Adventitious shoot regeneration and genetic transformation of *Prunus serotina* (black cherry) for reproductive sterility

Xiaomei Liu[1] and Paula M. Pijut[2]

[1] Purdue University, Dept. of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center (HTIRC),715 West State St., West Lafayette, IN 47907,
[2] USDA Forest Service, Northern Research Station, HTIRC, 715 West State St., West Lafayette, IN 47907.

Abstract

Black cherry (*Prunus serotina* Ehrh.) is one of the most valuable hardwoods in the eastern US, Canada, and Mexico. Its wood is highly prized for fine cabinets, furniture, architectural woodwork, and veneer. There has been an increase in demand for high quality black cherry wood and it is increasingly difficult to find large numbers of straight-stemmed black cherry trees in forest stands. Therefore, there is a need to establish black cherry plantations. However, genetically modified improved trees containing foreign genes are subject to government regulatory guidelines because of the potential for dispersal of transgenic pollen, and the environmental impact could be difficult to predict and control. To reduce the dispersion of all genes, engineering reproductive sterility will help simplify the impact analysis and thus facilitate regulatory and public approval. The objective of this research is to develop a reliable system for genetically engineering reproductive sterility in black cherry for three genotypes (F, #3, and #4). Black cherry leaves were transformed using *Agrobacterium tumefaciens* strain AGL1 carrying the *AGAMOUS* gene. Whole leaf explants with several wounds were co-cultured with *A. tumefaciens*, and selection and regeneration of transformed cells and shoots were carried out for 12 weeks on selection medium containing kanamycin. Shoot regeneration was achieved using woody plant medium supplemented with 9.1 µM thidiazuron (TDZ) plus1.1 µM naphthaleneacetic acid (NAA) plus 10 mg/L kanamycin. Timentin (300 mg/L) was used after three days of co-culture to kill the *Agrobacterium*. Late selection was carried out on the same medium except kanamycin was increased to 20 mg/L. Putative transgenic cherry shoots were achieved. PCR analysis is underway to confirm these putative transformants.

Introduction

Genetically modified improved trees containing foreign genes are subject to government regulatory guidelines because of the potential for dispersal of transgenic pollen from trees, and the environmental impact could be difficult to predict and control (Meilan et al., 2001). To reduce the dispersion of all genes, engineering reproductive sterility will help simplify the impact analysis and thus facilitate regulatory and public approval (Strauss et al., 1995). This will allow land owners to freely plant transgenic trees without concern for ecologically affecting an ecosystem. An efficient regeneration system is essential for genetic transformation. In this project, we will optimize a method previously established (Espinosa et al. , 2006) for black cherry regeneration in order to genetically transform and propagate elite black cherry with a reproductive sterility gene. With an efficient regeneration system (94.4%) that we optimized, we are using a RNAi construct which contains an *AGAMOUS* gene to impart reproductive sterility.

Objectives

1. Establish a highly efficient regeneration system (>90%) for three genotypes of black cherry.
2. Transform a reproductive sterility gene into black cherry via *Agrobacterium*.
3. Select transformants using kanamycin and confirm transformants by PCR.

Materials and Methods

Regeneration

Whole-leaf explants were cut transversely along the midrib and incubated on WPM supplemented with 9.08 µM TDZ in combination with 1.07 µM NAA. Cultures were incubated 3 weeks in the dark plus 2 weeks in light ,then transferred to MS + 4.44 µM BA + 0.49 µM IBA + 0.58 µM GA_3 + 20g•L^{-1} sucrose for 2 months.

Fig. 2 Shoots from #3 Fig. 3 Shoots regenerated for F Fig. 3 Shoots regenerated for #3

Kanamycin and Timentin sensitivity

Leaf explants with wounds were used.
Kan at 0, 5, 10, 15, 20, 25, or 30 mg/L were tested.
Tim at 0, 100, 200, 300, 400, or 500 mg/L were tested.

Timentin killing curve

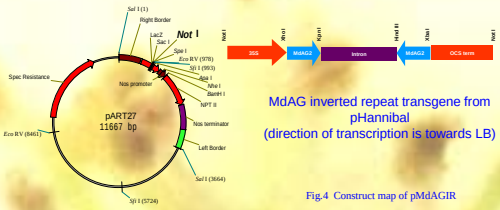
Agrobacterium was grown for 16 hours until OD_{600} was 0.8-1.0, Timentin at 0, 50, 100, 200, or 300 mg/L was added, then OD_{600} value was tested every 6 hours.

