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ORIGINAL PAPER

Agrobacterium-mediated genetic transformation of *Fraxinus americana* hypocotyls

Kaitlin J. Palla · Paula M. Pijut

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Abstract An *Agrobacterium tumefaciens*-mediated genetic transformation system was successfully developed for white ash (*Fraxinus americana*) using hypocotyls as the initial explants. Hypocotyls isolated from mature embryos germinated on Murashige and Skoog (MS) medium supplemented with 22.2 μM 6-benzyladenine (BA) and 0.5 μM thidiazuron (TDZ) were transformed using *A. tumefaciens* strain EHA105 harboring the binary vector pq35GR containing a fusion gene between neomycin phosphotransferase (*nptII)* and *gusA*, as well as an enhanced green fluorescent protein (EGFP). Explants were transformed in a bacterial suspension with 100 μM acetosyringone using 90 s sonication and 10 min vacuum infiltration. Putative transformed shoots representing seven independent lines were selectively regenerated on MS medium with 22.2 μM BA, 0.5 μM TDZ, 50 mg L^{-1} adenine sulfate, 10 % coconut water, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin. Timentin at 500 mg L^{-1} was optimal for controlling excess bacterial growth, and transformed shoots were selected using 30 mg L^{-1} kanamycin. The presence of GUS (β-glucuronidase), *nptII*, and EGFP in transformed plants was confirmed by polymerase chain reaction (PCR). Reverse transcription-PCR and fluorescence microscopy confirmed the expression of EGFP. Transgenic microshoots were rooted (80 %) on woody plant medium supplemented with 4.9 μM indole-3-butyric acid, 2.9 μM indole-3-acetic acid, and 500 mg L^{-1} timentin, and subsequently acclimatized to the culture room. This transformation protocol provides the framework for future genetic modification of white ash to produce plant material resistant to the emerald ash borer.

Keywords *Fraxinus* · Genetic transformation · Organogenesis · Regeneration · White ash

K. J. Palla
Department of Forestry and Natural Resources, Hardwood Tree Improvement and Regeneration Center (HTIRC), Purdue University, 715 West State St., West Lafayette, IN 47907, USA

Present Address:
K. J. Palla
Oak Ridge National Laboratory, MS-6407,
P.O. Box 2008, Oak Ridge, TN 37831, USA

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

Introduction

White ash (*Fraxinus americana* L.) is the most common and economically valuable of the 16 ash species native to North America. The wood of white ash is heavy, strong, hard, and stiff, and it has high resistance to shock (Forest Products Laboratory 2010). The wood is valued for the production of tool handles, furniture, flooring, boats, and baseball bats. Considered one of the best soil-improving ash species because of its calcium-rich leaves and earthworm palatability, white ash is also utilized as shelter and sustenance by a variety of wildlife. The vibrant fall color, relatively fast growth, and tolerance to a wide range of soil pH make it a favorite of the horticulture industry (Johnston 1939; Schlesinger 1990; Nesom 2001; Wallander 2008). In 2005, ash trees composed 5–29 % of all street trees in the Midwest, replacing many of the elm trees in urban settings since the advent of Dutch elm disease (MacFarlane and Meyer 2005).

North American ash resources are currently being threatened with extinction by the emerald ash borer (EAB),

Agrilus planipennis Fairmaire (Coleoptera: Buprestidae) (Herms and McCullough 2014). An aggressive wood-boring exotic, EAB has spread rapidly throughout at least 21 U.S. states and parts of Canada since being discovered in southeastern Michigan in 2002 (Yu 1992; Emerald Ash Borer Information Website 2014). Adult beetles feed on tree foliage and bore holes into the bark, but the most damage is a result of the larvae feeding on the phloem tissue, forming galleries under the bark, and disrupting the flow of nutrients (McCullough et al. 2008). EAB is fatal to ash trees, and there is neither any known innate resistance in native trees nor any means to completely eradicate the beetle. Over 99 % tree mortality in a stand was probable within 4 years of the first observed symptoms of infestation (Knight et al. 2012).

Biological control and eradication efforts have made limited impact on EAB populations or infestation rates, and the beetle's range is still rapidly expanding (Dobesberger 2002; Poland and McCullough 2006; Duan et al. 2012). Chemical controls were deemed expensive and inappropriate for protecting ash trees in forested or riparian areas, as well as environmentally undesirable (Poland and McCullough 2006; Bauer and Londoño 2011; Hahn et al. 2011). The predicted cost of treating, removing, and replacing urban forest trees affected by EAB was expected to exceed $10 billion by 2019 (Kovacs et al. 2010). Genetically engineering ash trees to express resistance to the EAB, such as the *Bacillus thuringiensis Cry* genes, is a desirable alternative for obtaining a cost-efficient, effective, and environmentally friendly means of controlling EAB damage in the North American landscape. To date, genetic transformation has been attempted for several *Fraxinus* spp., however transgenic shoots have only been reported for *F. pennsylvanica* and *F. profunda* (Roome 1992; Bates 1997; Du and Pijut 2009; Stevens and Pijut 2014). Bates (1997) obtained multiple shoots after transforming white ash with *Agrobacterium*, but no shoots were confirmed transgenic. Our study is the first report of successfully regenerating transgenic white ash, and provides a foundation for integrating EAB resistance genes into this species.

Materials and methods

Plant materials

Mature white ash seeds purchased in 2011 from Sheffield's Seed Co., Inc. (Locke, NY) were stored in a sealed container in the dark at 5 °C until used. Disinfestation, isolation of embryos, and adventitious shoot regeneration were achieved using our previously described protocol (Palla and Pijut 2011). Freshly isolated aseptic embryos were cultured vertically in Magenta GA-7 vessels (Magenta Corp., Chicago, IL; 25 embryos per vessel) containing 50 mL of pre-culture Murashige and Skoog (1962) (MS) medium (M499; *Phyto*Technology Laboratories, Shawnee Mission, KS) with organics (100 mg L^{-1} myoinositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 0.1 mg L^{-1} thiamine HCl, and 2 mg L^{-1} glycine), supplemented with 22.2 μM 6-benzyladenine (BA), and 0.5 μM thidiazuron (TDZ) for 5- to 15-d. Hypocotyls of in vitro germinated seedlings were then isolated and cultured horizontally on culture medium in order to determine explant sensitivity to kanamycin and timentin for transformation and selection procedures. All plant materials were cultured on medium containing 30 g L^{-1} sucrose and 7 g L^{-1} Bacto agar (No. 214030; Becton–Dickinson, USA), with the pH adjusted to 5.7 prior to autoclaving, and all plant cultures were incubated at 24 ± 2 °C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}), unless noted otherwise.

