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

Melissa Johnson and Paula M. Pijut
Purdue University
Forestry and Natural Resources
Hardwood Tree Improvement and Regeneration Center

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. *Bacillus 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.

Introduction

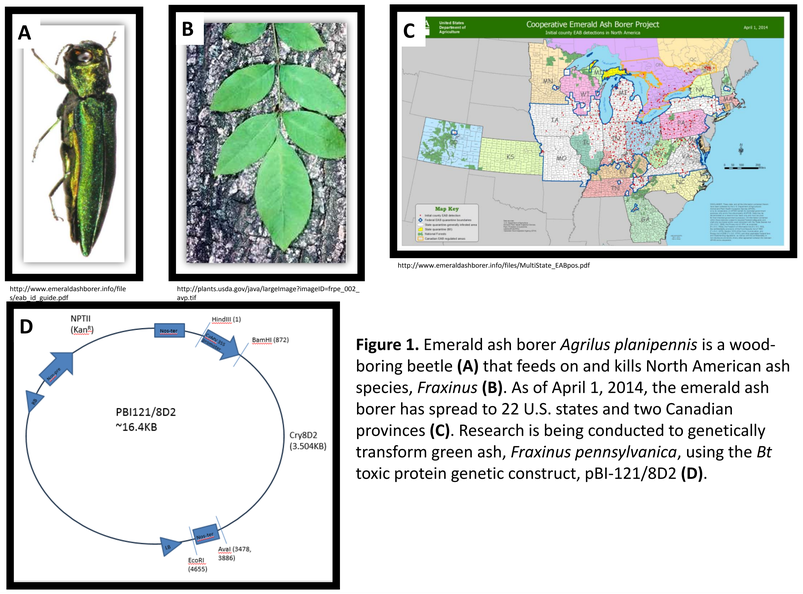

Figure 1. Emerald ash borer *Agrilus planipennis* is a wood-boring beetle **(A)** that feeds on and kills North American ash species, *Fraxinus* **(B)**. As of April 1, 2014, the emerald ash borer has spread to 22 U.S. states and two Canadian provinces **(C)**. Research is being conducted to genetically transform green ash, *Fraxinus pennsylvanica*, using the *Bt* toxic protein genetic construct, pBI-121/8D2 **(D)**.

Materials and Methods - Genetic Transformation

Seed surface-disinfestation

- Seeds are surface-disinfested in 70% ethanol, followed by a 20% bleach solution, and water rinses
- Seeds are soaked overnight in sterile water.

Excised embryos are placed on germination medium (MS medium + MS organics + 3% (w/v) sucrose + 0.7% (w/v) Bacto Agar) for 5 days. Hypocotyls are then isolated and cultured on pre-culture medium (MS medium + MS organics + 50 mg l^{-1} adenine hemisulfate + 13.2 µM 6-benzyladenine (BA) + 4.5 µM thidiazuron (TDZ) + 3% (w/v) sucrose + 10% coconut water (CW) + 0.7% (w/v) Bacto Agar) before transformation.

Agrobacterium

- *Agrobacterium tumefaciens* strain EHA105-pBI121/8D2 is streaked on a plate of solid YEP medium (10 g l^{-1} yeast extract, 10 g l^{-1} bacto peptone, 5 g l^{-1} NaCl, pH 7.0) containing 20 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin 4 days before transformation
- An individual colony of *Agrobacterium* is cultured in liquid YEP medium supplemented with 20 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin
- The *Agrobacterium* strain is cultured in the dark on an orbital shaker at 28 °C for 2 days

Transformation

- The previously cultured *Agrobacterium* is used to make a liquid suspension containing liquid MSBTAC (MS medium + MS organics + 50 mg l^{-1} adenine hemisulfate + 13.2 µM BA + 4.5 µM TDZ) with 100 µM acetosyringone
- Hypocotyls from the previously germinated embryos are sonicated for 90 s
- Sonicated hypocotyls are placed in the *Agrobacterium* suspension and vacuum infiltrated for 10 min at 62.5cm Hg
- Hypocotyls are then dried on sterile filter paper, cultured on pre-culture medium, and incubated in the dark at 28 °C for 3 days
- After co- cultivation, hypocotyls are rinsed with MSBTAC and cultured on selection medium (MS medium + MS organics + 50 mg l^{-1} adenine hemisulfate + 13.2 µM BA + 4.5 µM TDZ + 3% (w/v) sucrose) containing 100 mg l^{-1} timentin and 50 mg l^{-1} kanamycin Hypocotyls remain on selection medium for 6-8 weeks

Shoot Elongation

- Hypocotyls are cultured on shoot elongation medium (MS medium + B5G organics + 10 µM BA + 10 µM TDZ + 3% (w/v) sucrose) with addition of 100 mg l^{-1} timentin and 50 mg l^{-1} kanamycin
- Hypocotyls showing kanamycin resistance and shoot elongation will be transferred to rooting medium

