Genetic transformation of *Fraxinus profunda* hypocotyls and shoot regeneration

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ABSTRACT

Agrobacterium-mediated genetic transformation is an invaluable tool for conservation and preservation of *Fraxinus profunda*. Pumpkin ash (PA) is both an ecologically and economically important species. In addition to other endemic ash species, PA faces extirpation from attack by the emerald ash borer (EAB). To date there have been no reports of innate resistance among any North American *Fraxinus* species. Transgenic ash containing genes that impart resistance to EAB would be of great value. Although genetic transformation has been accomplished for green ash there have been no reports of regenerating transgenic PA. Therefore, the objective of this research was to develop a successful and efficient protocol for genetic transformation and regeneration of PA. Hypocotyls isolated from aseptic mature seed were pre-cultured for 5-7 days on a Murashige and Skoog medium supplemented with 22.2 µM 6-benzyladenine, 4.5 µM thidiazuron, 50 mg L^{-1} adenine hemisulfate, and 10% coconut water. Hypocotyls then were exposed to *Agrobacterium* strain EHA105 containing the pq35GR vector, with two selectable marker genes. Hypocotyl explants were transformed in a bacterial suspension with 100 µM acetosyringone, 90 s sonication, and 10 min vacuum-infiltration. Following a 2-3 day dark co-culture period, hypocotyls were washed four times to remove excess *Agrobacterium* prior to being cultured on a shoot regeneration medium. Adventitious shoots were regenerated following our previously established PA regeneration protocol with the addition of 50 mg L^{-1}adenine hemisulfate, 10% coconut water, 400 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin to the initial medium. Results of a replicated factorial experiment indicated that 400 mg L^{-1} timentin and 20 mg L^{-1} kanamycin were optimal for controlling *Agrobacterium* growth and selecting for transformed shoots, respectively. A β-glucuronidase (GUS) staining assay was used to test for positive transformation, and will be further supported by polymerase chain reaction and southern blotting. This research provides the basis for genetic transformation with genes specific for EAB resistance.

INTRODUCTION

- Pumpkin ash is a regionally native hardwood species that is both ecologically and economically important.
- Pumpkin ash, similar to other North American *Fraxinus* species, is vulnerable to the emerald ash borer (EAB) beetle, and combined with its limited range is listed as an endangered species (Fig. 1).
- With no known innate resistance to EAB, genetic transformation and in vitro regeneration is a powerful tool for preservation and conservation of this valuable species.
- Using an *Agrobacterium*-mediated transformation system EAB resistance genes can be imparted to pumpkin ash, and a successful transformation system has been reported for green ash (*F. pennsylvanica)* (Du and Pijut 2009).
- The objective of this research was to develop a successful and efficient transformation protocol for pumpkin ash.

MATERIALS AND METHODS

Agrobacterium Transformation

- Aseptic embryos were extracted and hypocotyls were cultured horizontally on Murashige and Skoog (MS) medium (1962) supplemented with 22.2 µM 6-benzyladenine (BA) plus 4.5 µM thidiazuron (TDZ), 50 mg L^{-1} adenine hemisulfate, 10% coconut water, 3% sucrose, and 0.7% Bacto agar for 5-7 days prior to transformation.
- Hypocotyls were sonicated in liquid MS medium for 90 s then vacuum-infiltrated with the *Agrobacterium* pq35GR vector (Fig. 2) suspension for 10 min. Hypocotyls were then co-cultured on the pre-culture medium for 2-3 d in the dark at 28°C.

Adventitious Shoot Regeneration and Selection of Transformed Shoots

- Adventitious shoots were recovered following the regeneration protocol for pumpkin ash (Stevens and Pijut 2012) with the addition of 50 mg L^{-1} adenine hemisulfate and 10% coconut water to initial shoot induction medium.
- Timentin at 400 mg L^{-1} and 20 mg L^{-1} kanamycin were added to the medium to control *Agrobacterium* growth and to select for transformed shoots, respectively. The histochemical β-glucuronidase (GUS) assay was used to test for positive transient GUS activity, and results will further be confirmed by polymerase chain reaction (PCR) and southern blotting.