Transformation

Leaf explants were pre-cultured for 3 days, exposed to *Agrobacterium* for 30 min, 4 days co-cultured for 4 days, washed, then cultured on selection medium with Kan and Tim.

Confirmation of transgenic plants

DNA was extracted from putative transgenic plants and from the plasmid to run PCR.



MdAG inverted repeat transgene from pHannibal
(direction of transcription is towards LB)

Fig.4 Construct map of pMdAGIR

Results

1) Kanamycin sensitivity

Table1 Shoot regeneration efficiency on Kan medium

Kan (mg/L)	Regeneration(%)
0	94.4
5	72.2
10	33.3
15	22.2
20	16.7
25	0
30	0

Fig.5 Regenerated shoots on Kan (top) and control (bottom)

2)Timentin sensitivity

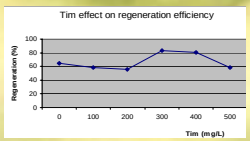


Fig. 6 Timentin effect on regeneration efficiency

Fig. 7 Control (left) and regenerated shoots on 300 mg/L Timentin (right)

3)Timentin kill curve

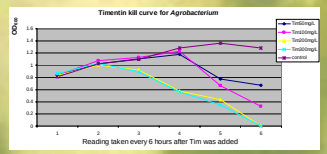


Fig. 8 Timentin kill curve for *Agrobacterium*

4) Transformants

Table2 Summary of transformation for two genotypes of black cherry

Genotype	Infected explants	Kan resistant shoots
F	72	10
#3	108	27

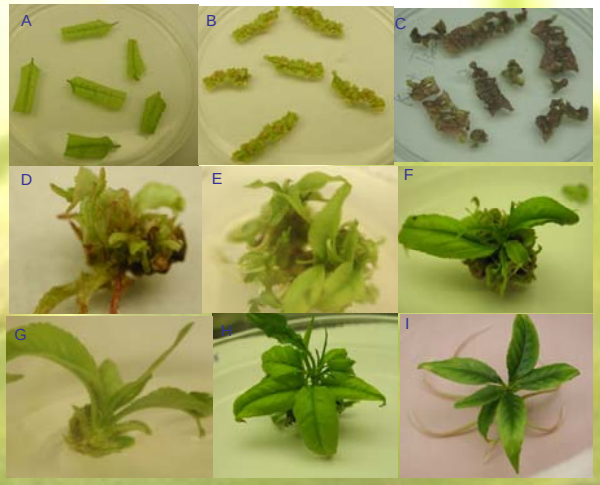


Fig. 9 Regeneration of black cherry
A. Leaf explants with wounds B. Callus formed after co-culture with *Agrobacterium*
C. Regeneration 3 weeks after transformation. D. Regenerated shoots of #3 after 6 weeks
E. Regenerated shoots of #3 after 8 weeks . F. Elongated transformants of #3 after 12 weeks.
G. Regenerated shoots of F after 6 weeks. H. Elongated transformants of F after 12 weeks.
I. Rooted shoot of F .

Conclusions

- Black cherry is very sensitive to kanamycin; 10 mg/L used for selection, 20 mg/L used for late selection of transformants.
- Timentin has a positive effect on regeneration of black cherry; 300 mg/L is effective to kill the *Agrobacterium* and produced a good regeneration efficiency.
- Black cherry can be transformed with foreign gene by *Agrobacterium*-mediated transformation. We obtained 27 kanamycin resistant putative transgenic plants.
- Black cherry transformation-regeneration is genotype dependent. For black cherry genotype F, only one shoot regenerated from one leaf explants. For genotype #3, several shoots regenerated from the leaf explants.

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Abbreviations: WPM-woody plant medium; MS- Murashige and Skoog (1962) medium; TDZ-thidiazuron; NAA-napthaleneacetic acid; BA-6-benzylaminopurine; Kan- kanamycin; IBA- indole-3-butyric acid; GA_3—gibberellic acid ; PCR-polymerase chain reaction; Tim-timentin.

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