a period of 2 weeks.

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 $\alpha = 0.05$.

Results and discussion

Confirmation of transgene integration and copy number determination

Three putative transgenic lines of 35S::PsTFL1, eight lines of 35S::MDL4-FLAG, three lines of rolC::MDL4-Flag, and two lines of rolC::PH3-FLAG were obtained. Genomic DNA was extracted from the wild-type and each independent line, and PCR analysis was conducted to confirm the presence of the transgenes. The fragments of *nptII* and full-length cDNA of *PsTFL1*, *MDL4*, or *PH3* were amplified from the genomic DNA, but not from the wild-type plant (Figs. 2, 3), indicating the presence of both the selectable marker gene and the target gene in the black cherry genome.

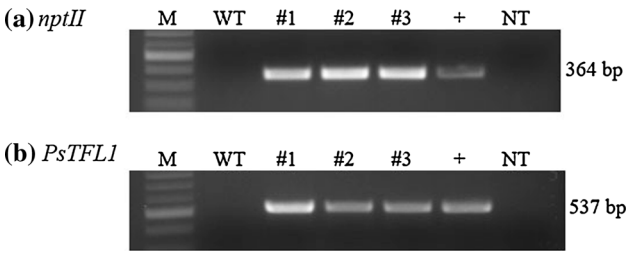

Fig. 2 PCR analysis of the integration of transgenes in the genome of black cherry plants. **a** 364 bp *nptII* fragment amplified from genomic DNA of transgenic plants **b** 537 bp *PsTFL1* cDNA amplified from genomic DNA of transgenic plants. *M* 100 bp DNA ladder, *WT* the wild-type, *#1–3* three transgenic plants, + positive control, *NT* a no-template control

The copy number of *nptII* in the 16 transgenic lines was determined by qPCR (Table 2) with the calculation based on the standard curve of both target and reference gene (Fig. 4). The three transgenic lines of 35S::PsTFL1 contained two or three copies of *nptII*, while the transgene copy number in the 13 lines of MDL4-FLAG and PH3-FLAG ranged from 4 to 7. The average copy number in the group of MDL4-FLAG and PH3-FLAG lines were higher than those in PsTFL1 lines. Low transgene copy number, especially a single copy of the transgene is desirable, as it was thought to have more stable gene expression than multicopy transgene inserts because the latter was more likely to induce gene silencing (Stam et al. 1997). In this study, the exact same transformation conditions, such as the duration of vacuum infiltration, concentration of *Agrobacterium* inoculum, and co-cultivation duration, were applied during the transformation process with different binary vectors. But, the minor difference among each replication of the transformation process (volume used) could possibly lead to the variation in virulence of *Agrobacterium* cells so that the gene copy number varied among different batches.

Expression analysis of target genes in transgenic black cherry shoots

The expression levels of *PsTFL1*, *MDL4*, and *PH3* in the leaves of the independent transgenic lines were quantified

