Regeneration and Genetic Transformation of Green Ash for Resistance to the Emerald Ash Borer

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Abstract

A regeneration protocol using 7-day-old hypocotyls from in vitro germinated seeds of green ash (Fraxinus *pennsylvanica*) was developed. The best regeneration medium for hypocotyls was MS medium supplemented with 13.3 µM BA plus 4.5 µM TDZ. Seventy-four percent of hypocotyl segments produced adventitious shoots, with a mean number of adventitious shoots per explant of 1.7 ±0.7. Adventitious shoots from hypocotyls were established as proliferating shoot cultures following transfer to MSB5 medium supplemented with 10 µM BA plus 10 µM TDZ. A high rooting percentage (73-90 %) on WPM containing IBA plus IAA was achieved for shoot cultures established from three seedling genotypes, when shoots were maintained for 10 days in the dark on root induction medium prior to transfer to a 16-hour photoperiod. The highest rooting (93%) of adventitious shoots from hypocotyls and number of roots per shoot (2.8 ± 0.2) were obtained with 4.9 µM IBA plus 5.7 µM IAA. Rooted plants were successfully acclimatized to the greenhouse and 100% survived overwintering in cold-storage. Kanamycin at 15 mg/L was determined suitable for use in selection medium. Further Polymerase Chain Reaction (PCR) analysis of kanamycin resistant shoots is in process. This regeneration and transformation system using hypocotyls provides a foundation for *Agrobacterium*-mediated genetic transformation of *Fraxinus pennsylvanica* for resistance to the emerald ash borer (EAB).

Materials and Methods

1. Regeneration

Adventitious shoot induction
Hypocotyl segments from 7-day-old embryos (Figure 2A) were cultured on Murashige and Skoog (MS) (1962) medium supplemented with 0, 4.4, 8.9, 13.3, or 22.2 µM 6-benzylademine (BA) in combination with 0, 0.5, 2.3, or 4.5 µM thidiazuron (TDZ) for adventitious shoot induction.

Shoot elongation
Adventitious shoots (Figure 2B) were transferred to MS basal salt, B5 vitamins (MSB5) medium (Gamborg et al., 1968) supplemented with 10 µM BA plus 10 µM TDZ for shoot elongation (Figure 2C).

Rooting of micropropagated shoots and elongated adventitious shoots
Microshoots were cultured on woody plant medium (WPM) (Lloyd and McCown, 1980) supplemented with 4.9 µM indole-3-butyric acid (IBA) in combination with 0, 2.9, 5.7, or 8.6 µM indole-3-aeetic acid (IAA) for root formation. Microshoots were maintained for 10 days in the dark on rooting medium prior to transfer to a 16-hour photoperiod. Elongated adventitious shoots were cultured on WPM supplemented with 4.9 µM IBA plus 5.7 µM IAA to induce root formation (Figure 2D).

Acclimatization of rooted plantlets
Plantlets were transferred to a peat moss, vermiculite, and perlite mixture (1:1:1) in 6- to 8-inch plastic pots (Figure 2E) . Plants were watered every two days. After 2 weeks of slowly acclimatizing the plants in the lab, plants were transferred to the greenhouse. After another 2 weeks, plantlets were transferred to larger pots for further roots elongation (Figure 2F). Then plantlets were transferred to cold-storage in chill dome for overwintering.

2. Genetic transformation

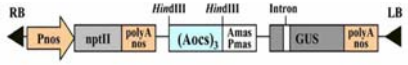


Figure 1. T-DNA region of binary vector pBISN1

Kanamycin sensitivity
7-day-old hypocotyls were cultured on regeneration medium containing kanamycin at 0, 5, 10, 15, 20, or 30 mg/L for 6 weeks.

Transformation
The effect of three different *Agrobacterium* strains (LBA4404, EHA105, and GV3103) with binary Vector (Figure 1) on transient expression of GUS were tested. Seven-day-old hypocotyls precultured for 2 days on regeneration medium (MS + 13.2 µM BA + 4.5 µM TDZ) were immersed in 20 ml bacterial solution (OD=0.6-1.0), vacuum infiltrated (25 inches of mercury) for 10 min plus 20 min on orbital shaker (100 rpm), blotted dry on sterilized paper, transferred to regeneration medium, and plates incubated in the dark for 2 days. Explants were then washed three times (5 min/wash) using liquid MS medium and blotted dry. Explants were transferred to regeneration medium + Kan 15 mg/L + Tim 300 mg/L for regeneration. Some explants were used for GUS assay.