Effect of kanamycin and timentin on hypocotyl regeneration

To determine the optimum concentration for selection of transformed tissue and controlling excess *Agrobacterium* growth (Cheng et al. 1998), hypocotyl explants from 5-d-old in vitro grown seedlings were cultured horizontally on co-culture MS medium with organics, 22.2 μM BA, 0.5 μM TDZ, 50 mg L^{-1} adenine hemisulfate (AS), 10 % (v/v) coconut water (CW) (C195; *Phyto*Technology Laboratories, Shawnee Mission, KS) supplemented with 0, 5, 10, 15, 20, 30, 40, or 50 mg L^{-1} kanamycin or 0, 100, 200, 300, 400, 500, or 600 mg L^{-1} timentin in Petri plates (100 × 25 mm; 50 mL medium). Antibiotics were each dissolved in sterile deionized water and filter-sterilized (0.22 μm) prior to being added to cooled autoclaved medium. Three replicates with 12 hypocotyls each were cultured for each treatment to determine the optimum concentration of antibiotics to be used in transformation and selection procedures. Regeneration response of callus and shoot formation frequency were evaluated and recorded after 6 weeks on initial induction medium.

Transformation vector and culture

The pq35GR vector (Fig. 1) was comprised of two independently arranged bi-directional enhancer repeats driven by the cauliflower mosaic virus 35S promoter (CaMV 35S). The marker genes incorporated into the vector were a fusion of neomycin phosphotransferase (*nptII*) and *gusA* (*β*-glucuronidase; GUS), and enhanced green fluorescent protein (*EGFP*) (Li et al. 2004). Introduced into *Agrobacterium tumefaciens* strain EHA105, this vector was also used for plant transformation studies with green and

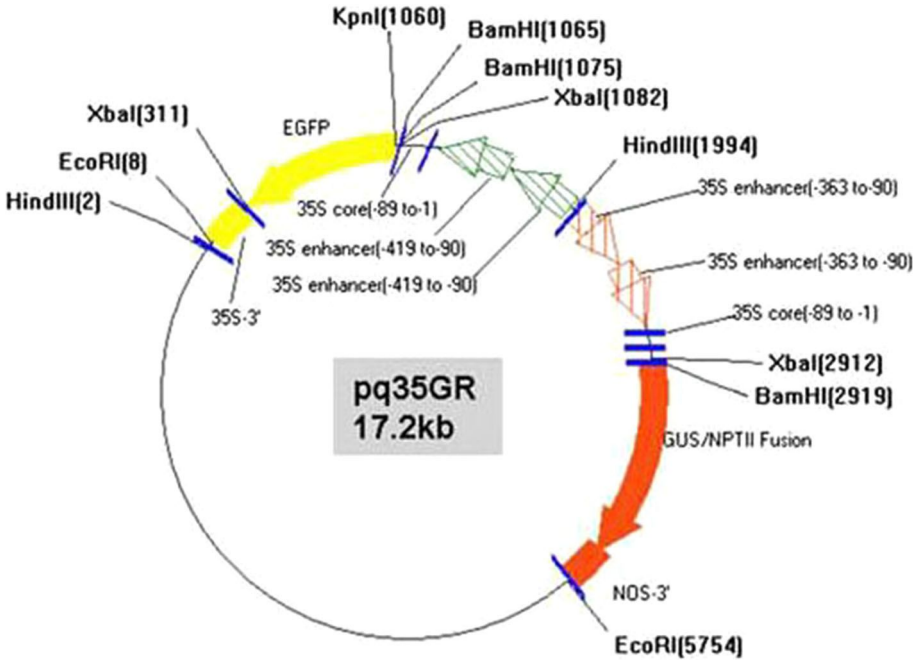

Fig. 1 pq35GR vector composed of two independent CaMV35S-driven enhancer repeats corresponding to an *nptII* and *gusA* fusion gene, and an *EGFP* gene (Li et al. 2004)

pumpkin ash (Du and Pijut 2009; Stevens and Pijut 2014). Single *Agrobacterium* colonies were cultured in the dark for 2 days on a rotary shaker (150 rpm) at 28 °C in 20 mL liquid YEP medium (10 g L^{-1} yeast extract, 10 g L^{-1} bacto-peptone, 5 g L^{-1} NaCl, at pH 7.0) supplemented with 20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin. The *Agrobacterium*-pq35GR suspension (OD_{600} = 0.4–0.6) was centrifuged at 3,000 rpm for 15 min, and the pellet was re-suspended in 20 mL liquid MS co-culture medium with organics, containing 22.2 μM BA, 0.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, with the addition of 100 μM acetosyringone. The bacterial suspension was placed on the rotary shaker (150 rpm) for 1 h prior to co-cultivation with hypocotyls.

