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Abstract

A reproducible transformation and regeneration system using hypocotyls of green ash (*Fraxinus pennsylvanica*) has been established. The best regeneration medium for freshly isolated hypocotyls was Murashige and Skoog (MS) supplemented with 13.3 μ M benzyladenine (BA) and 4.5 μ M thidiazuron (TDZ). Seventy-five percent of hypocotyl segments produced adventitious shoots. The effect of seedling age (0, 4, 7, 10, or 15-day-old) on adventitious shoot regeneration from hypocotyl explants was also studied. Shoot regeneration decreased with increasing age of the explants. Adventitious shoots from hypocotyls were established as proliferating shoot cultures following transfer to MSB5 medium supplemented with 10 μ M BA and 10 μ M TDZ. The highest rooting (93%) of adventitious shoots was obtained on woody plant medium containing 4.9 μ M indole-3-butyric acid (IBA) and 5.7 μ M indole-3-acetic acid (IAA). Rooted plants were successfully acclimatized to the greenhouse and 100% survived after overwintering in cold-storage. For genetic transformation, green ash was transformed using *Agrobacterium tumefaciens* strain EHA105:pq35GR carrying the NPTII and GUS fusion gene and EGFP gene. Ninety seconds sonication combined with 10 min vacuum-infiltration for co-cultivation produced the best results for transformation. GUS-, GFP-, and PCR-positive shoots from the cut ends of the hypocotyls close to the original cotyledon area were produced via an intermediate callus stage. This transformation and regeneration system using hypocotyls provides a foundation for *Agrobacterium*-mediated transformation of *F. pennsylvanica* for resistance to the emerald ash borer. Studies are underway using a construct containing the cry8Da protein (or derivatives) of *Bacillus thuringiensis*.

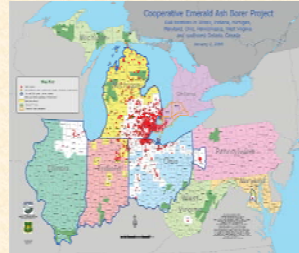


Figure 1 The distribution and infection of emerald ash borer (EAB)

Objectives

- Develop adventitious shoot regeneration system for green ash.
- Develop *agrobacterium*-mediated transformation protocol for green ash.

Introduction

Green ash (*Fraxinus pennsylvanica*), also called red ash, swamp ash, and water ash is the most widely distributed of the North American ash species. Green ash is very popular as a shade tree because of its good form and adaptability. The wood is used for specialty products such as tool handles, oars, crates, and baseball bats because of the strength, hardness, shock resistance, and excellent bending qualities of the wood. Green ash is normally relatively free from insect and diseases, but the emerald ash borer (EAB), an aggressive exotic beetle from Asia, recently was reported to attack and kill all ash trees (Haack et al., 2002). A pest risk assessment completed in Canada (Dobesberger, 2002) concluded that the EAB could potentially spread throughout the entire range of ash and cause significant economic losses and environmental damage. To date there is no known efficient means to completely eradicate the EAB. The development of transgenic green ash exhibiting resistance to attack by the EAB is urgently needed and promising.

Materials and Methods

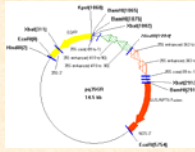
Regeneration of green ash from hypocotyls of mature embryos

Seeds of *Fraxinus pennsylvanica* were surface disinfected and stored in sterile, distilled water overnight. The following day, the embryos were isolated and hypocotyl explants were excised and cultured on MS medium supplemented with 0, 4.4, 8.9, 13.3, or 22.2 μ M BA in combination with 0, 0.5, 2.3, or 4.5 μ M TDZ for adventitious shoot induction. After 4 weeks, adventitious shoots from hypocotyls were transferred to MS basal salt, B5 vitamins (MSB5) medium (Gamborg et al., 1968) supplemented with 10 μ M BA plus 10 μ M TDZ to induce shoot elongation. Elongated shoots were rooted on woody plant medium (Lloyd and McCown, 1980) (WPM) supplemented with 4.9 μ M IBA in combination with 0, 2.9, 5.7, or 8.6 μ M IAA. Plantlets were transplanted into plastic pots containing a moist, autoclaved peat moss:vermiculite:perlite mixture (1:1:1).

Agrobacterium-mediated transformation and regeneration from hypocotyls of green ash

• *A. tumefaciens* strain EHA105 with binary vector pq35GR which has bi-directional duplex promoters with duplicated enhancers was used for transformation. Four-day-old hypocotyls from mature embryos were pre-cultured for one day on pre-culture medium (MS medium supplemented with 13.2 μ M BA, 4.5 μ M TDZ, 50 mg/L adenine hemisulfate, and 10% coconut milk). Overnight cultures of *Agrobacterium* were collected by centrifugation, 15 min 3,000 x g and then re-suspended to a final concentration OD600 0.6-1.0 in co-cultivation medium

(same as pre-culture medium) with 100 μ M acetosyringone and cultured at 28 °C for 1hr with rotary shaking (150 rpm). Pre-cultured hypocotyls were immersed in *Agrobacterium* with 90 sec sonication plus 10 min vacuum-infiltration, and then blotted dry, transferred to co-cultivation medium, and incubated in the dark for two days. After two days co-cultivation, hypocotyl explants were washed three times in sterilized water, and then blotted dry. Inoculated hypocotyls were placed on selection medium (pre-culture medium plus 20 mg/L kanamycin and 300 mg/L timentin).



