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GENETIC TRANSFORMATION AND HYBRIDIZATION

Agrobacterium-mediated transformation of *Fraxinus pennsylvanica* hypocotyls and plant regeneration

Ningxia Du · Paula M. Pijut

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Abstract A genetic transformation protocol for green ash (*Fraxinus pennsylvanica*) hypocotyl explants was developed. Green ash hypocotyls were transformed using *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pq35GR containing the neomycin phosphotransferase (*nptII*) and β-glucuronidase (GUS) fusion gene, and an enhanced green fluorescent protein gene. Pre-cultured hypocotyl explants were transformed in the presence of 100 μM acetosyringone using 90 s sonication plus 10 min vacuum-infiltration. Kanamycin at 20 mg l^{-1} was used for selecting transformed cells. Adventitious shoots regenerated on Murashige and Skoog medium supplemented with 13.3 μM 6-benzylaminopurine, 4.5 μM thidiazuron, 50 mg l^{-1} adenine sulfate, and 10% coconut water. GUS- and polymerase chain reaction (PCR)-positive shoots from the cut ends of hypocotyls were produced via an intermediate callus stage. Presence of the GUS and *nptII* genes in GUS-positive shoots were confirmed by PCR and copy number of the *nptII* gene in PCR-positive shoots was determined by Southern blotting. Three transgenic plantlets were acclimatized to the greenhouse. This transformation and regeneration system using hypocotyls provides a foundation for *Agrobacterium*-mediated transformation of green ash. Studies are underway using a construct containing the Cry8Da protein of *Bacillus thuringiensis* for genetic transformation of green ash.

Keywords Genetic engineering · Green ash · Sonication · Transgenic · Vacuum-infiltration

Abbreviations

BA	6-Benzylaminopurine
EGFP	Enhanced green fluorescent protein
GUS	β-Glucuronidase
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
*npt*II	Neomycin phosphotransferase
PCR	Polymerase chain reaction
TDZ	Thidiazuron

Introduction

Green ash (*Fraxinus pennsylvanica* Marsh.), also called red and swamp ash, is native to the eastern U.S. and parts of Canada (Kennedy 1990), and is the most widely distributed North American ash species. The wood is used for furniture, boxes, tool handles, oars, and crates because of its strength, hardness, shock resistance, and excellent bending qualities. It is also a good source of fiber for making high-grade paper. Green ash is historically resistant to insects and diseases. However, the emerald ash borer (EAB), an aggressive, exotic, wood-boring beetle (Yu 1992), has been a major threat to ash tree species in North America in recent years (Haack et al. 2002). In 2002, it was identified as the cause of extensive ash (*Fraxinus* spp.) mortality in

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N. Du
Department of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center (HTIRC), Purdue University, 715 West State Street, West Lafayette, IN 47907, USA

P. M. Pijut (✉)
USDA Forest Service, Northern Research Station, HTIRC, 715 West State Street, West Lafayette, IN 47907, USA
e-mail: ppijut@purdue.edu; ppijut@fs.fed.us

southeastern Michigan and nearby Ontario, Canada. EAB has killed approximately 25 million ash trees in lower Michigan since its arrival in North America (Cappaert et al. 2005; Poland and McCullough 2006). EAB infestations have also been found in Michigan's Upper Peninsula, Ohio, Indiana, Illinois, Maryland, western Pennsylvania, Virginia, West Virginia, Wisconsin, and Missouri. The risk EAB poses to the ash resources in North America is substantial. In addition to the multi-million dollar U.S. consumption of ash wood for the period 1999–2003, the U.S. exported ash lumber to 78 countries (151,154.8 m^3; $70,038,131; average per year) and ash logs to 45 countries (35,979.6 m^3; $9,929,298; average per year) (USDA FAS 2004). A pest risk assessment concluded that the EAB could potentially spread throughout the entire range of ash and cause significant economic losses and environmental damage (Dobesberger 2002). All native ash species are susceptible and there are no known innate resistance genes in North American ash species.

Currently there is no efficient means to completely eradicate EAB, and genetic engineering offers a promising way to combat the insect. The development of transgenic *Fraxinus* spp. exhibiting resistance to attack by EAB will have great economic benefits to landowners, the forest products industry, and to the U.S. foreign trade of ash lumber and logs. There are two reports of genetic transformation of *Fraxinus*, but neither of them regenerated transgenic plants (Bates 1997; Roome 1992). The first report on ash transformation used mature hypocotyls of *F. pennsylvanica* and kanamycin as the selection agent (Roome 1992). Transformed callus confirmed by polymerase chain reaction (PCR) and Southerns were obtained, but no stable, transgenic plants were produced. Roome (1992) also found that there was no *Agrobacterium* strain dependence for achieving green ash transformation. Bates (1997) tried to develop protocols to genetically transform white ash (*F. americana*) using *Agrobacterium*. The results showed that white ash was susceptible to infection by *Agrobacterium* and hypocotyls produced multiple shoots in vitro, but none of them were confirmed to be transgenic plants by molecular analysis. There is a need to develop a reliable and efficient transformation system to make genetic engineering possible.

