Efficient *Agrobacterium*-mediated Transformation of *Prunus serotina* via Sonication and Vacuum-Infiltration

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Abstract

An improved transformation protocol was developed for in vitro leaf explants of black cherry (*Prunus serotina* Ehrh.) through *Agrobacterium*-mediated transformation. *Agrobacterium tumefaciens* strain EHA105 harbouring an RNAi construct for the *AGAMOUS* (*AG*) gene (silencing for reproductive sterility) was used for co-cultivation. Based on a protocol established in our lab, the leaf explants were sonicated, vacuum- infiltrated, or both with *A. tumefaciens* before co-cultivation on culture medium containing 100 μM acetosyringone for 3 d. Explants were cultured for 3 wk in darkness and then 3 d in the light on woody plant medium containing 9.08 μM thidiazuron, 1.07 μM napthaleneacetic acid, 60 μM silver thiosulphate, 3% sucrose, and 200 mg L^{-1} timentin. The regenerating shoots were then selected on Murashige and Skoog medium supplemented with 30 mg L^{-1} kanamycin, 3% sucrose, 8.88 μM 6-benzylaminopurine, 0.49 μM indole-3-butyric acid, 0.29 μM gibberellic acid, and 200 mg L^{-1} timentin for at least five subcultures. The integration of the *nptII* gene into the black cherry genome was confirmed by polymerase chain reaction, and the transformation efficiency was determined by the number of explants that formed transgenic shoots relative to the total number of leaf explants tested. Among the different combinations of sonication and vacuum-infiltration, a high transformation efficiency (60%) was obtained when leaves were vacuum-infiltrated for 15 min with *A. tumefaciens*. This transformation system will be useful for future genetic modification and over-expression of two genes encoding the enzymes, prunasin hydrolase (PH) and mandelonitrile lyase (MDL), that are involved in cyanogenesis in black cherry for possible insect pest resistance. Both PH and MDL cDNA were cloned from black cherry and will be inserted into the binary vector pBI121 and pCAMBIA2301, respectively under the control of the *Agrobacterium rhizogenes RolC* promoter for phloem-specific expression (Sugaya et al. 1989). The two binary vectors will be transformed into *A. tumefaciens* strain EHA105 for future transformation of black cherry.

Introduction

✓ **Black cherry** (*Prunus serotina* Ehrh.), also known as wild cherry, rum cherry, and mountain black cherry, is the only species of its genus which provides commercial lumber valuable for cabinetry and veneer (Hough 1960).

✓ **Gummosis** is a non-specific defensive response of trees caused by insect attack (e.g. **peach bark beetle**) or infection by fungi. It is prevalent in members of the Rosaceae (e.g. *Prunus* spp.) (Barnd and Ginzel 2008).

✓ **Sonication** is known to create micro-wounding on the tissue and facilitate *Agrobacterium* cells to enter into plant cells, whereas **vacuum-infiltration** also improves penetration of *Agrobacterium* cells into the plant tissue layers (Subramanyam et al. 2010). Both of these treatments have been used in transformation systems of various plant species, and proven to increase the transformation efficiency.

✓ **Cyanogenesis** is a natural defensive response of *Prunus* sp. to insect pests. Once cells are damaged or ingested, hydrogen cyanide (HCN) is released when the catabolic enzymes (**prunasin hydrolase** and **mandelonitrile lyase**) mix with the cyanogenic glycosides.

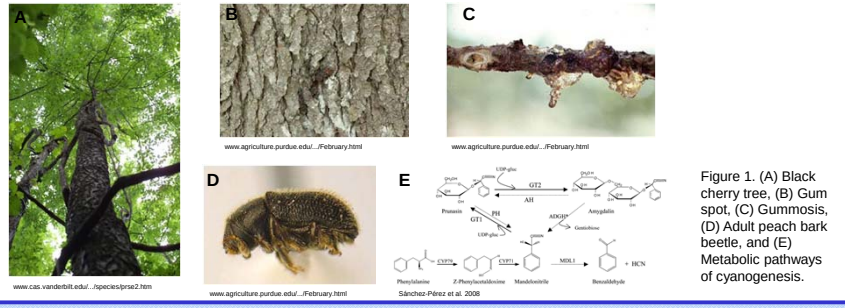

Figure 1. (A) Black cherry tree, (B) Gum spot, (C) Gummosis, (D) Adult peach bark beetle, and (E) Metabolic pathways of cyanogenesis.