Agrobacterium transformation and transgenic shoot regeneration

Hypocotyls excised from 5- to 15-d-old germinated embryos were placed in 20 mL liquid medium [MS medium with organics, 22.2 μM BA, 0.5 μM TDZ, 50 mg L^{-1} AS, and 10 % CW] and sonicated for 90 s before being transferred to the liquid *Agrobacterium*-pq35GR suspension and vacuum infiltrated (62.5 cm Hg) for 10 min. Explants were then blotted dry on sterile filter paper and cultured on semi-solid MS co-culture medium in Petri plates (100 × 15 mm; 30 mL; 50 explants per plate) and cultured in the dark for 2–3 days at 27 °C. Hypocotyls were then rinsed three times with liquid MS co-culture medium to remove excess *Agrobacterium*, blotted dry, and cultured horizontally on selection medium [MS medium with organics, supplemented with 22.2 μM BA, 0.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin; 15–20 explants per plate] for up to 8 weeks. Explants regenerating shoot primordia were transferred to selection-elongation medium [MS medium with Gamborg et al. (1968) B5 vitamins plus 2 mg L^{-1} glycine (MSB5G) supplemented with 10 μM BA, 10 μM TDZ, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin]. Elongating, kanamycin-resistant shoots were evaluated for the presence of marker genes after six or more subcultures (4 week interval) on selection-elongation medium. Shoots confirmed to contain transgenes were moved to multiplication medium [MSB5G medium supplemented with 10 μM BA, 10 μM TDZ, and 500 mg L^{-1} timentin] to increase shoot proliferation rates.

Effect of cytokinin concentration on shoot elongation

To maximize the number of microshoots elongating from transformed hypocotyls, a factorial experiment was performed to determine the optimal concentrations of BA and TDZ. Explants initiating shoot buds on selection medium (4–8 weeks) were transferred to MSB5G medium supplemented with 30 mg L^{-1} kanamycin, 500 mg L^{-1} timentin, 0, 10, 15, or 20 μM BA in combination with 0, 2.5, 5, or 10 μM TDZ to induce shoot elongation. Magenta GA-7 vessels containing 50 ml of medium were used, with one explant per vessel; eight explants per treatment were tested. Cultures were incubated for 6 weeks under a 16 h photoperiod, and the number of shoots elongated was recorded.

Rooting of transgenic microshoots

Elongated transgenic shoots (3–5 cm in length; with at least two nodes) were pooled and randomly cultured on woody plant medium (Lloyd and McCown 1981) [WPM; L154; *Phyto*Technology, Shawnee Mission, KS with organics (100 mg L^{-1} myoinositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 1 mg L^{-1} thiamine HCl, and 2 mg L^{-1} glycine] supplemented with 500 mg L^{-1} timentin, 4.9 μM indole-3-butyric acid (IBA) plus 0, 2.9, 5.7, or 8.6 μM indole-3-acetic acid (IAA). Magenta GA-7 vessels containing 50 mL of medium were used, with one explant per vessel. Cultures were placed in the dark at 26 °C for 5 days, then cultured up to 12 weeks in the light. An overlay of 5 mL liquid rooting medium was added to cultures each week after 6 weeks in light culture. Rooting percentage, the number of roots per shoot, and root length were recorded once roots were determined comparable to previous experiments (Palla and Pijut 2011). Three replications with five shoots per treatment per replication were conducted. Rooted microshoots were acclimatized to ambient culture room conditions as described previously (Palla and Pijut 2011).

Histochemical GUS assay

Transformed plant tissue (callus, hypocotyl, and leaf primordia) regenerating on selection medium containing kanamycin was incubated overnight at 37 °C 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 the procedures described by Jefferson et al. (1987). Chlorophyll was removed from the tissue after staining using 20 % (v/v) commercial bleach solution (5.25 % sodium hypochlorite) for 15–20 min to allow for visualization and documentation of GUS expression.

Visualization of enhanced green fluorescent protein

The presence of GFP was detected using a fluorescence stereomicroscope (Leica MZFLIII) equipped with a 470/40 nm excitation filter (GFP-Plant) and a 525/50 nm barrier filter, and lit with an HBO 100 W mercury bulb. Leaves from transgenic plants confirmed to contain the *nptII*, GUS, and EGFP genes were examined along with control (non-transgenic) leaves, and the presence or absence of green fluorescence was compared. No interference filter was used to block chlorophyll autofluorescence, and the Spot™ imaging software was used.

Molecular analysis of transgenic plant lines

Genomic DNA was isolated from leaves of seven independent putative transgenic lines and from one control (non-transformed) plant following either the protocol of the Qiagen DNeasy Plant Mini Kit (Qiagen, USA) or the procedures described by Lefort and Douglas (1999). Polymerase chain reaction (PCR) was designed to specifically amplify *nptII*, GUS, and EGFP. Primers (forward *nptII*-F 5′-TGCTCCTGCCGAGAAAGTAT-3′ and reverse *nptII*-R 5′-AATATCACGGGTAGCCAAGC-3′) were designed to amplify a 364 bp PCR fragment for the *nptII* gene. Primers (forward GUS-F 5′-TGCTGTCGGCTTTA ACCTCT-3′ and reverse GUS-R 5′-GGCACAGCACATC AAAGAGA-3′) were designed to amplify a 332 bp PCR fragment for GUS. Primers (forward EGFP-51 5′-ATGGT GAGCAAGGGCGAGGAGCTGT-3′ and reverse EGFP-32 5′-TTACTTGTACAGCTCGTCCATGCCG-3′) were designed to amplify a 720 bp PCR fragment for the EGFP.

The PCR reaction (25 μL) consisted of 2.5 μL 10× PCR buffer (Invitrogen), 1 μL 50 mM Mg^{2+}, 1 μL 50 mM dNTP, 1 μL each of 10 μM *nptII*-F and *nptII*-R or 10 μM GUS-F and GUS-R or 10 μM EGFP-51 and EGFP-32 primers, 2 μL DNA template (100 ng μL^{-1}), 0.25 μL 5 U μL^{-1} Taq polymerase, and 16.25 μL sterile-deionized water. Plasmid DNA was used as a positive control; DNA from a non-transformed plant and sterile-deionized water served as negative controls. The *nptII* PCR reaction included 2 min at 94 °C, followed by 35 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 2 min, with a final 10 min cycle at 72 °C. The GUS PCR reaction varied in the number of cycles run (45) and the annealing temperature used (55 °C), but was otherwise identical. The EGFP PCR reaction included 4 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min, with a final 7 min cycle at 72 °C. Amplified products of the PCR were visualized under UV light after gel electrophoresis (1 % w/v agarose plus 0.5 μg mL^{-1} ethidium bromide).