We present here what we believe is the first report of transgenic green ash plants. Explants were transformed using *Agrobacterium tumefaciens* strain EHA105 harboring the pq35GR binary vector containing the fusion gene between neomycin phosphotransferase (*nptII*) and β-glucuronidase (GUS) genes, and an enhanced green fluorescent protein (EGFP) gene. This transformation and regeneration system using hypocotyls provides a foundation for *Agrobacterium*-mediated transformation of *F. pennsylvanica*. Studies are underway using a construct containing the Cry8Da protein of *Bacillus thuringiensis* for genetic transformation.

Materials and methods

Plant materials

Disinfection of mature green ash seeds (F.W. Schumacher Co., Inc., Sandwich, MA), isolation of embryos, regeneration of adventitious shoots, and rooting were achieved as described by Du and Pijut (2008). Freshly isolated embryos were cultured vertically in Magenta GA-7 vessels (Magenta Corp., Chicago, IL) containing 50 ml Murashige and Skoog (1962) (MS; M499, *Phyto*Technology Laboratories; Shawnee Mission, KS) basal medium, plus organics, 30 g l^{-1} sucrose, and 7 g l^{-1} Difco-Bacto agar. Hypocotyls (0.5–1.0 cm) were excised from 4- to 5-day-old in vitro-grown seedlings and cultured (16-h photoperiod; 80 μmol m^{-2} s^{-1}) horizontally on MS medium to determine explant sensitivity to the antibiotics and for transformation experiments.

Transformation vector and bacterial strain

The pq35GR vector (Fig. 1) 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. The plasmid was introduced into *A. tumefaciens* strain EHA105 and used for plant transformation (Li et al. 2004). The *Agrobacterium* culture was grown overnight in 20 ml YEP medium (10 g l^{-1} yeast extract,

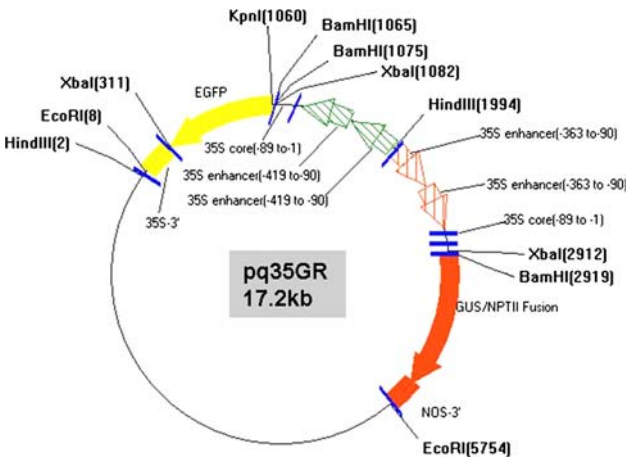

Fig. 1 The pq35GR vector consisted of the cauliflower mosaic virus (*CaMV*) 35S promoter-derived bi-directional promoters containing two divergently arranged enhancer repeats, a fusion between the *nptII* and GUS genes, and the EGFP gene (Li et al. 2004)

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 on a rotary shaker (200 rpm) at 28°C. Overnight cultures of *Agrobacterium*-pq35GR ($OD_{600} = 0.6–1.0$) were centrifuged at 3,000 rpm for 15 min, the pellet was re-suspended in 20 ml liquid MS medium containing 100 μM acetosyringone, and then placed on a shaker (100 rpm) at 28°C for 1 h prior to co-cultivation.

Effect of kanamycin and timentin on hypocotyl regeneration

To investigate the effect of selection agent type and concentration on callus induction and shoot formation, hypocotyl explants (3–5 mm in length) from 4- to 5-day-old in vitro-grown seedlings were placed on regeneration medium [MS medium supplemented with 13.2 μM 6-benzylaminopurine (BA), 4.5 μM thidiazuron (TDZ), 50 mg l^{-1} adenine hemisulfate, and 10% (v/v) coconut water (C195, *Phyto*Technology Laboratories; Shawnee Mission, KS)] with kanamycin (0, 5, 10, 15, 20, 30, 40, or 50 mg l^{-1}) or timentin (0, 100, 200, 300, 400, 500, or 600 mg l^{-1}) in Petri plates (100 × 25 mm; 45 ml medium); kanamycin and timentin were dissolved in sterile, deionized water, filter-sterilized (0.22 μm), and added to the medium after autoclaving. Three replicates with 12 hypocotyls each were used. The regeneration response was evaluated under the selection conditions after 4 weeks of culture in vitro. All media included 30 g l^{-1} sucrose and 7 g l^{-1} Difco-Bacto agar, and the pH of the medium was adjusted to 5.8 prior to autoclaving. All cultures were incubated at 24 ± 2°C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}). Callus induction and shoot formation were recorded after 6 weeks of culture.