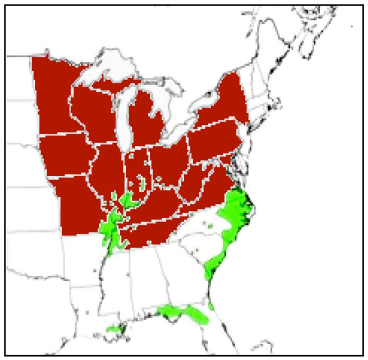

Figure 1. Native range of pumpkin ash (green) (*Fraxinus profunda*) (USDA Forest Service), and EAB infestation as of July 2011 (red). (http://www.emeraldashborerinfo.com/)

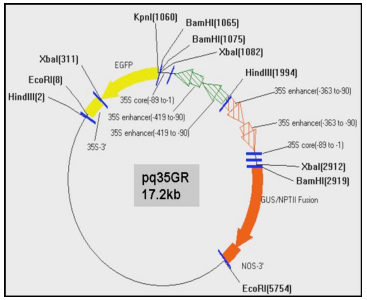

Figure 2. The pq35GR construct containing GUS-*nptII* fusion genes to select for positive transformation.

RESULTS AND CONCLUSIONS

- Pumpkin ash hypocotyls were successfully transformed (Fig. 3 A), and putative transgenic shoots were regenerated (Fig. 3 B).
- The addition of 20 mg L^{-1} kanamycin in the selection medium was sufficient to prevent callogenesis and shoot formation, thus suitable for transformation selection (Table 1; Fig. 3 C).
- Timentin at 400 mg L^{-1} was sufficient to control the *Agrobacterium* without a deleterious effect on adventitious shoot regeneration (Table 2; Fig. 3 D).
- Pumpkin ash will be further transformed and confirmed through PCR and a southern blot analysis. Adventitious rooting of transgenic plants will also be examined.
- This transformation protocol provides the basis for future genetic engineering studies including the insertion of EAB specific resistance genes.

Table 1. Effect of kanamycin on *Fraxinus profunda* regeneration.

Kanamycin (mg L^{-1})	Callus Formation (%)	Shoot Formation (%)	Mean No. Shoots
0	91.7 ± 4.7a	41.7 ± 8.3ab	0.8 ± 0.2a
5	83.3 ± 6.3a	47.2 ± 8.4a	1.2 ± 0.3a
10	36.1 ± 8.1b	22.2 ± 7b	0.2 ± 0.1b
15	5.6 ± 3.9c	0c	0b
20	0c	0c	0b
30	0c	0c	0b
40	0c	0c	0b
50	0c	0c	0b

Means in each column followed by the same letter were not significantly different according to Tukey's multiple comparison test (P ≤ 0.05).

Table 2. Effect of timentin on *Fraxinus profunda* regeneration.

Timentin (mg L^{-1})	Callus Formation (%)	Shoot Formation (%)	Mean No. Shoots
0	88.9 ± 5.3ab	44.4 ± 8.4a	1.3 ± 0.5a
50	100 ± 0a	55.6 ± 8.4a	1.0 ± 0.2a
100	97.2 ± 2.8ab	41.7 ± 8.3a	0.9 ± 0.2a
200	100 ± 0a	55.6 ± 8.4a	1.2 ± 0.3a
300	94.4 ± 3.9ab	61.1 ± 8.2a	1.4 ± 0.3a
400	100 ± 0a	72.2 ± 7.6a	2.2 ± 0.4a
500	94.4 ± 3.9ab	58.3 ± 8.3a	1.5 ± 0.3a
600	83.3 ± 6.3b	41.7 ± 8.3a	1.4 ± 0.5a

Means in each column followed by the same letter were not significantly different according to Tukey's multiple comparison test (P ≤ 0.05).

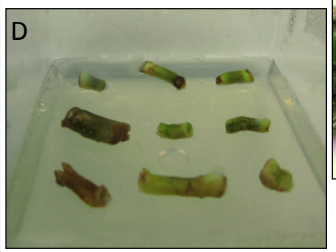

Figure 3. *Agrobacterium*-mediated transformation of pumpkin ash hypocotyls (*Fraxinus profunda*) with the pq35GR vector. (A) Hypocotyls 4 wk after transformation showing GUS-positive callus (arrow), (B) Putatively transformed shoot* in the middle of two non-transformed hypocotyls, (C) Normal shoot regeneration and elongation in the presence of 400 mg L^{-1} timentin after 12 wk, (D) Hypocotyls failing to initiate callus after 10 wk exposure to 20 mg L^{-1} kanamycin.

ACKNOWLEDGEMENTS

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