RNA isolation and reverse transcription-PCR analysis

Reverse transcription-PCR (RT-PCR) was performed on transgenic plant lines to confirm transgene expression. RNA was isolated from leaves (100 mg) of microshoots confirmed by PCR to contain transgenes, and one control (non-transformed) plant following the protocol of the Qiagen RNeasy Plant Mini Kit (Qiagen, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove genomic DNA. DNase-treated RNA (5 μg) was used as a template for reverse transcription to synthesize complementary DNA (cDNA) with AccuScript High Fidelity 1st Strand cDNA Synthesis kit (Agilent Technologies, USA)

according to the manufacturer's instructions. PCR was performed using cDNA created with Oligo (dT) primers to amplify sequences specific to the EGFP gene. PCR was also performed to amplify sequences specific to a housekeeping 26S ribosomal RNA (rRNA) gene using cDNA created with random primers. PCR to amplify the 720 bp product for the EGFP gene was carried out using the respective primers and thermal cycle as described previously. Primers (forward 26S-F 5′-GTCCTAAGATGAGC TCAA-3′ and reverse 26S-R 5′-GGTAACTTTTCTGA CACCTC-3′) were designed to amplify a 160 bp PCR product for the 26S rRNA gene. The PCR reaction (25 µL) consisted of 2.5 µL 10× PCR buffer (Invitrogen), 1 µL 50 mM Mg^{2+}, 1 µL 50 mM dNTP, 1 µL each of 10 µM 26S-F and 26S-R or 10 µM EGFP-51 and EGFP-32 primers, 3 µL cDNA template (5 ng μL^{-1}), 0.25 µL 5 U μL^{-1} Taq polymerase, and 15.25 µL sterile–deionized water. Plasmid DNA was used as a positive control, cDNA from a non-transformed plant and sterile–deionized water served as negative controls, and DNase-treated RNA that had not been reverse transcribed served as template control to monitor DNA contamination. The 26S PCR reaction included 4 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, with a final 7 min cycle at 72 °C. Amplified products of the PCR were visualized under UV light after gel electrophoresis (1 % agarose (w/v) plus 0.5 µg mL^{-1} ethidium bromide).Statistical analysis SPSS (Software Version 20) (SPSS 2011) was used to analyze data. An analysis of variance (ANOVA) was performed using the General Linear Model procedure on the individual replicate means by treatment for percent shoot formation, number of shoots per hypocotyl, percent root formation, number of roots per shoot, root length, and number of lateral roots. When the ANOVA indicated statistical significance, a Duncan's comparison test with an alpha level of 0.05 was used to distinguish the differences between treatments.

Results and discussion

Effect of kanamycin and timentin on ash hypocotyl regeneration

Hypocotyls were exposed to various concentrations of kanamycin and timentin in order to determine the effect that the antibiotics had on regeneration of white ash adventitious shoots. Kanamycin is commonly used in the selection of transformed cells, inhibiting the growth of cells that do not have the *nptII* gene integrated, and concentration is critical for this selection. White ash callus and shoot regeneration were significantly inhibited when kanamycin concentrations reached 30 mg L^{-1} (Table 1). No shoot primordia were able to regenerate at that concentration and callus production was severely limited, with the majority of hypocotyls turning chlorotic and necrotic after 6 weeks of culture. Thus, we determined 30 mg L^{-1} kanamycin to be the optimal concentration to use for subsequent transformation and selection, as it provided a high selection pressure to ensure regenerating shoots would be stable transformants instead of potential escapes. This agrees with the previous study on white ash, where Bates (1997) noted organogenic inhibition in seedlings cultured on kanamycin concentrations ≥20 mg L^{-1}. Other ash studies reported kanamycin inhibited organogenesis at concentrations of 20 mg L^{-1} (Du and Pijut 2009; Stevens and Pijut 2014). Roome (1992) observed that kanamycin as low as 10 mg L^{-1} limited green ash shoot production, and was unable to regenerate any shoots at higher concentrations. Timentin was used in this study to inhibit growth of *Agrobacterium* after transformation. White ash hypocotyls were not significantly inhibited by timentin, and the highest shoot primordia regeneration (80.6 %) occurred on medium containing 500 mg L^{-1} (Table 2). Similar high concentrations (300–400 mg L^{-1}) were noted to have little effect on hypocotyl regeneration for other ash and hardwood species (Gonzalez Padilla et al. 2003; Andrade et al. 2009; Du and Pijut 2009; Stevens and Pijut 2014).

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

Kanamycin (mg L^{-1})	Callus formation[a] (%)	Shoot formation[a,b] (%)
0	100.0 ± 0.0a	83.3 ± 9.6a
5	66.7 ± 8.3b	50.0 ± 4.8b
10	47.2 ± 10.0bc	27.8 ± 7.3c
15	52.8 ± 12.1bc	25.0 ± 9.6c
20	41.7 ± 8.3c	11.1 ± 4.8 cd
30	19.4 ± 4.8d	0.0 ± 0.0d
40	0.0 ± 0.0d	0.0 ± 0.0d
50	0.0 ± 0.0d	0.0 ± 0.0d

Hypocotyls were placed on Murashige and Skoog medium supplemented with 22.2 µM 6-benzyladenine, 0.5 µM thidiazuron, 50 mg L^{-1} adenine hemisulfate, and 10 % coconut water plus different concentrations of kanamycin. Data taken after 6 weeks of culture

[a] Mean ± standard error for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$)

[b] Mean ± standard error for hypocotyls that produced leaf primordia

Transformation and regeneration of plants

Germination of mature white ash seedlings was previously noted to be variable, with a delay of 11- to 21-days before being sorted to use for transformation (Bates 1997). By

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

Timentin (mg L^{-1})	Callus formation[a] (%)	Shoot formation[a,b] (%)
0	100.0 ± 0.0a	91.7 ± 4.8a
50	100.0 ± 0.0a	50.0 ± 12.7c
100	100.0 ± 0.0a	72.2 ± 7.4abc
200	100.0 ± 0.0a	75.0 ± 4.8abc
300	100.0 ± 0.0a	75.0 ± 4.8abc
400	100.0 ± 0.0a	61.1 ± 10.0bc
500	100.0 ± 0.0a	80.6 ± 10.0ab
600	100.0 ± 0.0a	71.5 ± 3.4abc