Transformation and regeneration of transgenic adventitious shoots

Hypocotyl segments from 4- to 5-day-old seedlings were not pre-cultured or 1 day pre-cultured on regeneration medium and then vacuum-infiltrated (62.5 cm Hg) in 20 ml bacterial suspension for 10, 20, or 30 min, or 90 s sonication plus 10 min vacuum-infiltration. Explants were blotted on sterile filter paper to remove excess bacterial solution before transfer to co-cultivation media and incubated in the dark at 25°C for 2 days. Two different media were tested for co-cultivation. Co-cultivation medium I consisted of MS medium supplemented with 13.2 μM BA, 4.5 μM TDZ, 50 mg l^{-1} adenine hemisulfate, and 10% coconut water. Co-cultivation medium II consisted of MS medium supplemented with 13.2 μM BA, 4.5 μM TDZ without adenine hemisulfate and coconut water. After 2 days co-cultivation, hypocotyl segments were washed three times with sterile, distilled water (5 min each) to remove excess bacteria, blotted on sterile filter paper, and transferred to selection media (regeneration medium containing 20 mg l^{-1} kanamycin and 300 mg l^{-1} timentin). After 10 days on selection media, hypocotyl explants were histochemically stained for GUS activity. The frequency of transient GUS expression was recorded. For each treatment, 50 explants were used and each treatment was replicated twice.

Following the optimized conditions (1 day pre-culture, 2 day co-culture on MS medium supplemented with 13.2 μM BA, 4.5 μM TDZ, 50 mg l^{-1} adenine hemisulfate, 10% coconut water, and 90 s sonication plus 10 min vacuum-infiltration) for transformation and co-cultivation, hypocotyl explants were transferred to selection media and cultured under a 16-h photoperiod (80 μmol m^{-2} s^{-1}). Four weeks after selection, kanamycin-resistant callus obtained from the infected hypocotyls along with the original hypocotyls were transferred to shoot-regeneration/-elongation media, MSB5 medium [MS basal medium with Gamborg et al. (1968) B5 vitamins] supplemented with 10 μM BA, 10 μM TDZ, 20 mg l^{-1} kanamycin, and 300 mg l^{-1} timentin. Kanamycin-resistant shoots were regenerated and proliferated on elongation media. Shoots, 2–3 cm in length, were transferred to root induction medium, which was woody plant medium (WPM: L154, *Phyto*Technology Laboratories; Shawnee Mission, KS) (Lloyd and McCown 1981) plus organics supplemented with 4.9 μM indole-3-butyric acid (IBA), 5.7 μM indole-3-acetic acid (IAA), 20 mg l^{-1} kanamycin, and 300 mg l^{-1} timentin). Rooted plantlets were gently rinsed in distilled water to remove any agar from the roots and then transplanted into 10 × 9-cm plastic pots containing a moist, autoclaved peat moss:vermiculite:perlite mixture (1:1:1). Two weeks after acclimatization, plantlets were moved to the greenhouse and then transplanted into tall Treepots™ (Stuewe and Sons Inc.; Corvallis, OR) and watered daily with fertilizer water (15N-5P-15K; Miracle Gro Excel Cal-Mag; The Scotts Co., Marysville, OH).

Histochemical GUS assay

Transient GUS expression and stable GUS activity were monitored on hypocotyl explants placed on selection medium for 10 days, kanamycin-resistant callus, regenerating shoots from kanamycin-resistant callus, and roots from kanamycin-resistant plants. Tissue was incubated in a staining solution (0.1 M $NaHPO_4$ buffer (pH 7.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA, 800 mg l^{-1} X-Gluc, 0.06% (v/v) Triton X-100) following standard procedure as described by Jefferson et al. (1987). The reaction was carried out overnight at 37°C. Chlorophyll was removed from the tissues using 20% (v/v)

commercial bleach solution (5.25% sodium hypochlorite) for 10–20 min prior to visualization and photography.