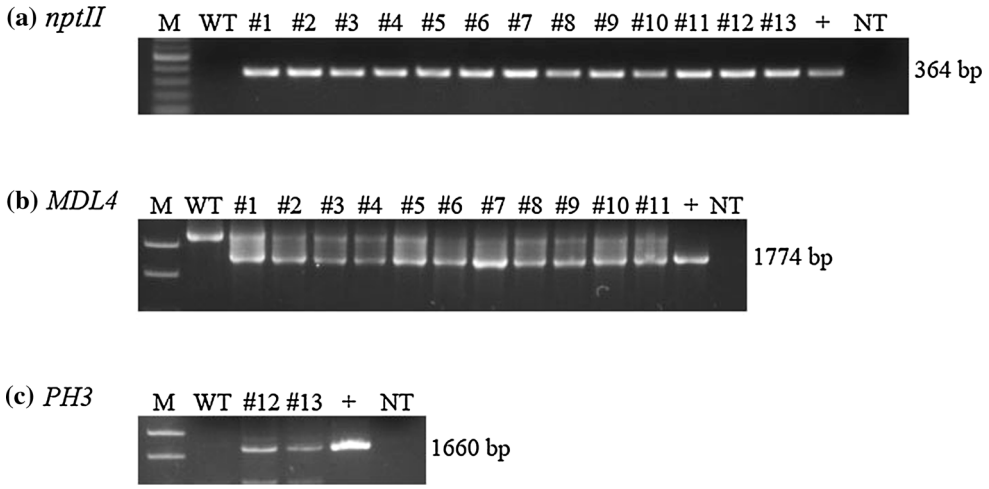

Fig. 3 PCR analysis of the integration of transgenes in the genome of black cherry plants. **a** 364 bp *nptII* fragment amplified from genomic DNA of transgenic plants, **b** 1,774 bp *MDL4* cDNA amplified from genomic DNA of transgenic plants, **c** 1,660 bp *PH3* cDNA amplified from genomic DNA of transgenic plants. *M* 100 bp DNA ladder, *WT* the wild-type, *#1–8* eight transgenic lines of 35S:MDL4-FLAG, *#9–11* three transgenic lines of rolC:MDL4-FLAG, *#12–13* two transgenic lines of rolC:PH3-FLAG, + positive control, *NT* a no-template control

Table 2 C_T value of the reference gene *mdl2* and the target gene *nptII* for each transgenic line and the estimated copy number of *nptII* for each line

Sample	C_T (mdl2)	C_T (nptII)	4′ (X_0/R_0)	Estimated no. of copies
35S::PsTFL1-1	18.69 ± 0.11	18.98 ± 0.27	2.22 ± 0.29	2
35S::PsTFL1-2	20.99 ± 0.37	21.09 ± 0.19	2.02 ± 0.42	2
35S::PsTFL1-3	20.74 ± 0.18	20.33 ± 0.14	2.88 ± 0.23	3
35S::MDL4-FLAG-1	18.65 ± 0.25	17.55 ± 0.15	4.87 ± 0.29	5
35S::MDL4-FLAG-2	19.72 ± 0.32	18.51 ± 0.23	5.50 ± 0.40	6
35S::MDL4-FLAG-3	19.47 ± 0.21	18.67 ± 0.14	4.12 ± 0.25	4
35S::MDL4-FLAG-4	19.47 ± 0.20	18.20 ± 0.10	5.67 ± 0.23	6
35S::MDL4-FLAG-5	19.35 ± 0.05	18.42 ± 0.10	4.49 ± 0.12	4
35S::MDL4-FLAG-6	18.17 ± 0.15	17.15 ± 0.01	4.52 ± 0.15	5
35S::MDL4-FLAG-7	19.83 ± 0.07	18.59 ± 0.55	5.63 ± 0.55	6
35S::MDL4-FLAG-8	18.53 ± 0.08	17.91 ± 0.16	3.52 ± 0.18	4
rolC::MDL4-FLAG-1	18.99 ± 0.20	18.33 ± 0.03	3.71 ± 0.20	4
rolC::MDL4-FLAG-2	18.64 ± 0.14	17.70 ± 0.22	4.40 ± 0.26	4
rolC::MDL4-FLAG-3	19.05 ± 0.04	18.06 ± 0.12	4.63 ± 0.12	5
rolC::PH3-FLAG-1	19.75 ± 0.12	18.18 ± 0.13	7.0 ± 0.17	7
rolC::PH3-FLAG-2	18.01 ± 0.15	17.03 ± 0.16	4.40 ± 0.22	4

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

by RT-qPCR and were compared with that of the wild-type. The relative *PsTFL1* mRNA levels of three independent lines had 3.7-, 5.1-, and 5.8-fold increase compared with that of the wild-type (Fig. 5). In this study, the black cherry cDNA fragment of *TFL1, MDL4,* or *PH3* was introduced into its own genome, respectively. This could cause the cosuppression of transgene and endogenous gene if there was a multicopy transgene locus, as the transgene sequence was fully homologous with its endogenous gene (Stam et al. 1997). However, in this case, the expression levels of *PsTFL1* in the three transgenic lines were significantly higher than that in the non-transgenic control, even though there were multiple copies of transgenes integrated in the genome. This could be a result of multiple copies inserted into different loci, and the promoters that drive transgene expression were not heavily methylated, so that the transgene expression was not silenced.