• Histochemical GUS assay of kanamycin-resistant callus, elongated kanamycin-resistant shoots, and rooted kanamycin-resistant plantlets were performed as described by Jefferson et al. (1987). All tissue were stained in X-gluc overnight at 37 °C. Chlorophyll was removed from the tissue using commercial bleach rinses.

• Total genomic DNA was isolated from leaves of GUS-positive lines for polymerase chain reaction (PCR). The PCR primers were designed to amplify a 332 bp fragment of the GUS gene a 364 bp fragment of the NPTII gene.

Results

1. Regeneration of plants from *Fraxinus pennsylvanica* hypocotyls

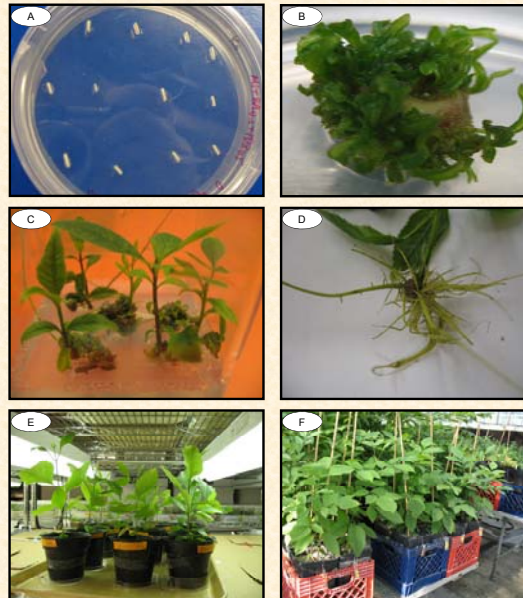


Fig. 3 Plant regeneration from hypocotyls of *Fraxinus pennsylvanica*. (A) Freshly isolated hypocotyls, (B) Adventitious shoot induction from hypocotyls on MS with 13.3 μ M BA and 4.5 μ M TDZ after 4 weeks, (C) Adventitious shoot elongation on MSB5 with 10 μ M BA and 10 μ M TDZ, (D) Rooting of shoots on WPM with 4.9 μ M IBA and 5.7 μ M IAA, (E) Normal growth of potted green ash plants in culture room after 2 weeks, and (F) Acclimatization of plantlets to the greenhouse after 4 weeks.

2. Obtainment of transgenic *Fraxinus pennsylvanica* and histochemical analysis of transgenic plants

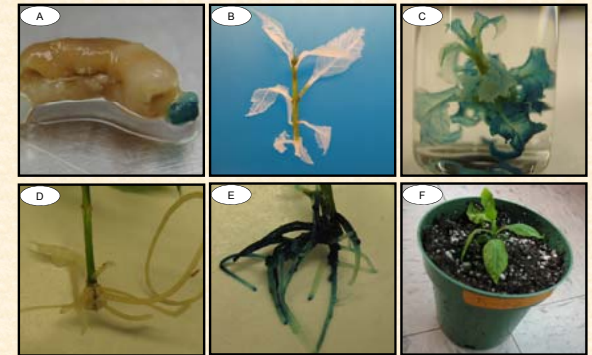


Fig. 4 Regeneration of transgenic *Fraxinus pennsylvanica* and GUS assay of transgenic plant. (A) Callus formation from transformed cell of hypocotyl, (B) and (C) GUS analysis of elongated non-transgenic plant and kanamycin-resistant shoot, respectively, (D) and (E) GUS analysis of rooted non-transgenic plant and transgenic plant, respectively, and (F) Normal growth of potted transgenic plant in culture room.

3. PCR analysis of transgenic *Fraxinus pennsylvanica*



Fig. 4 Amplified fragment from GUS gene (332 bp), M: 100 bp marker, 1: Plasmid DNA, 2: H2O control, 3: Non-transformed plant, 4, 5, 6: Transgenic lines (F1, F2, and F3), Amplified fragment from NPTII gene (364 bp), 7: Plasmid DNA, 8: H2O control, 9: Non-transformed plant, 10,11,12: Transgenic lines (F1, F2, and F3).

Conclusions

- Adventitious shoot regeneration and rooting protocol was successfully developed for green ash (*Fraxinus pennsylvanica*) seedling explants.
- *Agrobacterium*-mediated transformation protocol was developed for green ash.

Future work

- Produce transgenic green ash plants with resistance to the EAB using a cry gene construct based on *Agrobacterium*-mediated transformation protocol as reported here.

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