Molecular Analysis

- Total genomic DNA will be isolated from kanamycin-resistant shoots (leaves)
- Polymerase Chain Reaction (PCR) will be performed to amplify specific DNA fragments in transgenic shoots corresponding to the cry8D2 gene
- A primer set (forward primer; cry8D2-F 5'-AGGGCCCGGATTCGCTACG -3' and reverse primer; cry8D2-R 5'-GCCGCTTTGCCTCCTTGACG-3') was designed to amplify a 457 bp PCR product for the cry8D2 gene
- A 25 µl PCR reaction will be prepared containing 2.5 µl of 10 x PCR buffer, 1 µl 10 mM dNTP, 0.8 µl 50 mM Mg2+, 1 µl 10 µM cry8D2-F and cry8D2-R primers, 1 µl 200 ng/µl DNA template, respectively, and 0.2 µl 5U/µl Taq polymerase
- Plasmid DNA used in transformation will serve as a positive control and DNA from non-transformed shoots as 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 10 min

Rooting

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

Results

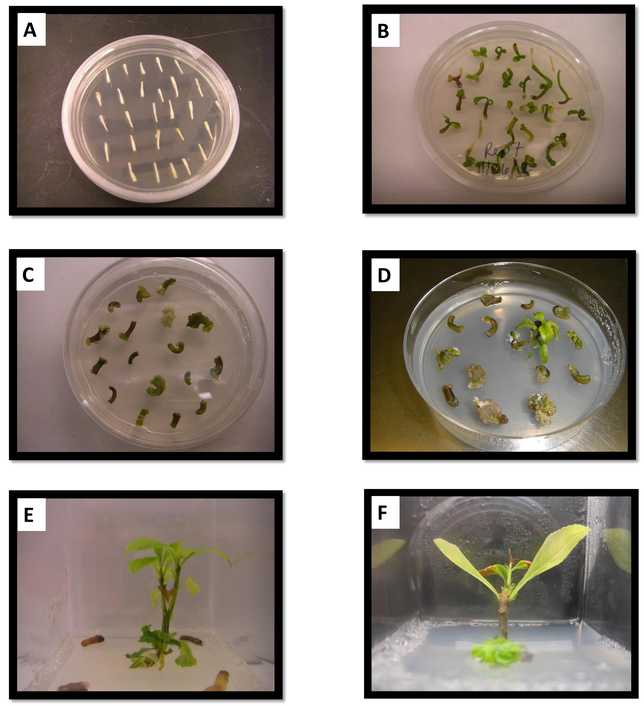

Figure 2. Excised embryos are cultured on germination medium for 5 days **(A)** to germinate **(B)**. After hypocotyls have been isolated and transformed, they are placed on selection medium containing kanamycin and timentin for 3 weeks **(C)** and then transferred to fresh medium for an additional 3 weeks **(D)**. Hypocotyls that show resistance to kanamycin and shoot 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)**.

Materials and Methods – In Vitro Regeneration

- Green ash in vitro shoot cultures
- Internode sections
- Leaf sections
- Murashige and Skoog medium, B5G 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

BA and NAA FACTORIAL

BA (µM)	0	4.4	8.9	13.3	17.8	22.2	26.6
NAA (µM)							
0	----------	2	3	4	5	6	7
0.5	8	9	10	11	12	13	14
1.1	15	16	17	18	19	20	21
1.6	22	23	24	25	26	27	28
2.15	29	30	31	32	33	34	35

Results

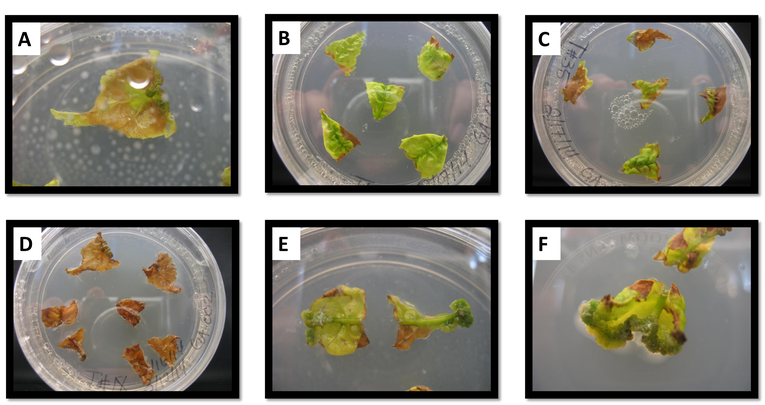

Figure 3. Leaf explants on treatment (T) 18 showed callus growth after 3 weeks **(A)**, but after 7 weeks on T 18 medium, the leaf explants became necrotic **(D).** Leaf explants on T 27 showed little regeneration after 3 weeks **(B)**, but after 7 weeks on T 27, callus growth increased **(E)**. Leaf explants on T 35 also showed little change after 3 weeks **(C)** and after 7 weeks on T 35, there is much more callus growth **(F)**.

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 replications 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

Feeding Bioassays

- Any confirmed transgenic *Bt* green ash will be propagated and rooted for feeding assays with adult emerald ash borer

References

Du, N. and Pijut, P.M. 2008. Regeneration of plants from *Fraxinus pennsylvanica* hypocotyls and cotyledons. Scientia Horticulturae 118:74-79

Du, N. and Pijut, P.M. 2009. *Agrobacterium*-mediated transformation of *Fraxinus pennsylvanica* hypocotyls and plant regeneration. Plant Cell Reports 28:915-923

Acknowledgement

This research is supported by funding from Purdue Department of Forestry and Natural Resources and Sloan Foundation Indigenous Graduate Partnership.