However, transgene silencing was observed in the transgenic lines 35S::MDL4-FLAG, rolC::MDL4-FLAG, and rolC::PH3-FLAG. None of the 13 transgenic lines exhibited significant change of the *MDL4*/*PH3* mRNA level compared to the wild-type (Fig. 6). It was interesting

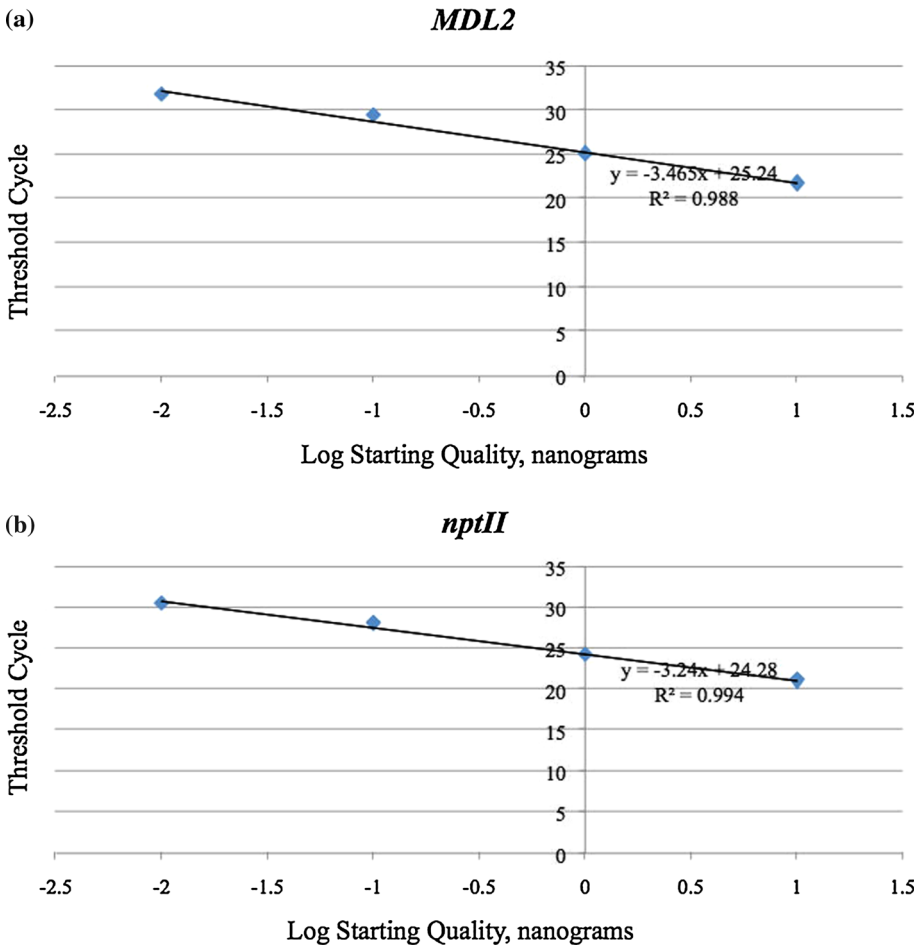

Fig. 4 qPCR relative standard curve of the reference gene, *MDL2*, and the target gene, *nptII*, obtained by plotting the threshold cycle (C_T) value versus the log of each initial concentration of genomic DNA

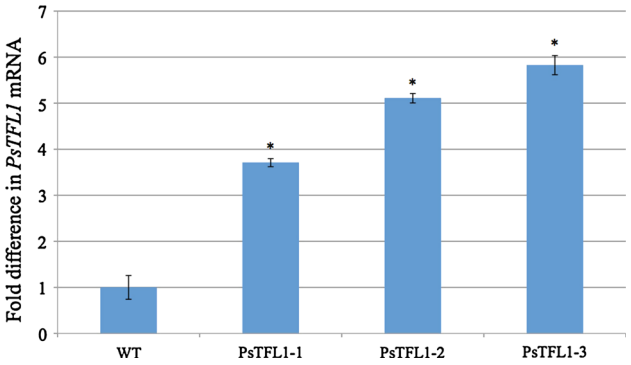

Fig. 5 The relative expression levels of *PsTFL1* in each transgenic plant compared to the wild-type. *Actin* in black cherry was used as a reference gene. Each sample was replicated three times