Objectives

✓ To improve the transformation efficiency of *Prunus serotina* by testing the effects of sonication and vacuum-infiltration.

✓ To clone the genes encoding the two enzymes, prunasin hydrolase (PH) and mandelonitrile lyase (MDL), and develop constructs for future transformation of black cherry for insect pest resistance.

Materials and Methods

✓ *Agrobacterium*-mediated transformation of an elite mature genotype (#3) of black cherry using an RNAi construct for the *AGAMOUS* (*AG*) gene was conducted as described by Liu and Pijut (2010). Zero, 30, 60, or 90 sec sonication were tested on leaf explants harvest from in vitro cultures. Then the leaves were immersed in the bacterial suspension and vacuum-infiltrated (62.5 cm Hg) for 0, 5, 10, or 15 min before co-cultivation.

✓ Total DNA was extracted from 100 mg leaves of putative transgenic shoots and a non-transgenic control using the modified CTAB (cetyltrimethyl ammonium bromide) method (Murray and Thompson 1980). PCR was conducted to yield a 364 bp fragment of the *nptII* gene.

✓ The genes encoding PH and MDL were cloned from in vitro cultures of black cherry (mature genotype #3). PCR products were harvested after agarose gel electrophoresis and ligated into pGEM-T easy vector (Promega). The phloem specific promoter *RolC* and the nopaline synthase (NOS) terminator will be assembled into the vector. The fragment RolC-PH-NOS and RolC-MDL-NOS will be ligated into the binary vector pBI121 and pCAMBIA2301, and transferred into *A. tumefaciens* strain EHA105 for future transformation of black cherry.

Results and Conclusions

✓ Fifty-five out of 58 surviving shoots were confirmed to have the *nptII* gene integrated into the genome (Table 1).

✓ Fifteen min vacuum-infiltration alone had the highest transformation efficiency (60%) and generated 22 independent transgenic lines (Fig. 2F).

✓ We are in the process of developing the transformation vectors (Fig. 2G). The next step will be to transfer into all three elite mature genotypes of black cherry using the improved transformation system.

Table 1. Effects of sonication and vacuum-infiltration on transformation of black cherry.

Vacuum Infiltration (min)	Sonication (sec)	No. of leaf explants infected	No. of shoots regenerated	No. of transgenic shoots
-	-	20	0	0
5	-	20	3	2
10	-	20	10	10
15	-	20	22	22
-	30	20	0	0
-	60	20	3	3
-	90	20	14	14
5	30	20	0	0
10	30	20	0	0
15	30	20	2	1
5	60	20	0	0
10	60	20	0	0
15	60	20	0	0
5	90	20	0	0
10	90	20	0	0
15	90	20	4	3

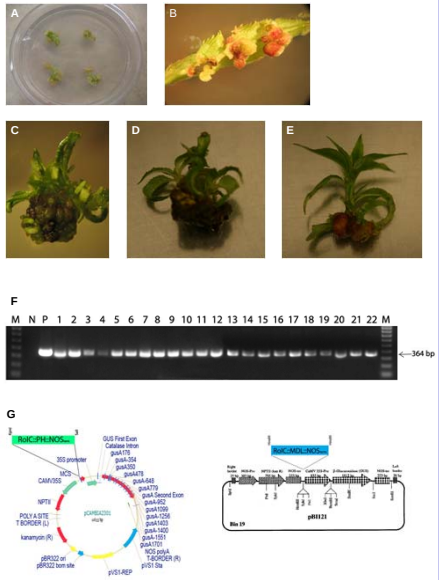

Figure 2. (A) Leaf explants on woody plant medium, (B) Callus (C) Putative transgenic shoots regenerated from callus, (D) Elongated shoots, (E) Transgenic shoots, (F) PCR analysis for transgenic black cherry with the construct PsAGRNAi; a 364 bp fragment for the *nptII* gene was amplified, and (G) Maps of the two constructs for insect pest resistance.

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