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

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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. This will allow land owners to freely plant transgenic trees without concern for ecologically affecting an ecosystem. 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) by using a RNAi construct which contains the *AGAMOUS* gene. To conduct genetic transformation, we need to establish a highly efficient regeneration system (90-100%) via adventitious shoots from leaves. Shoot regeneration was achieved from leaves of in vitro cultures of *P. serotina* using woody plant medium (WPM) supplemented with 0, 2.27, 4.54, 6.81, or 9.08 µM thidiazuron (TDZ) in combination with 0, 0.27, 0.54, or 1.07 µM naphthaleneacetic acid (NAA). Percent regeneration was influenced by plant growth regulators and by genotype. For genotype F, TDZ had a significant effect on regeneration efficiency which increased as the concentration of TDZ was increased. Optimal regeneration (94.4%) was observed with whole-leaf explants cut transversely along the midrib and incubated abaxial side up on media supplemented with 9.08 µM TDZ plus 1.07 µM NAA. The highest mean number of shoots (8.15) was regenerated on media containing 9.08 µM TDZ plus 0.54 µM NAA. The regeneration efficiency of the three genotypes was significantly different. For genotype #3 and #4, the regeneration efficiency was lower than that of genotype F. Genotype #3 had a regeneration of 50% with a mean number of shoots (4.83) on media supplemented with 6.81 µM TDZ plus 0.54 µM NAA. Genotype #4 had a 58.3% regeneration efficiency when explants were cultured on media containing 9.08 µM TDZ plus 1.07 µM NAA with the greatest mean number of shoots (4.67) on media with only 9.08 µM TDZ. Experiments are underway to determine the selection and transformation protocols to be used for genetic modification of black cherry.

Introduction

Black cherry is a highly valued species for timber and sawlog production .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. Few studies on adventitious shoot regeneration have been conducted for black cherry. The first successful regeneration of adventitious shoots from black cherry leaves of a specific genotype was reported by Hammatt and Grant (1998). In this project, we will optimize a method previously established (Espinosa , 2004) for black cherry regeneration in order to genetically transform and propagate elite black cherry with a reproductive sterility gene. We will use a RNAi construct which contains an *AGAMOUS* gene to impart 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 identify transformants by PCR or southern blot .
4. Root and acclimatize transformed plantlets to the greenhouse.
5. Induce early flowering in the greenhouse to observe the phenotype of transgenics.

Materials and Methods

Micropropagation

Nodal sections (2 cm) from three genotypes (F , #3, #4) of black cherry were cultured on Murashige and Skoog (1962) medium (MS) + 4.44 µM 6-benzyladenine (BA)+ 0.49 µM Indole-3-butyric acid (IBA) + 0.29 µM gibberellic acid (GA_3) +20g•L^{-1} sucrose (Tricoli et al. 1985) 25 °C under a 16-h photoperiod (80 µmol•m^{-2}•s^{-1}) and transferred to fresh media every 3 wk.

Regeneration

Whole-leaf explants were cut transversely along the midrib and incubated abaxial side up on WPM supplemented with 0, 2.27, 4.54, 6.81, or 9.08 µM TDZ in combination with 0, 0.27, 0.54, or 1.07 µM NAA. Cultures were incubated 3 wks in the dark plus 2 wk 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.

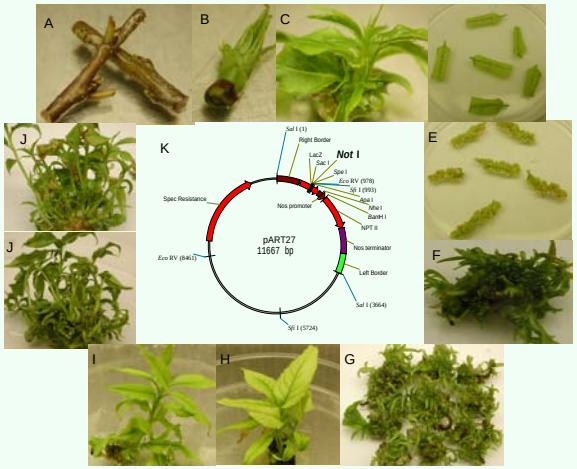

Fig.1 In vitro propagation of black cherry. A, B. Nodal explants. C. Shoot culture. D. Leaf explants E. Callus induction F. Adventitious shoots . G. Adventitious shoots arising from one leaf. H,I,J. Elongated adventitious shoots for genotype # 3, # 4, and F. K. RNAi construct which contains the *AGAMOUS* gene for sterility.

Table1. Analysis of variance for adventitious shoot regeneration

Genotype	No of leaves tested	No of leaves response	No. of shoots regenerated	Source of variation	DF	Mean square	F Value	Pr>F
F	720	340	2201	TDZ	4	38.6	2.16	0.071
				NAA	3	31.5	2.21	0.125
3	240	50	157	TDZ	4	7.23	2.76	0.055
				NAA	3	7.28	2.76	0.0542
				TDZ*NAA	7	8.61	3.29	0.008
4	240	37	132	TDZ	4	2.02	0.43	0.655
				NAA	3	8.14	1.73	0.182

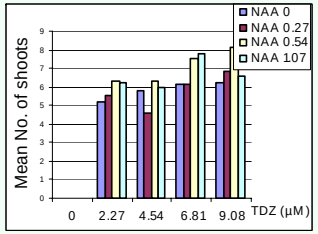

Fig.2 The effect of TDZ on mean number of shoots.