Molecular analysis of transgenic plant lines

Total genomic DNA was isolated from young leaves of three GUS-positive transgenic lines and a non-transformed plant as described by LeFort and Douglas (1999). PCR was performed to amplify specific DNA sequences in transgenic plants corresponding to the *nptII* and GUS genes. A primer set (forward primer *nptII*-F 5′-TGCTCCTGCCGA GAAAGTAT-3′ and reverse primer *nptII*-R 5′-AATAT CACGGGTAGCCAAGC-3′) was designed to amplify a 364 bp PCR product for the *nptII* gene. Another primer set (forward primer GUS-F 5′-TGCTGTCGGCTTTAACC TCT-3′ and reverse primer GUS-R 5′-GGCACAGCA CATCAAAGAGA-3′) was designed to amplify a 332 bp PCR product for the GUS gene. A 25 µl PCR reaction was prepared containing 2.5 µl of 10× PCR buffer (Invitrogen), 1 µl 10 mM dNTP, 0.8 µl 50 mM Mg^{2+}, 1 µl 10 µM *nptII*-F and *nptII*-R primers or 10 µM GUS-F and GUS-R primers, 1 µl 200 ng μl^{-1} DNA template, respectively, and 0.2 µl 5 U μl^{-1} Taq polymerase. Plasmid DNA used in transformation served as a positive control, DNA from a non-transformed plant and distilled water were used as negative controls. The PCR reaction included a denaturing step of 94°C for 2 min followed by 30 cycles of 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. Amplified PCR products were visualized and photographed under UV light after electrophoresis through a 1.0% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide.

For Southern blot analysis, green ash genomic DNA (20 µg) from leaves was digested with *Xba*I, separated by electrophoresis in a 0.8% (w/v) agarose gel, and transferred onto a Hybond N+ nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Blots were hybridized with PCR-generated probes for *nptII* gene (364 bp) labeled with [α-^{32}P] dCTP Random Prime Labeling System (Rediprime II; GE Healthcare Bio-Sciences Corp.). Purified PCR product of NPTII (364 bp) (1 ng) served as a positive control, and DNA from non-transformed plant as a negative control. Pre-hybridization and hybridization were carried out at 65°C overnight in buffer solution containing 6× SSC containing 0.9 M NaCl and 0.09 M sodium citrate, 5× Dehart's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 mg ml^{-1} denatured salmon sperm DNA. The membrane was then washed at 65°C twice for 20 min in 2× SSC, 0.1% SDS, and once for 10 min in 0.5× SSC, 0.1% SDS. The membrane was exposed overnight to a Phosphor Imaging Plate (Fuji Photo Film Co.; Tokyo, Japan) and developed in a related scanner system.

Statistical analyses

Data were analyzed with an analysis of variance (ANOVA) using the GLM procedure of SAS (Version 8; SAS Institute 1999). When the ANOVA indicated statistical significance, a Tukey's comparison test was used to distinguish differences between treatments at the 5% probability level.

Results and discussion

Effect of kanamycin and timentin on ash hypocotyl regeneration

Kanamycin is widely used for selecting transformed cells and plants in plant transformation studies. It is very important to determine the appropriate concentration of kanamycin to select putative transformants. In our study, dose-response hypocotyl explants of green ash were very sensitive to kanamycin. Both callus and shoots regenerated from hypocotyls were severely affected by kanamycin. Callus formation was significantly affected (19.4%) by kanamycin at 5 mg l^{-1} and growth was completely inhibited at 10 mg l^{-1} or higher (Table 1). There were no shoots regenerated from hypocotyls when kanamycin concentration was higher than 15 mg l^{-1}. Kanamycin at 20 mg l^{-1} was effective in inhibiting callus induction and shoot formation and was then used for selecting transformed cells. Kanamycin at 20 mg l^{-1} had been previously tested to select transformed cells of green and white ash although no transgenic plants were regenerated (Bates 1997; Roome

Table 1 Effect of kanamycin concentration on percent callus formation and shoot regeneration of *Fraxinus pennsylvanica* hypocotyls

Kanamycin (mg l^{-1})	Callus formation[a] (%)	Adventitious shoot formation[a] (%)
0	100 ± 0a	61.1 ± 4.8a
5	19.4 ± 4.8b	47.2 ± 4.8b
10	0c	13.9 ± 9.6c
15	0c	2.7 ± 4.8cd
20	0c	0d
30	0c	0d
40	0c	0d
50	0c	0d

Hypocotyls were placed on MS medium supplemented with 13.2 µM BA, 4.5 µM TDZ, 50 mg l^{-1} adenine hemisulfate, and 10% coconut water with different concentrations of kanamycin. Data were collected after 6 weeks of culture

[a] Values represent means for 12 explants per treatment, replicated three times. Mean ± SE in each column followed by the same letter are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)

1992). Roome (1992) found that adventitious shoot production was repressed at a concentration of kanamycin as low as 10 mg l^{-1} with only one shoot produced, and no shoots were produced at higher concentrations. Bates (1997) found that epicotyl or hypocotyl elongation was inhibited when seedlings of white ash were cultured on medium containing 10 mg l^{-1} kanamycin with the greatest inhibition at 20 mg l^{-1} kanamycin. Callus size and formation were inhibited on medium containing 10 mg l^{-1} kanamycin. Shoot organogenesis was also inhibited at 20 mg l^{-1} kanamycin, and as the kanamycin concentration was increased, shoot organogenesis decreased.

