Genetic Transformation and Regeneration of Green Ash (Fraxinus pennsylvanica) from Hypocotyl and Leaf Explants

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Abstract

The emerald ash borer (EAB), Agrilus planipennis, is an exotic and invasive beetle from Asia that has been threatening North American Fraxinus populations since it was discovered in the United States in 2002. EAB larvae feed on the phloem of ash trees and can kill a tree within a few years of the initial infestation. Balsillus thuringiensis (Bt) is a naturally-occurring soil bacterium that is commonly used as a microbial pesticide and is non-lethal to mammals. The Cry genes of Bt produce crystalline inclusion bodies that, when ingested by EAB larvae, cause death by disrupting the membrane of the midgut. The ash species native to North America have not co-evolved with EAB, therefore these species do not possess natural defenses to EAB. In order to produce green ash with the ability to resist the EAB, research has begun to develop transgenic Bt green ash. Protocols for the transformation of green ash have already been established in our lab. In this project, the transformation protocols have been modified to include the Bt toxic protein genetic construct. In addition to developing transgenic Bt ash, this project focuses on developing a protocol for adventitious shoot regeneration of green ash from in vitro leaf explants. A protocol for regeneration of green ash from hypocotyls has already been established. However, because of the rapid loss of green ash in native forest populations, as well as other important ash species such as white (F. americana), pumpkin (F. profunda), and black (F. nigra), it is likely that seed repositories will become depleted. Development of a new adventitious shoot regeneration method from in vitro leaf explants will allow us to transform any selected ash species, and continue conservation and propagation of ash for future research opportunities and outplanting.

Materials and Methods – In Vitro Regeneration

• Green ash in vitro shoot cultures
  - Intermode sections
  - Leaf sections
  - Murashige and Skoog medium, B5 organics, 3% sucrose, 10% coconut water, 0.7% agar
  - Factorial including cytokinin: 6-benzyladenine (BA) and auxin: naphthaleneacetic acid (NAA)
  - Observe number of explants forming callus, shoots, or shoot buds
  - Explants transferred to fresh treatment medium every 3 weeks

Results

Genetic Transformation

• Polymerase Chain Reaction (PCR) will be performed to amplify specific DNA fragments in transgenic shoots corresponding to the cryB2D gene
  - A primer set (forward primer: cryB2D F 5’-AGGCGGCGGATTGCGTAGG -3’ and reverse primer: cryB2D R 5’-GGCCGTTGCCTCTGTTAGC-3’) was designed to amplify a 957 bp PCR product for the cryB2D gene
  - A 2% agar PCR will be performed containing 2.5 µl of 50x PCR buffer, 1 µl 50 mM MgCl2, 0.5 µl 50 mM MgCl2, 0.1 µl PCR product, 1 µl 200 ng/µl DNA template, respectively, and 0.2 µl 5′/3′ Taq polymerase
  - Plasmid DNA used in transformation will serve as a positive control and DNA from non-transformed shoots will serve as a negative control
  - PCR reaction will include a denaturing step of 94°C for 2 min, 30 cycles 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final cycle at 72°C for 5 min

Leaf Regeneration

• Elongated kanamycin-resistant shoots will be transferred to woody plant medium with 4.9 µM indole-3-butyric acid and 5.7 µM indole-3-acetic acid to stimulate adventitious root formation

Further Study

• Genetic Transformation
  - Polymerase Chain Reaction will be performed on putative transgenic shoots to confirm the presence of the Bt and nptII gene
  - Additional replicates will be performed to produce more putative transgenic shoots and to increase efficiency of transformation

• Leaf Regeneration
  - Observation of leaf explants on treatment media will continue
  - Adjustments to treatment media may be made to produce adventitious shoot regeneration

• Seed surface disinfection
  - Seeds are surface-disinfected in 70% ethanol, followed by a 20% bleach solution, and water rinses
  - Seeds are soaked overnight in sterile water

• Excised embryos are cultured on germination medium for 5 days
  - After co-cultivation, hypocotyls are rinsed with MSBTAC and cultured on selection medium containing MS medium + MS organics + 50 mg l−1 adenine hemisulfate + 13.2 µM BA + 4.5 µM thidiazuron (TDZ) + 3% (w/v) sucrose + 10% coconut water + 0.7% (w/v) Bacto Agar before transformation.

• The emerald ash borer (EAB), Agrilus planipennis, is a wood-boring beetle (A) that feeds on and kills North American ash species, Fraxinus (B). As of April 1, 2014, the emerald ash borer has spread to 22 U.S. states and two Canadian provinces (C). Research is being conducted to genetically transform green ash, Fraxinus pennsylvanica, using the Bt toxic protein genetic construct, pB122/B2D (D).

• Excised embryos are cultured on germination medium (MS medium + MS organics + 50 mg l−1 adenine hemisulfate + 13.2 µM BA + 4.5 µM TDZ) with 100 µM kanamycin. A overlay of liquid shoot elongation medium without thidiazuron is also placed on selection medium containing kanamycin and timentin for 3 weeks (E).

• Shoots elongated on shoot elongation medium (MS medium + B5 organics + 10 µM BA + 100 µM TDZ + 3% (w/v) sucrose) with addition of 100 mg l−1 timentin and 50 mg l−1 kanamycin. Hypocotyls remain on selection medium for 6-8 weeks (F).

• Elongated kanamycin-resistant shoots will be transferred to woody plant medium with 4.9 µM indole-3-butyric acid and 5.7 µM indole-3-acetic acid to stimulate adventitious root formation

• Leaf explants on treatment (T) 7 showed callus growth after 3 weeks (A), but after 7 weeks on T 18 medium, the leaf explants became necrotic (B). Leaf explants on T 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99.

• T1, T3, and T5 showed little change after 3 weeks (C) and after 7 weeks on T 35, there is much more callus growth (D).

• Leaf explants on T 35 also showed little change after 3 weeks (E) and after 7 weeks on T 35, there is much more callus growth (F).

References


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Figure 1. Emerald ash borer: Agrilus planipennis. A wood-boring beetle (A) that feeds on and kills North American ash species, Fraxinus (B). As of April 1, 2014, the emerald ash borer has spread to 22 U.S. states and two Canadian provinces (C). Research is being conducted to genetically transform green ash, Fraxinus pennsylvanica, using the Bt toxic protein genetic construct, pB122/B2D (D).

Figure 2. Excised embryos are cultured on germination medium for 5 days (A) to germinate (B). After hypocotyls have been isolated and transformed, they are placed on selection medium containing kanamycin and timentin for 3 weeks (C) and then transferred to fresh medium for an additional 3 weeks (D). Hypocotyls that show resistance to kanamycin and show growth are placed on stem elongation medium containing kanamycin and timentin (E). Putative transgenic shoots that display stunted growth are cultured on shoot elongation medium without thidiazuron and kanamycin. A overlay of liquid shoot elongation medium without thidiazuron is also added to this culture medium (F).