Conclusions

The best hypocotyl regeneration medium was MS supplemented with 13.3 µM BA plus 4.5 µM TDZ. The greatest percentage of hypocotyls regenerating was 76.3% and the mean number of adventitious shoots induced per hypocotyl was 1.7±0.7.
A high rooting percentage (73-90 %) on WPM containing IBA plus IAA was achieved for three seedling genotypes and 93% rooting was achieved on WPM supplemented with 4.9 µM IBA plus 5.7 µM IAA for adventitious shoots.
Rooted plants were successfully acclimatized to the greenhouse and 100% survived overwintering in cold-storage.
Kanamycin at 15 mg/L was suitable for use in selection medium.
Strain EHA105 was most suitable for transformation of hypocotyls.

Objective

Develop a stable *Agrobacterium*-medium transformation system for genetically engineering green ash with resistance to the EAB.

Introduction

Green ash (*F. pennsylvanica*) (Oleaceae) is an important North American tree species. The wood is used for solid wood products, crates, boxes, and for specialty products such as tool handles, oars, and baseball bats because of the strength, hardness, shock resistance, and excellent bending qualities of the wood. Green ash is also very popular as a shade tree because of its good form, adaptability to various soil conditions, and normally relatively free from insect and diseases (Kennedy, 1990). But the emerald ash borer (EAB), an aggressive exotic beetle from Asia, has been found infesting green ash, white ash (*F. americana*), black ash (*F. nigra*), as well as several horticultural varieties of ash in Ohio, Indiana, Illinois, Michigan, Maryland and Ontario, and this devastation results in significant economic loss and environmental damage in these areas with EAB infestation (Dobesberger, 2002; Haack et al., 2002). To date there is no efficient means to completely eradicate the EAB. Genetic engineering is an efficient and feasible tool to produce plants with resistance to pests compared to conventional breeding strategies. Adventitious shoot regeneration in tissue culture from plant organs is an important prerequisite for genetic modification via *Agrobacterium*-mediated transformation.

Results

1. Regeneration

Figure 2. Regeneration of plants from hypocotyls of green ash

Figure 3. Effect of BA and TDZ on shoot regeneration from hypocotyls

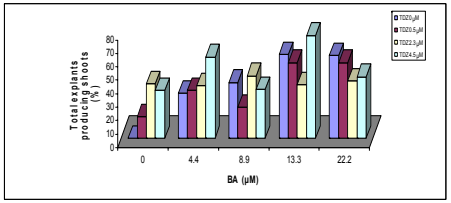


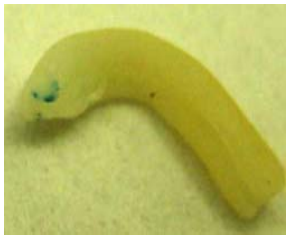
Table 1. Effect of *Fraxinus pennsylvanica* genotypes and auxin level on root formation of microshoots

Auxin (µM)		Rooting (%)	Mean No. roots	Mean root length (cm)	Mean No. lateral roots
IBA	IAA				
control		0	0	0	0
4.9	0	73.15±0.12b	2.87±1.39a	3.03±1.49a	19.13±7.68a
4.9	2.9	88.89± 0.06a	2.64±1.12a	2.91±1.84a	18.08±7.14a
4.9	5.7	89.81±0.10a	2.99±1.01a	3.02±1.89a	17.76±6.47a
4.9	8.6	88.89±0.11a	2.8±1.09a	3.36±2.05a	17.62±7.12a
Effect of genotype					
GAS-001		95.83±0.05a	3.34±1.51a	2.92±2.03b	16.72±6.96b
GAS-002		81.94±0.10b	2.89±1.23ab	2.38±1.65b	16.67±6.71b
GA-JP03-10		77.78±0.15b	2.25±0.73b	3.94±1.78a	21.04±7.65a

Values represent the pooled means ± standard error of three independent experiments. Means in each column followed by the same letter are not significantly different according to Tukey's Multiple Comparison Test ($P \leq 0.05$). Control = hormone-free WPM medium.

2. Primary result on transformation


GUS expression on the surface of hypocotyl


GUS expression at the end of hypocotyl


Kanamycin resistant shoots


Elongation of kanamycin resistant shoots

Table 2. Effect of *Agrobacterium* strains on transient expression of GUS

Strain	No. of hypocotyls for transformation	No. hypocotyls showing GUS transient expression	Transient expression (%)
LBA4404	45	10	22.0
EHA105	48	16	33.3
GV3101	50	16	32.0

Table 3. Shoot regeneration from hypocotyl on Kanamycin medium

Kanamycin (mg/L)	Regeneration (%)
0	58
5	46
10	8
15	0
20	0
30	0

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