In this study, timentin was used to inhibit *Agrobacterium* overgrowth. Results showed that timentin at 300 and 400 mg l^{-1} did not noticeably affect callus formation and subsequent shoot regeneration from hypocotyls (Table 2). Thus, timentin at 300 mg l^{-1} was used in subsequent experiments. Similar concentrations of timentin were also found effective at controlling *Agrobacterium* overgrowth in other species (Gonzalez-Padilla et al. 2003; Koroch et al. 2002; Park et al. 2005).

Transformation and regeneration

The lack of prior knowledge of successful transformation of green ash using *Agrobacterium* prompted us to examine factors that may limit transformation efficiency. We investigated the effect of pre-culture duration, inoculation duration, and co-cultivation medium on the level of GUS expression in hypocotyls after transformation.

Table 2 Effect of timentin concentration on percent callus formation and shoot regeneration of *Fraxinus pennsylvanica* hypocotyls

Timentin (mg l^{-1})	Callus formation[a] (%)	Adventitious shoot formation[a] (%)
0	100 ± 0a	42.8 ± 23.3b
50	100 ± 0a	70.6 ± 4.2ab
100	96.7 ± 5.8a	50 ± 0b
200	100 ± 0a	47.2 ± 12.7b
300	100 ± 0a	73.3 ± 2.9ab
400	100 ± 0a	88.9 ± 12.7a
500	100 ± 0a	52.8 ± 4.8ab
600	100 ± 0a	59.4 ± 15.5ab

Hypocotyls were placed on MS medium supplemented with 13.2 μM BA, 4.5 μM TDZ, 50 mg l^{-1} adenine hemisulfate, and 10% coconut water with different concentrations of timentin. Data were collected after 6 weeks of culture

[a] Values represent means for 12 explants per treatment, replicated three times. Mean ± SE in each column followed by the same letter are not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)

It has been reported that pre-culture prior to inoculation with *Agrobacterium* improved the regeneration of transformed cells in *Brassica oleraceae* spp. *capitata* (Lee et al. 2000), but decreased in *Perilla frutescens* (Kim et al. 2004). Chakrabarty et al. (2002) found that hypocotyl explants of *B. oleraceae* var. *botrytis* were hypersensitive to the bacterial culture when no pre-culture was allowed. Hypocotyl explants showed necrosis and 97.6% hypocotyl mortality was observed. Green ash hypocotyls were also hypersensitive to *Agrobacterium* strain EHA105 without any pre-culture on regeneration media. Wounded explants became swollen, browned, necrotic, and finally died when explants were directly inoculated with *Agrobacterium* without pre-culture. Accordingly, the effect of pre-culture on regeneration of transformed cells of green ash was examined using hypocotyl explants cultured on MS medium supplemented with 13.2 μM BA, 4.5 μM TDZ, 50 mg l^{-1} adenine hemisulfate, and 10% coconut water. One day pre-culture of hypocotyl explants on regeneration media decreased the number of explants which turned brown and became necrotic (data not shown). The viability of hypocotyls was also improved following *Agrobacterium* inoculation. Based on these results, 1 day of pre-culture of hypocotyls on regeneration medium was used in subsequent transformation experiments.

Two different co-cultivation media were tested in this study. Pre-cultured hypocotyl explants were transferred to either co-cultivation medium I or II after 10 min *Agrobacterium* inoculation. The bacterium could be seen around the hypocotyl explants after 1 day co-cultivation, and it became thick around the hypocotyl explants after 2 days on co-cultivation medium I. No bacterial growth was seen around hypocotyl explants or on the surface of the medium 10 days after co-cultivation on medium II. Adenine hemisulfate and coconut water additives in co-cultivation medium I favored *Agrobacterium* growth. Adenine hemisulfate and coconut water increased GUS expression efficiency from 22 to 71% for green ash hypocotyl explants in this study (Table 3). The growth-promoting effect of coconut water replaceable wholly or in part by an extract of tumors induced by *A. tumefaciens* in carrot tissue has been reported (Steward et al. 1955). Coconut water has also been used to enhance shoot formation from different types of explants, including hypocotyl, stem, and petiole of *Plumbago zeytlanica* (Wei et al. 2006).

Inoculation time is an important factor that affects transformation efficiency. It can affect the degree of attachment of *Agrobacterium* to explants and the degree of damage of explants. We tested 10, 20, or 30 min inoculation times on transient GUS expression in hypocotyl explants. Hypocotyl explants were not injured in all three inoculation durations and no significant differences on transient GUS expression were observed when hypocotyl

Table 3 Effect of different transformation factors on transient GUS expression frequency of green ash hypocotyl explants

Factors	GUS expression (%)
Inoculation time[a] (min)	
10 min vacuum-infiltration	76b
20 min vacuum-infiltration	70b
30 min vacuum-infiltration	58b
90 s sonication with	100a
10 min vacuum-infiltration	
Co-cultivation medium[b]	
Co-cultivation medium I	22b
Co-cultivation medium II	71a