that overexpression of the *PsTFL1* gene could be easily obtained, whereas in all of the MDL4-FLAG and PH3-FLAG lines, the mRNA level of the target genes showed no significant difference than the wild-type. This might be attributed to the higher transgene copy number in the MDL4-FLAG and PH3-FLAG lines than the PsTFL1 lines, as higher copy number was more likely to cause gene silencing (Cogoni and Macino 2000). Schubert et al. (2004) reported that silencing occurs when a transgene copy number exceeds a gene-specific threshold. To study if the expression level of the target gene was correlated with higher copy number, transgene copy number and *MDL4* mRNA levels in the eight 35S::MDL4-FLAG lines were plotted as x–y pairs. The result suggested that higher copy number was not associated with lower expression level (Fig. 7). Dutt et al. (2012) compared four phloem-specific promoters including the *rolC* promoter and used the 35S promoter as a control. The expression level of the β-glucuronidase (*GUS*) gene driven by the 35S promoter was found to be higher than that driven by the *rolC* promoter in the leaves of transgenic citrus (*Citrus aurantifolia* Swingle). It is believed that there was a positive correlation between the silencing frequency and promoter strength (Que et al. 1997; Mishiba et al. 2005). Mishiba et al. (2005) compared the transgene expression driven by 35S and *A. rhizogenes rolC* gene promoter in transgenic gentian (*Gentiana triflora* × *Gentiana scabra*) plants, and transgene silencing only occurred in 35S-*GtMADS4* gentian lines, not in rolCp-*GtMADS4* lines. Furthermore, Lee et al. (2012) reported that a virus-derived promoter might induce

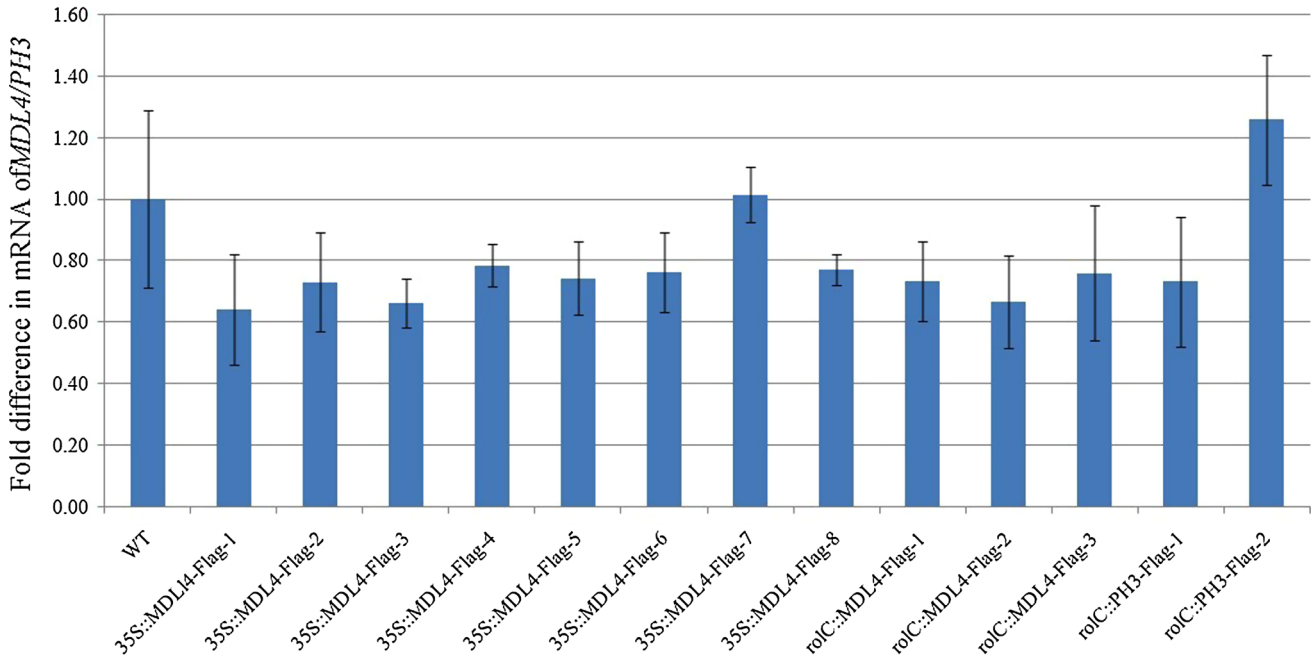

Fig. 6 The relative expression levels of *MDL4* or *PH3* in each transgenic plant compared to the wild-type. *Actin* in black cherry was used as a reference gene. Each sample was replicated three times