Hypocotyls were placed on Murashige and Skoog medium supplemented with 22.2 μM 6-benzyladenine, 0.5 μM thidiazuron, 50 mg L^{-1} adenine hemisulfate, and 10 % coconut water plus different concentrations of timentin. Data taken after 6 weeks of culture

[a] Mean ± standard error for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$)

[b] Mean ± standard error for hypocotyls that produced leaf primordia

culturing aseptically extracted embryos on pre-culture medium, not only were embryos ready for transformation in 5 days, but germination was almost entirely uniform. Embryos could be grown on this medium for up to 15 days to allow for further hypocotyl growth. This extended pre-culture may help the tissue better survive the transformation process (data not shown). Green ash hypocotyls were seen to be hypersensitive to *Agrobacterium*, but a brief pre-culture period prior to inoculation attenuated the response (Du and Pijut 2009). The medium used to germinate the embryos in this study appeared to serve the same purpose as a pre-culture period, allowing hypocotyls to be freshly isolated and used for transformation. Combining embryo germination with a pre-culture step was shown to be beneficial for plum (Gonzalez Padilla et al. 2003) and pumpkin ash (Stevens and Pijut 2014).

Since *Agrobacterium* was shown to be attracted to the compounds released by wounded plants (Satchel et al. 1985), the use of freshly isolated hypocotyls increased the number of sites where inoculation could potentially occur. Roome (1992) used abrasive celites to wound explants in one transformation study, while freshly cut hypocotyls proved effective for Gonzalez Padilla et al. (2003). Sonication was used in this study to further increase the number of superficial wound sites where gene transfer could take place, and was combined with vacuum-infiltration to force the *Agrobacterium* cells into further contact with the white ash hypocotyl cells. Sonication in conjunction with vacuum-infiltration has proven valuable for the transformation of many species, particularly when thicker explant tissue such as hypocotyls have been used (Andrade et al. 2009; Du and Pijut 2009; Subramanyam et al. 2011; Stevens and Pijut 2014).

White ash hypocotyls were co-cultivated for 2- to 3-days after being exposed to an *Agrobacterium* solution with an $OD_{600} = 0.4–0.6$ to allow for gene transfer and integration. Higher *Agrobacterium* concentrations and longer co-culture periods resulted in an excess of bacterial growth during the selection process, regardless of timentin concentration, resulting in necrosis of explant tissue (data not shown). Similar bacterial concentrations (Sun et al. 2011) and co-culture times (Du and Pijut 2009; Stevens and Pijut 2014) have been used with success. Six weeks after culture on selection medium, 105 explants out of 537 were producing callus or shoot primordia, and were moved to selection and elongation medium. Seven independent transgenic lines were recovered after 8 weeks (1.3 % transformation efficiency). Adventitious shoots that elongated in the presence of kanamycin were continuously cultured every 3 or 4 weeks on selection–elongation medium for six or more subcultures. Shoot elongation occurred at a slow rate while on medium containing kanamycin, particularly for three out of the seven independent lines, although shoots were green and normal in appearance (Fig. 2a). The optimal concentration of BA and TDZ for shoot elongation was examined and determined to be 10 μM each (37.5 %; 1.3 ± 0.6 shoots per explant; Table 3). Liquid overlays of elongation medium have been shown to increase the growth rate of transformed shoots (Stevens and Pijut 2014), but failed to promote white ash shoot growth, instead causing excess callus formation at the base of microshoots. Once microshoots had grown large enough to remove leaf material without damaging growth, PCR was performed to confirm the presence of transgenes, and then all transgenic shoots were cultured on elongation medium without kanamycin (Fig. 2b). Shoot elongation recovered to a normal rate after two to three subcultures and were propagated in vitro for use in rooting trials (Palla and Pijut 2011).

Rooting (80 %) of the seven transgenic white ash lines recovered in this study occurred on WPM supplemented with 500 mg L^{-1} timentin, 4.9 μM IBA, and 2.9 μM IAA (Table 4). These results follow what was observed in previous regeneration work (Palla and Pijut 2011). Kanamycin has been known to negatively affect rooting (Gonzalez Padilla et al. 2003), and was observed to limit shoot callusing, root initiation, and root elongation in initial rooting tests with white ash transformants obtained in our lab (data not shown). All shoots used in the current rooting trial were derived from lines PCR-confirmed to contain transgenes, therefore kanamycin was omitted from the rooting medium to enhance root initiation and elongation. As with shoot elongation, root formation and development was slow for the transformed shoots. Liquid overlays of medium were observed to enhance rooting in green ash (Kim et al. 1998). A liquid overlay of rooting medium (5 mL) every week