[a] One-day pre-cultured hypocotyls inoculated with pq35GR: EHA105 for different time periods

[b] One-day pre-cultured hypocotyls inoculated with EHA105: pq35GR and co-cultured on different co-cultivation medium for 2 days in dark. Co-cultivation medium I: MS medium supplemented with 13.2 μM BA, 4.5 μM TDZ without adenine hemisulfate and coconut water. Co-cultivation medium II: MS medium supplemented with 13.2 μM BA, 4.5 μM TDZ, 50 mg l^{-1} adenine hemisulfate, and 10% coconut water

explants were inoculated with bacterial suspension for 10–30 min (Table 3). We also compared a 90-s sonication plus 10-min vacuum-infiltration of hypocotyls to 10 min bacterial soaking of hypocotyls for GUS expression. The results indicated that sonication plus vacuum-infiltration greatly increased the level of GUS expression from 76 to 100% (Table 3). Liu et al. (2005) also found that sonication combined with vacuum-infiltration produced an efficient *Agrobacterium*-mediated transformation system.

Blue foci or sectors of GUS staining were found at the cut ends of hypocotyl explants of injured tissue and also on the area of hypocotyl where the surface layer was removed (Fig. 2a, b). This suggested that wounded hypocotyl tissue lacking the superficial layer of tissue was a target site of *Agrobacterium*, and removal of the surface layer can increase the number of transformed cells. Our findings were very similar to reports by Murray et al. (1977) and Dong and McHughen (1993) in flax. They found transformed cells not only on the cut ends of hypocotyls, but also on the area of hypocotyl explants where most of the epidermis was removed.

Wounding of explant materials may allow *Agrobacterium* to better access plant cells. Wounding treatment also stimulates the production of *vir* gene inducers, such as phenolic substances, and enhances the plant cell competence for transformation (Stachel et al. 1985). Shin and Park (2006) treated 3-day-old seedlings of *Brassica juncea* with hydrogen peroxide or sodium hydrosulfite solution as a chemical abrasive, prior to *Agrobacterium*-infiltration. Such treatments greatly enhanced transformation efficiency compared to transformation via *Agrobacterium*-infiltration without abrasive treatment. Besides chemical abrasives, mechanical treatments were used to break cell walls and enhance transformation efficiency. Abrasive celites were used to damage the cell wall to increase the number of potential binding sites of *A. tumefaciens* in Roome's study (1992), and superficial wounding of explants was a crucial part for completing their transformation system. Needles were also used to injure plant material to enhance transformation efficiency (Rohini et al. 2005; Supartana et al. 2006). It has been recently reported that sonication-assisted *Agrobacterium* transformation (SAAT) can be used to promote transformation efficiency in several different plant species (Pathak and Hamzah 2008; Wang et al. 2006; Yookongkaew et al. 2007). Micro-wounding both on the upper and lower surfaces of plant tissue produced by SAAT facilitate the accessibility of plant cells to *Agrobacterium*, leading to enhanced transformation efficiency (Trick and Finer 1997).

Optimized transformation procedures (consisting of 1 day pre-culture, 90-s sonication plus 10-min vacuum-infiltration, and co-cultivation for 2 days in the dark on co-cultivation medium I) were used to stably transform green ash. Four weeks after infected explants were transferred to selection medium, 98 explants out of 544 (18%) produced callus from the cut ends of hypocotyls and also from the area of the hypocotyl with the surface layer removed. Histochemical analysis confirmed the presence of GUS activity in the callus tissues (Fig. 2c). Adventitious shoots appeared on the kanamycin-resistant callus after the hypocotyls were transferred to shoot-elongation media (MSB5 medium containing 10 μM BA and 10 μM TDZ) supplemented with 20 mg l^{-1} kanamycin and 300 mg l^{-1} timentin. Twenty-four out of 544 (4.4%) explants produced shoots directly from the surface layer of the hypocotyl. Shoots regenerated directly from the cell surface layer around the cut ends and injured cells on the surface of hypocotyls under selection medium did not show blue color following GUS staining, and these were considered escapes. It is thought that transformed cells on inoculated explants after co-cultivation with *Agrobacterium* could protect non-transformed cells from the selection agents and cause the production of escapes (Dong and McHughen 1993). However, adventitious shoots obtained from intermediate kanamycin-resistant callus were all confirmed to be transgenic shoots. Kanamycin-resistant callus along with hypocotyl explants were transferred to shoot-elongation media 4 weeks after culture on selection media. Three shoots regenerated out of 98 (3.1%) kanamycin-resistant callus 4 weeks after culture on elongation media. Microshoots were then propagated in vitro for the three kanamycin-resistant lines