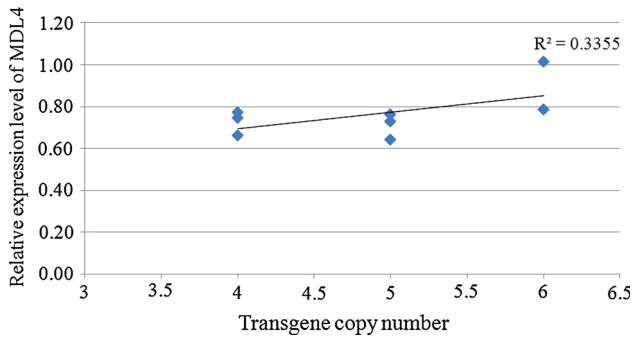

Fig. 7 Regression analysis between the mRNA level of *MDL4* and transgene copy number. *Dots* represent individual transgenic lines of 35S:MDL4-FLAG

gene silencing through a plant's defense mechanism after being recognized as parasitic sequences. In this study, both 35S- and *rolC* promoter-driven transgenes were silenced.

PsTFL1 is a single-copy gene in the genome of black cherry (Wang and Pijut 2013), while either *MDL4* or *PH3* have many isoforms that share a high similarity in their gene sequences respectively even though the expression levels of each isoform in different tissues was different (Hu and Poulton 1999; Zhou et al. 2002). The differences in the nature of coding regions between *PsTFL1* and *MDL4*/*PH3* might have played an important role in their gene-specific threshold for the onset of silencing (Schubert et al. 2004). Furthermore, the tissue level compartmentation and restricted subcellular localization of the two enzymes were found which contributed to the prevention of large-scale cyanogenesis in undamaged black cherry tissues (Poulton and Li 1994, Swain and Poulton 1994). Zheng and Poulton (1995) reported that MDL expression may be under transcriptional control in black cherry seeds during fruit maturation. Taken together, it was possible that there was also tight regulation of the two enzymes at the post-transcriptional level because self-protection from large-scale cyanogenesis in intact cells is essential. To avoid gene silencing, chloroplast transformation would be a good solution, but this requires the

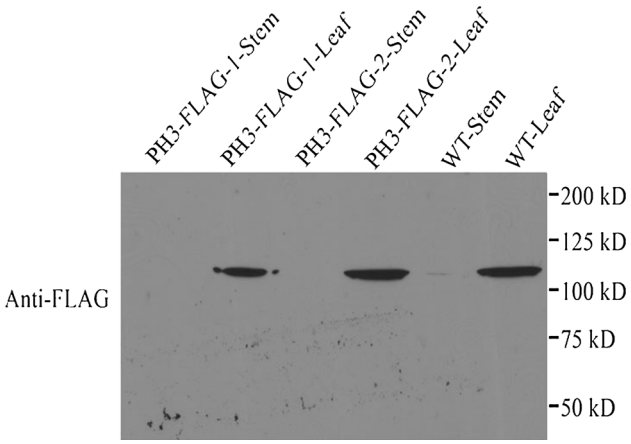

Fig. 8 Western blot assay conducted with total proteins isolated from the stem and leaf tissue of the transgenic line rolC::PH3-FLAG-1, rolC::PH3-FLAG-2, and the wild-type. The anti-FLAG antibody was not able to detect the expression of PH3 (55 kD)