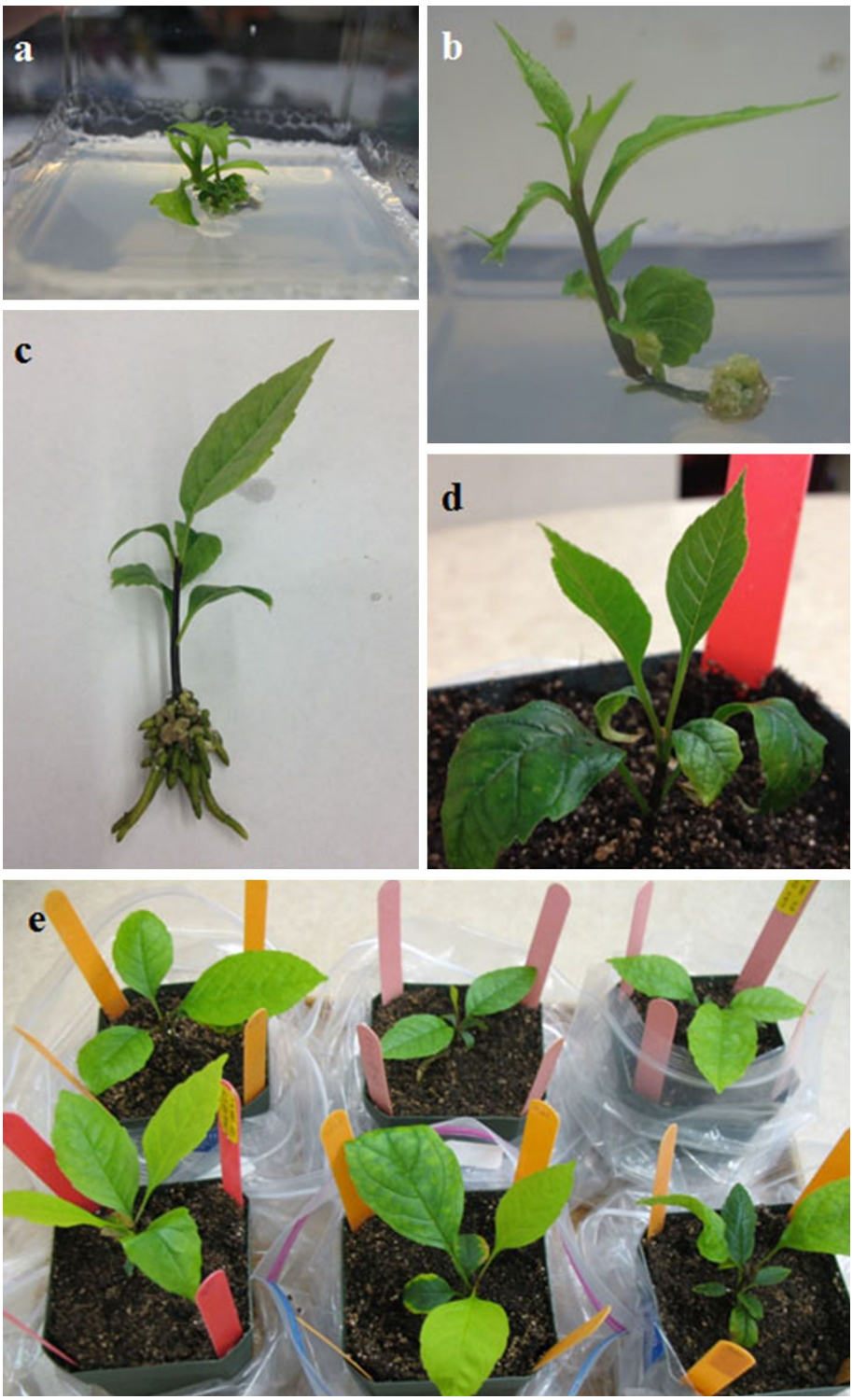

Fig. 2 Kanamycin-resistant adventitious *Fraxinus americana* shoots. **a** Shoot elongating on selection–elongation medium [MSB5G plus 10 μM BA, 10 μM TDZ, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin], **b** PCR-confirmed transgenic shoot proliferating on elongation medium without kanamycin, **c** root formation on PCR-confirmed transgenic shoot, **d**, **e** PCR-confirmed transgenic plants acclimatized to ambient culture room conditions

after the initial 6 weeks on rooting medium proved beneficial for elongating transgenic white ash roots, as well as maintaining the health of the shoot during culture. All roots that formed were firmly attached (Fig. 2c), although callus formation was more abundant at the base of the transgenic shoot and roots were thicker than had been observed during previous regeneration work on white ash (Palla and Pijut 2011). Some shoots exhibited root initiation from nodal or internodal stem portions in contact with the rooting medium, rather than from the basal end of the shoot. Rooted

Table 3 Effect of cytokinin concentration on elongation of transgenic *Fraxinus americana* microshoots

BA (μM)	TDZ (μM)	Shoot elongation[a,b] (%)	Mean no. shoot[a,b]
0.0	0.0	0.0 ± 0.0b	0.0 ± 0.0b
10.0	0.0	0.0 ± 0.0b	0.0 ± 0.0b
15.0	0.0	12.5 ± 70.7ab	2.0 ± 0.0a
20.0	0.0	12.5 ± 35.4ab	1.0 ± 0.0ab
0.0	2.5	0.0 ± 0.0b	0.0 ± 0.0b
10.0	2.5	0.0 ± 0.0b	0.0 ± 0.0b
15.0	2.5	12.5 ± 35.4ab	1.0 ± 0.0ab
20.0	2.5	0.0 ± 0.0b	0.0 ± 0.0b
0.0	5.0	0.0 ± 0.0b	0.0 ± 0.0b
10.0	5.0	0.0 ± 0.0 b	0.0 ± 0.0b
15.0	5.0	12.5 ± 70.7ab	2.0 ± 0.0a
20.0	5.0	0.0 ± 0.0b	0.0 ± 0.0b
0.0	10.0	0.0 ± 0.0b	0.0 ± 0.0b
10.0	10.0	37.5 ± 75.6a	1.3 ± 0.6ab
15.0	10.0	0.0 ± 0.0b	0.0 ± 0.0b
20.0	10.0	12.5 ± 35.4ab	1.0 ± 0.0ab

Hypocotyls forming shoot primordia were placed on Murashige and Skoog medium with Gamborg et al. (1968) B5 vitamins plus 2 mg L^{-1} glycine supplemented with 30 mg L^{-1} kanamycin, 500 mg L^{-1} timentin, and various concentrations of 6-benzyladenine (BA) and thidiazuron (TDZ). Data taken after 6 weeks of culture

[a] Values represent the means ± standard errors for eight explants per treatment

[b] Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$)

plantlets were acclimatized (100 %) to ambient culture room conditions (Fig. 2d, e).

Analysis of transgenic plant lines

Transformed plant tissue on selection medium containing kanamycin was exposed to a histochemical GUS assay 4- to 6-weeks after initial culture. Transient GUS expression was observed as a bright blue coloration in the tissue after chlorophyll removal. Staining was apparent within hypocotyl tissue, showing the successful transformation with *Agrobacterium* into the plant tissue, as well as on regenerating callus and leaf primordia, visualizing the transient expression of the GUS gene (Fig. 3a–c).

