Agrobacterium-Mediated Transformation of White Ash (*Fraxinus americana* L.) Hypocotyls

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ABSTRACT

White ash trees provide both economical and ecological benefits. Ash is a valuable hardwood for wood products, and provides food and shelter for wildlife. The emerald ash borer (EAB) is an invasive pest that poses substantial risk to the ash resource in North America. There are no means of complete eradication or of any innate resistance in ash. Therefore, the development of white ash with resistance to the EAB is an urgent goal. White ash hypocotyls were transformed using *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pq35GR containing the neomycin phosphotransferase (*nptII*) and β-glucuronidase (GUS) genes. Mature embryos were cultured on Murashige and Skoog (MS) medium with Gamborg B5 vitamins containing 10 µM 6-benzylaminopurine (BA) plus 10 µM thidiazuron (TDZ) for 5 d, before hypocotyls were excised and pre-cultured on MS medium containing 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} adenine sulfate, and 10% coconut water (CW). Pre-cultured hypocotyls were transformed in the presence of 100 µM acetosyringone using 90 s sonication plus 10 min vacuum-infiltration. Hypocotyls were then co-cultured for 2 d in the dark. Kanamycin-resistant shoots were selected on MS medium containing 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} adenine sulfate, 300 mg L^{-1} timentin, 20 mg L^{-1} kanamycin, and 10% CW, then transferred to shoot elongation medium. Shoot elongation was optimized on MSB5 medium containing 0-20 µM BA plus 0-10 µM TDZ, 300 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin. The GUS and *nptII* genes were confirmed in hypocotyls, and three kanamycin-resistant shoots are in the process of being confirmed.

Figure 1. EAB larvae (A), adult beetle (B), larval galleries (C)

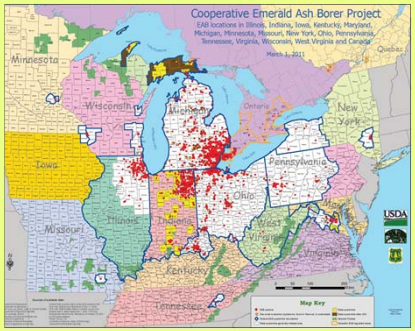

Figure 2. Locations of EAB infestation as of March 1, 2011

BACKGROUND

White ash, *Fraxinus americana* L. (family Oleaceae) provides both ecological and economic benefits. Endemic to North America, this species is integral to many ecosystems and has been widely planted as an urban street tree. White ash trees provide shelter and nesting sites for a variety of wildlife. The seeds are food for several bird and mammal species, and its bark was found to be utilized by rabbits, deer, beaver, and porcupine. White ash, a highly valued commercial hardwood, is used in the production of baseball bats, furniture, flooring, crates, boats, and doors. The estimated value of the 3.8 billion urban ash trees in the U.S. was $2.4 trillion in 2007, with an additional $282.3 billion in compensatory value from the 7.5 million ash trees growing on U.S. timberlands. White ash is threatened by the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), an aggressive wood-boring beetle indigenous to Asia. The adult beetles feed on the foliage of ash trees (Fig. 1B), but most of the damage is produced by the larval stage (Fig. 1A) which bores through the bark and into the cambial region, feeding on the tree phloem and producing galleries (Fig. 1C) that disrupt the flow of nutrients; eventually killing the tree. Over one-half of the tree canopy can be lost within the first 2 yr, and the entire tree is usually dead within 3- to 4-yr after the first observed symptoms. EAB is fatal to ash trees once the tree is infested, and there is no known innate resistance in native trees, nor any means to completely eradicate the EAB. All North American ash species are susceptible, and there is evidence that EAB is spreading quickly throughout the native range (Fig. 2), placing white ash in eminent danger. Bates (1997) attempted to develop a white ash transformation system using *Agrobacterium*, based on previous regeneration work, but was unable to confirm any transgenic plants. A complete plant regeneration and rooting protocol was developed using hypocotyls and cotyledons of green ash (Du and Pijut 2008), and has successfully translated into an *Agrobacterium*-mediated transformation and plant regeneration system (Du and Pijut 2009). This regeneration and rooting protocol has since been optimized for white ash (Fig. 4), providing a starting framework for development of a genetic transformation system for this species.

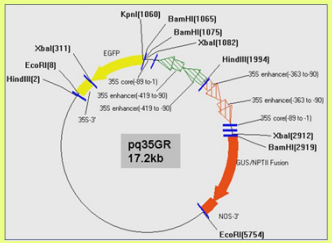

Figure 3. The pq35GR vector consisted of the cauliflower mosaic virus (*CaMV*) 35S promoter-derived bi-directional promoters containing two divergently arranged enhancer repeats, a fusion between the *nptII* and GUS genes, and the EGFP gene (Li et al. 2004).