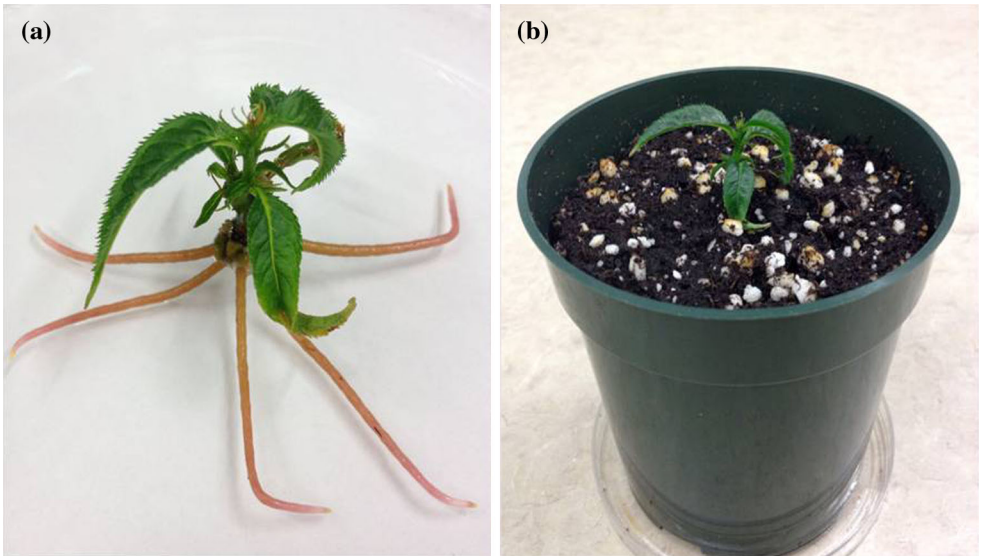

Fig. 9 Rooting and acclimatization of transgenic black cherry overexpressing *PsTFL1*. **a** Roots developed from the basal end of an in vitro transgenic shoot after 1 month. **b** The plantlet obtained after acclimatization

chloroplast genome sequence information which was currently not available in black cherry (Hasunuma et al. 2009; Clarke and Daniell 2011). Therefore, more applicable approaches would be to reduce transgene copy number by using low selective pressure, or to incorporate genes from other species that encode enzymes catalyzing the same reaction into the black cherry genome. It was reported that higher concentration of antibiotics could result in the regeneration of plants that contain higher transgene copy numbers (Dalton et al. 1995), and decreased level of homology in nucleotide sequences might alleviate post-transcriptional gene silencing (Schubert et al. 2004).

Western blot analysis was conducted to detect the presence of PH3-FLAG in the leaf or stem total protein sample. The total proteins from the wild-type and rolC::PH3-FLAG-1 with lower *PH3* mRNA level were used as controls. But, at the 55 kDa region where PH3 was supposed to be present, no band was detected using the anti-FLAG antibody (Fig. 8). This result further suggested the occurrence of transgene silencing in these lines.

Rooting and acclimatization of 35S::PsTFL1 lines

After micropropagation, the transgenic shoots of three 35S::PsTFL1 lines were transferred to rooting medium and treated for 5 days in darkness for root induction. Usually, transgenic black cherry shoots were often difficult and slow to root (Liu and Pijut 2010). Surprisingly, roots formed quickly from these transgenic lines and all shoots started to root in 3 weeks. After acclimatization, the plantlets appeared normal and healthy (Fig. 9).

Conclusions

Three independent transgenic black cherry lines overexpressing *PsTFL1* were obtained using the improved protocol for *Agrobacterium*-mediated transformation (Wang and Pijut 2014). The presence of transgenes and the transgene copy number was investigated by PCR and qPCR analysis. The transgenic shoots were successfully rooted and acclimatized using the improved rooting protocol. This work of using endogenous *TFL1* for reproductive sterility in black cherry provided useful materials for studying floral genes in the genomic background of tree species. Further evaluation of the expression of floral-related genes in these transgenic lines may give us a better understanding of the molecular mechanisms underlying flower initiation and vegetative-to-reproductive transition in trees. Although the approach of overexpressing endogenous *MDL4* or *PH3* in black cherry for pest resistance was not successful because of the high frequency of gene silencing of these two genes in the transgenic shoots, it provided useful information and insights to guide our future studies of genetic engineering in black cherry and other species.

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