Genomic DNA extracted from the leaves of seven putative transgenic lines was used in PCR analysis along with a non-transformed white ash plant. PCR amplification resulted in 332, 364, and 720 bp (Fig. 4, lanes 4–10) DNA fragments from all seven independent lines, corresponding to GUS, *nptII*, and EGFP, respectively. PCR products of the corresponding size were found in the plasmid control (Fig. 4, lane 1), but absent in both the non-transformed plant (Fig. 4, lane 3) and the water control (Fig. 4, lane 2). This confirmed the successful integration of the foreign genes of interest into *F. americana*. Lines confirmed to be transgenic were further analyzed using a stereomicroscope with a 470/40 nm excitation filter to visualize fluorescence

Table 4 Effect of auxin concentration on in vitro root formation of transgenic *Fraxinus americana* microshoots

Treatment IAA + IBA (μM)	Rooting[a,b] (%)	Mean no. roots[a,b]	Mean root length[a,b] (cm)	Mean no. lateral roots[a,b]
0.0 + 4.9	13.3 ± 13.3b	0.5 ± 0.4b	0.8 ± 0.1b	0.2 ± 0.1b
2.9 + 4.9	80.0 ± 11.5a	3.8 ± 0.8a	1.2 ± 0.3a	0.8 ± 0.3a
5.7 + 4.9	66.7 ± 13.3a	4.1 ± 1.3a	1.0 ± 0.2a	0.6 ± 0.2ab
8.6 + 4.9	53.3 ± 6.7a	3.1 ± 1.1ab	0.7 ± 0.2ab	0.2 ± 0.1b

Microshoots were placed on woody plant medium supplemented with 500 mg L^{-1} timentin, 4.9 μM indole-3-butyric acid (IBA), and various concentrations of indole-3- acetic acid (IAA). An overlay of liquid medium was added every week after 6 weeks of culture in the light. Data taken after 5 day dark culture followed by 8–12 weeks light culture

[a] Values represent the means ± standard errors for 15 explants per treatment

[b] Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$)

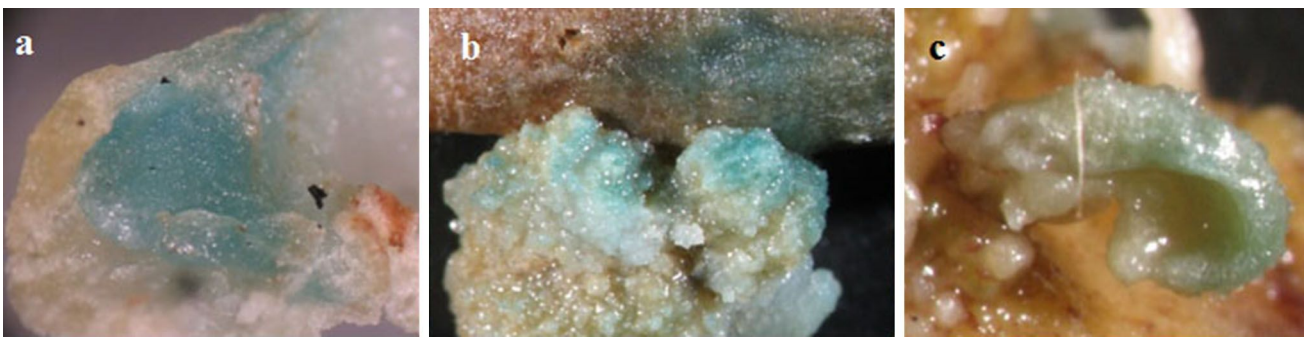

Fig. 3 Transient GUS expression in *Fraxinus americana*. Expression in **a** hypocotyl tissue, **b** callus, and **c** leaf primordia

associated with the presence of the EGFP gene. Young leaves from PCR-confirmed shoots were compared in vivo against non-transformed control leaves. Bright green portions of fluorescence were apparent only on leaves from transgenic shoots (Fig. 5), confirming both the presence and the expression of the EGFP gene.

The expression of the EGFP gene in leaves of transgenic plants was also determined by RT-PCR analysis. The amplification of the 720 bp fragment of EGFP was found in all seven transgenic plant lines (Fig. 6, lanes 5–11). A corresponding band was found in the plasmid control (Fig. 6, lane 4), but not in the water control (Fig. 6, lane 1), the RNA control (Fig. 6, lane 2), or the non-transformed plant (Fig. 6, lane 3). This confirmed the expression of the EGFP gene in all seven transformed lines, as well as the success of the reverse transcription of RNA into cDNA and the removal of DNA contamination. Yang et al. (2006) noted that *Agrobacterium* cells can potentially survive selection, persisting around the cells in leaves, stems, and roots of transgenic plants for 24 months while still in the presence of kanamycin. The highly conserved region of the housekeeping 26S rRNA found in plants was used as a control to validate RT-PCR results, as was used to evaluate the stable transformation of *Cornus* (Liu et al. 2013). All seven transgenic lines (Fig. 7, lanes 5–11) as well as the non-transformed plant (Fig. 7, lane 4) showed amplification of the 26S rRNA fragment, confirming that the gene

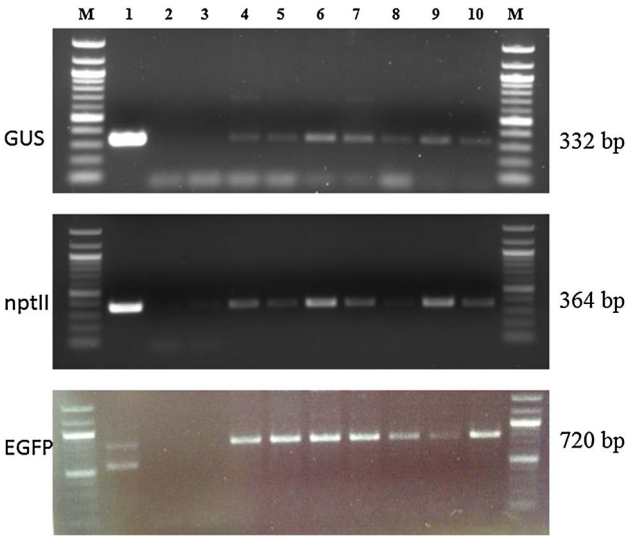

Fig. 4 PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic white ash for amplification of 332-bp GUS, 364-bp *nptII* gene, and 720-bp EGFP, respectively. M 100 bp molecular marker, *lane* 1 positive control (pq35GR), *lane* 2 water control, *lane* 3 negative control (non-transformed plant), *lanes* 4–10 (putative transgenic shoots), M 100 bp molecular marker