MATERIALS AND METHODS

- Mature embryos were extracted from aseptic seed and cultured vertically on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with Gamborg B5 vitamins (Gamborg et al. 1968) containing 10 µM 6-benzylaminopurine (BA) plus 10 µM thidiazuron (TDZ) for 5 d germination.
- *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pq35GR containing the neomycin phosphotransferase (*nptII*) and β-glucuronidase (GUS) genes (Fig. 3) was cultured in 20 mL liquid YEP medium (10 g L^{-1} yeast extract, 10 g L^{-1} Bacto peptone, 5 g L^{-1} NaCl) with 20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin for 2 d prior to transformation.
- Hypocotyls were excised from germinated embryos and pre-cultured on MS medium containing 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} adenine sulfate, and 10% coconut water (CW) for 1 d prior to transformation.
- *Agrobacterium* was suspended in liquid pre-culture medium with 100 µM acetosyringone. Pre-cultured hypocotyls were sonicated for 90 s in liquid pre-culture medium, and then vacuum-infiltrated for 10 min in the presence of *Agrobacterium* (OD_{600} 0.6- 1).
- Hypocotyls were co-cultured for 2 d in the dark, rinsed three times with liquid pre-culture medium before being transferred to selection medium.
- Kanamycin-resistant shoots were selected on MS medium containing 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} adenine sulfate, 300 mg L^{-1} timentin, 20 mg L^{-1} kanamycin, and 10% CW, then transferred to shoot elongation medium (Fig. 5).
- Shoot elongation was optimized on MS medium containing 0-20 µM BA plus 0-10 µM TDZ, 300 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin (Table 1).
- Transient GUS expression, and the presence of the *nptII* gene in two putative transgenics were confirmed via PCR analysis (Fig. 6, 7).

RESULTS

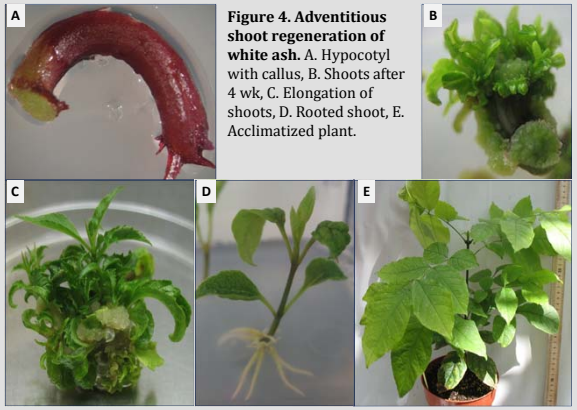

Figure 4. Adventitious shoot regeneration of white ash. A. Hypocotyl with callus, B. Shoots after 4 wk, C. Elongation of shoots, D. Rooted shoot, E. Acclimatized plant.

Table 1. Effect of BA and TDZ on shoot elongation

BA (µM)	TDZ (µM)	Shoot Elongation (%)	Avg Shoot No.
10	0	0.0 ± 0.0 b	0.0 ± 0.0 b
15	0	12.5 ± 70.7 ab	2.0 ± 0.0 a
20	0	12.5 ± 35.4 ab	1.0 ± 0.0 ab
0	2.5	0.0 ± 0.0 b	0.0 ± 0.0 b
10	2.5	0.0 ± 0.0 b	0.0 ± 0.0 b
15	2.5	12.5 ± 35.4 ab	1.0 ± 0.0 ab
20	2.5	0.0 ± 0.0 b	0.0 ± 0.0 b
0	5	0.0 ± 0.0 b	0.0 ± 0.0 b
10	5	0.0 ± 0.0 b	0.0 ± 0.0 b
15	5	12.5 ± 70.7 ab	2.0 ± 0.0 a
20	5	0.0 ± 0.0 b	0.0 ± 0.0 b
0	10	0.0 ± 0.0 b	0.0 ± 0.0 b
10	10	37.5 ± 75.6 a	1.3 ± 0.6 ab
15	10	0.0 ± 0.0 b	0.0 ± 0.0 b
20	10	12.5 ± 35.4 ab	1.0 ± 0.0 ab

Figure 5. Putative transgenic white ash shoots

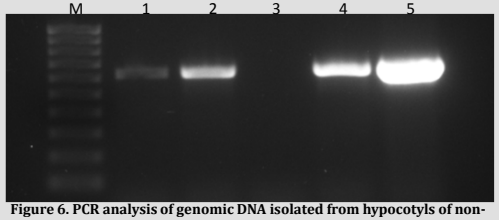

Figure 6. PCR analysis of genomic DNA isolated from hypocotyls of non-transformed and transgenic white ash for amplification of 400-bp *nptII* gene. M 100 bp molecular marker, Lanes 1-2 putative transgenics, Lane 3 negative control, Lane 4-5 positive controls.

Figure 7. Transient GUS expression. A. Hypocotyl, B. Leaf, and C. Callus.

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