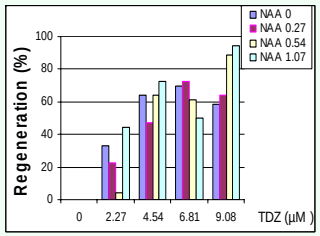

Fig.3 The effect of TDZ on regeneration efficiency.

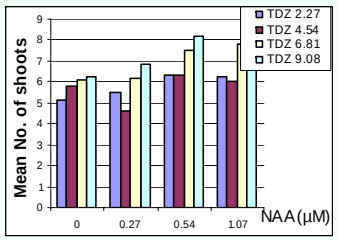

Fig.4 The effect of NAA on mean number of shoots.

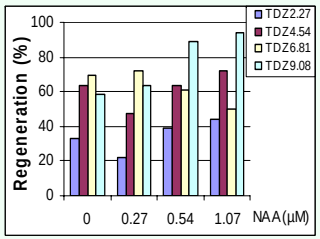

Fig.5 The effect of NAA on regeneration efficiency.

Table 2 Adventitious shoot regeneration for three genotypes of black cherry

Growth regulators (µM)		F		3		4	
TDZ	NAA	Regeneration (%)	Mean No. of shoots	Regeneration (%)	Mean No. of shoots	Regeneration (%)	Mean No. of shoots
0	0	0	0	0	0	0	0
0	0.27	0	0	0	0	0	0
0	0.54	0	0	0	0	0	0
0	1.07	0	0	0	0	0	0
2.27	0	33.3	5.17AB	0	0	0	0
2.27	0.27	22.2	5.5AB	25	1	0	0
2.27	0.54	38.9	6.36AB	8.3	6	0	0
2.27	1.07	44.4	6.25AB	16.7	4.5	0	0
4.54	0	63.9	5.78AB	41.7	3.2	0	0
4.54	0.27	47.2	4.59AB	0	0	0	0
4.54	0.54	63.9	6.35AB	8.3	3	0.5	3.67
4.54	1.07	72.2	6AB	25	2.67	33.3	5.25
6.81	0	69.4	6.12AB	41.7	1.8	33.3	3.75
6.81	0.27	72.2	6.15AB	50	4.83	16.7	1.33
6.81	0.54	61.1	7.55AB	41.7	4.8	16.7	3.5
6.81	1.07	50	7.78A	8.3	2	25	2.67
9.08	0	58.3	6.24AB	50	4	25	4.67
9.08	0.27	63.9	6.87AB	16.7	1	0	0
9.08	0.54	88.9	8.16A	41.7	1.4	41.7	2.8
9.08	1.07	94.4	6.59AB	41.7	3	58.3	3.86

Genotype F, n=36, #3 and #4, n=12. Means within a column followed by the same letter are not significantly different by Duncan test.

Results

1. Genotype F: highest shoot regeneration percentage (94.4%) was obtained on medium supplemented with 9.08 µM TDZ and 1.07 µM NAA (Table 2) .The highest mean number of shoots (8.15) was obtained with 9.08 µM TDZ and 0.54 µM NAA (Fig.1, J). Shoots became clusters when NAA was increased to 1.07 µM.
2. Genotype #3: highest regeneration of 50% was obtained with 6.81 µM TDZ plus 0.54 µM NAA (Fig.1.H) with a mean number of shoots 4.83.
3. Genotype #4: 58.3% regeneration efficiency was achieved when explants were cultured on media containing 9.08 µM TDZ plus 1.07 µM NAA, with a mean number of shoots (4.67) on media with 9.08 µM TDZ (Fig.1.I).
4. No adventitious shoots were regenerated when explants were exposed to media without TDZ.
5. Regeneration efficiency was influenced by plant growth regulators. TDZ had a significant effect on regeneration efficiency which increased as the concentration of TDZ was increased (Fig.2,3).
6. There was no interaction between TDZ and NAA except for genotype #3 (Table1).

Conclusions

- The regeneration efficiency of the three genotypes was significantly different. For genotype #3 and #4 (elite mature lines), the regeneration efficiency was much lower than that of genotype F (seedling).
- 9.08 µM TDZ +0.54 µM NAA will be used for kanamycin selection because it produced the highest mean number of shoots with a relatively ideal regeneration efficiency.
- More replications are needed for genotype #3 and #4 to determine the optimal growth regulator combination for regeneration.

References

Espinosa, C.2004.Adventitious shoot regeneration and rooting of *Prunus serotina* in vitro cultures. M.S. thesis. Purdue University, West Lafayette, IN.50p.

Hammatt , N. and Grant, N.J.1998. Shoot regeneration from leaves of *Prunus serotina* Ehrh. (black cherry) and *P. avium* L. (wild cherry). Plant Cell Rep. 17: 526–530.

Meilan, R., Brunner, A., Skinner, J., and Strauss, S. 2001. Modification of flowering in transgenic trees. Molecular Breeding of Woody Plants. Progress in Biotechnology series. 247-256.

Murashige, T. and Skoog, F.1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.

Strauss, S.H . Rottmann, W. H., Brunner, A.M. and Sheppard, L.A. 1995.Genetic engineering of reproductive sterility in forest trees. Molecular Breeding. 1:5-26.

Tricoli, D.M ,Maynard, C.A., and Drew, A. 1985.Tissue culture propagation of mature trees of *Prunus serotina* I. Establishment , multiplication , and rooting in vitro. Forest Science 31(1):201-208.