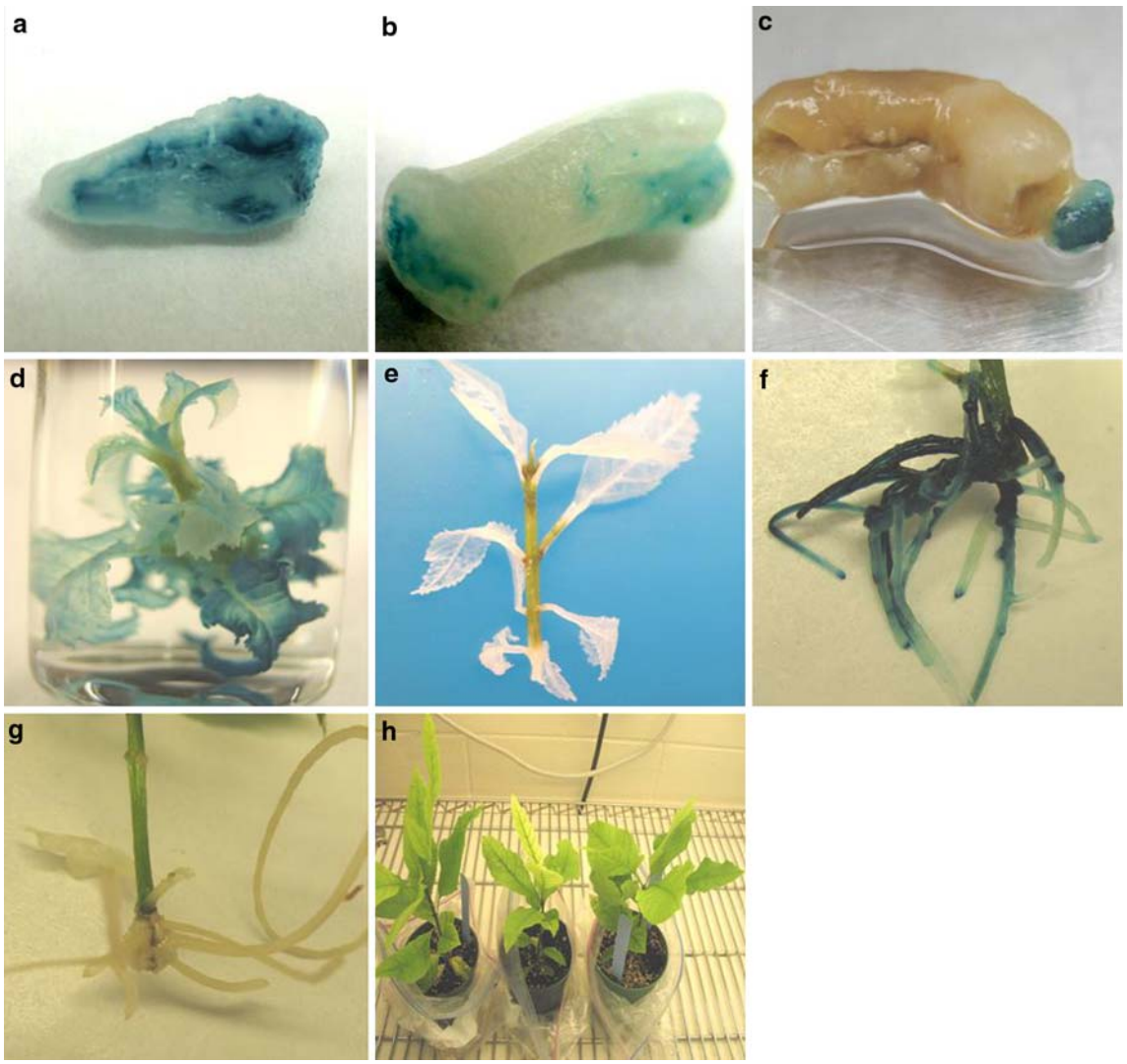

Fig. 2 Transient GUS expression and regeneration of transgenic *Fraxinus pennsylvanica* plants and GUS assay of transgenic plants. **a** Transient GUS expression on area of hypocotyl where the surface layer was removed, **b** transient GUS expression on ends of hypocotyl, **c** GUS staining of callus formed from hypocotyl, **d** and **e** GUS expression of elongated, kanamycin-resistant shoots and non-transformed shoot, respectively, **f** and **g** GUS analysis of rooted transgenic plant and non-transformed plant, respectively, and **h** normal growth of acclimatized transgenic plants in a culture room

before being transferred to rooting media (Du and Pijut 2008). Staining of shoots and roots from these kanamycin-resistant lines showed strong expression of the GUS gene, which was absent in non-transformed shoots and roots (Fig. 2d–h).

Molecular analysis of transgenic plant lines

Genomic DNA was extracted from three independently regenerated transgenic green ash lines and a non-transformed plant line and used in PCR amplification to verify transgene insertion. A 364 bp fragment corresponding to the *nptII* gene target region was found in all three transgenic lines (Fig. 3, lanes 4–6) and plasmid control (Fig. 3, lane 1), but not in the non-transformed plant (Fig. 3, lane 3). The 332 bp fragments corresponding to the GUS gene were present in all three transgenic lines (Fig. 3, lanes 10–12) and plasmid control (Fig. 3, lane 7), but was absent in the non-transformed plant (Fig. 3, lane 9).