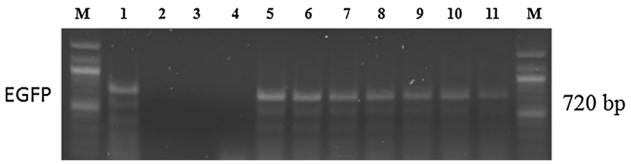

Fig. 6 RT-PCR analysis of cDNA isolated from leaves of non-transformed and transgenic white ash for amplification of 720-bp EGFP. M 100 bp molecular marker, *lane* 1 water control, *lane* 2 DNase-treated RNA template control, *lane* 3 non-transformed plant (negative control), *lane* 4 pq35GR plasmid DNA (positive control), *lanes* 5–11 transgenic shoots, M 100 bp molecular marker

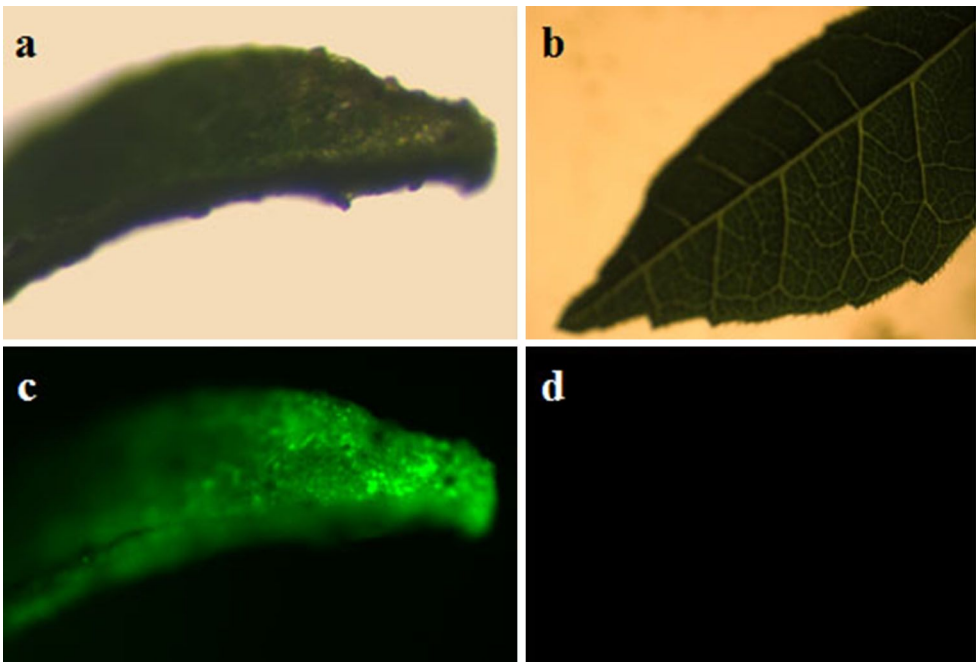

Fig. 5 EGFP visualization of transformed leaf. **a** Transformed leaf from microshoot confirmed to harbor GUS, *nptII*, and EGFP and **b** control non-transformed leaf under white light. Same **c** transformed leaf and **d** non-transformed leaf as seen through EGFP excitation filter (470/40 nm)

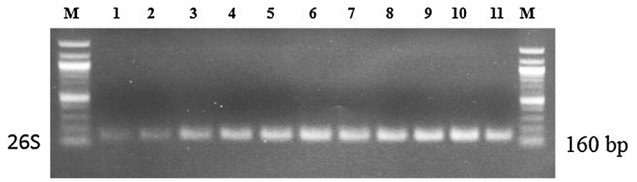

Fig. 7 RT-PCR analysis of cDNA isolated from leaves of non-transformed and transgenic white ash for amplification of 160-bp 26S gene. M 100 bp molecular marker, *lane* 1 pq35GR plasmid DNA (negative control), *lane* 2 water control, *lane* 3 DNase-treated RNA template control, *lane* 4 non-transformed plant, *lanes* 5–11 transgenic shoots, M 100 bp molecular marker

expression detected during RT-PCR was the result of an integrated transgene rather than *Agrobacterium* contamination. Contamination of the PCR reaction was apparent however, resulting in faint banding for the water control (Fig. 7, lane 2) and plasmid control (Fig. 7, lane 1). Several attempts were made to eliminate the source of this contamination and obtain a clearer image.

Conclusions

Transgenic white ash plants were successfully developed using an *Agrobacterium*–mediated transformation and regeneration protocol for hypocotyl explants. Hypocotyls germinated up to 15 days on pre-culture medium containing cytokinins were exposed to sonication and vacuum-infiltrated with *Agrobacterium* strain EHA105 harboring the pq35GR vector. Selection of transformed cells was optimal with 30 mg L^{-1} kanamycin and excess *Agrobacterium* growth was controlled with 500 mg L^{-1} timentin without limiting organogenic potential. The integration of marker genes was confirmed through PCR analysis, GUS staining, GFP visualization, and RT-PCR analysis. Optimal rooting of transgenic shoots (80 %) occurred on WPM with 500 mg L^{-1} timentin, 4.9 μM IBA, plus 2.9 μM IAA. This is the first report of successfully regenerating transgenic white ash plants. Similar methods have been reported successful for green ash and pumpkin ash (Du and Pijut 2009; Stevens and Pijut 2014). This protocol provides a method for future development of EAB-resistant white ash trees. Studies are currently underway to integrate the *Bacillus thuringiensis* Cry8Da toxin protein into *F. americana*, with the goal of imparting resistance to the beetle.

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