For Southern analysis, genomic DNA from three transgenic lines and a non-transformed plant line was digested with restriction endonuclease *Xba*I. This enzyme recognizes three sites within the T-DNA region of pq35GR. The membrane was hybridized with a 364 bp fragment corresponding to the *nptII* gene to analyze transgene integration. No hybridization was detected in the non-transformed control plant (Fig. 4, lane 1). One hybridization band

Fig. 3 PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic *Fraxinus pennsylvanica* plants using primer sets specific for amplification of 364-bp *nptII* gene and 332-bp GUS gene. *M* 100 bp molecular marker, *lanes 1–6* PCR amplification for *npt*II gene, *lanes 7–12* PCR amplification for GUS gene. *Lane 1* positive control, pq35GR, *lane 2* water control, *lane 3* negative control non-transformed plant, *lanes 4–6* putative transgenic lines, *lane 7* positive control, pq35GR, *lane 8* water control, *lane 9* negative control, non-transformed plant, *lanes 10–12* putative transgenic plants

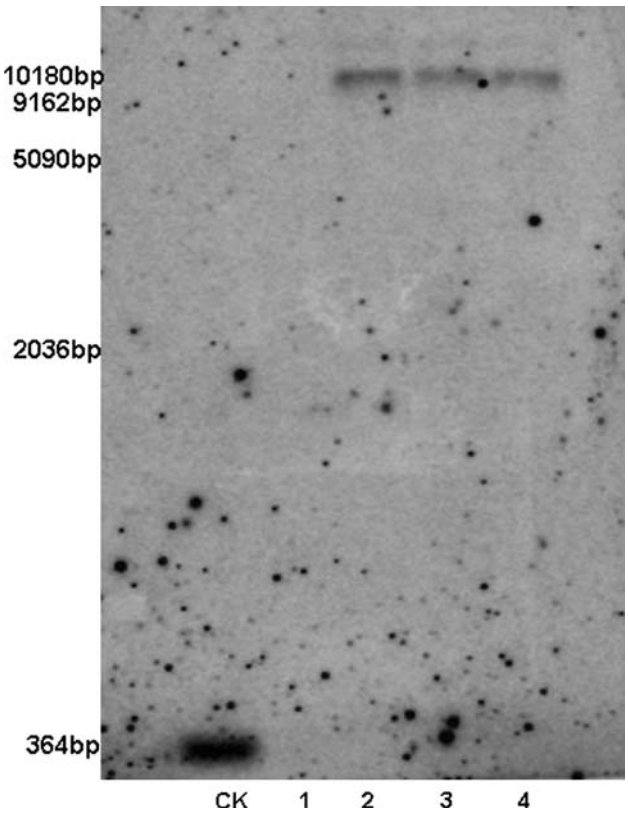

Fig. 4 Southern blot analysis of transgenic lines of green ash for *nptII* gene. DNA samples (20 μg) were digested with *Xba*I. *CK* positive control of *nptII*, *lane 1* negative control non-transformed plant, *lanes 2–4* putative transgenic plants

was detected in each of the transgenic lines (Fig. 4, lanes 2–4) confirming that the regenerated lines were stably transformed.

Conclusions

A successful *A. tumefaciens*-mediated transformation and regeneration protocol for hypocotyl explants of green ash was developed. The transformation protocol was (1) 3- to 5-mm long hypocotyl explants from 4- to 5-day-old in vitro seedlings were pre-cultured for 1 day on regeneration medium; (2) one-day pre-cultured hypocotyl explants were wounded by 90 s sonication and then inoculated with *Agrobacterium* strain EHA105 harboring pq35GR for 10 min under vacuum-infiltration; (3) co-cultivation was carried out for 2 days in the dark on co-cultivation medium I; (4) selection and regeneration of transformed cells was conducted on regeneration media containing 20 mg l^{-1} kanamycin and 300 mg l^{-1} timentin; (5) proliferation of regenerants was carried out on elongation medium containing 20 mg l^{-1} kanamycin and 300 mg l^{-1} timentin; and (6) rooting of regenerants was carried out on rooting media containing 20 mg l^{-1} kanamycin and 300 mg l^{-1} timentin, and plantlets transplanted into soil. Following this protocol, stably transformed plants of green ash were obtained after 12 weeks from culture initiation. This transformation and regeneration protocol provides a foundation for *F. pennsylvanica* improvement via genetic transformation, and will facilitate production of EAB-resistant ash. Studies are underway using a construct containing a Cry8Da protein of *B. thuringiensis* for genetic transformation of green ash.

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