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ORIGINAL PAPER

Agrobacterium-mediated genetic transformation and plant regeneration of the hardwood tree species *Fraxinus profunda*

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Abstract
Key message **This transformation and regeneration protocol provides an integral framework for the genetic improvement of *Fraxinus profunda* (pumpkin ash) for future development of plants resistant to the emerald ash borer.**
Abstract Using mature hypocotyls as the initial explants, an *Agrobacterium tumefaciens*-mediated genetic transformation system was successfully developed for pumpkin ash (*Fraxinus profunda*). This transformation protocol is an invaluable tool to combat the highly aggressive, non-native emerald ash borer (EAB), which has the potential to eliminate native *Fraxinus* spp. from the natural landscape. Hypocotyls were successfully transformed with *Agrobacterium* strain EHA105 harboring the pq35GR vector, containing an enhanced green fluorescent protein (*EGFP*) as well as a fusion gene between neomycin phosphotransferase (*nptII*) and *gusA*. Hypocotyls were cultured for 7 days on Murashige and Skoog (MS) medium with 22.2 μM 6-benzyladenine (BA), 4.5 μM thidiazuron (TDZ), 50 mg L^{-1} adenine hemisulfate (AS), and 10 % coconut water (CW) prior to transformation. Hypocotyls were transformed using 90 s sonication plus 10 min vacuum infiltration after *Agrobacterium* was exposed to 100 μM acetosyringone for 1 h. Adventitious shoots were regenerated on MS medium with 22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 400 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin. Timentin at 400 and 20 mg L^{-1} kanamycin were most effective at controlling *Agrobacterium* growth and selecting for transformed cells, respectively. The presence of *nptII*, GUS (β-glucuronidase), and EGFP in transformed plants was confirmed using polymerase chain reaction (PCR), while the expression of EGFP was also confirmed through fluorescent microscopy and reverse transcription-PCR. This transformation protocol provides an integral foundation for future genetic modifications of *F. profunda* to provide resistance to EAB.

Keywords *Fraxinus* · Genetic transformation · Pumpkin ash · Regeneration · Vitro

Abbreviations

AS	Adenine hemisulfate
BA	6-Benzyladenine
CaMV 35S	Cauliflower mosaic virus 35S promoter
CW	Coconut water
EAB	Emerald ash borer
EGFP	Enhanced green fluorescent protein
GUS	β-Glucuronidase
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
MSB5G	Murashige and Skoog medium with Gamborg B5 vitamins and glycine
nptII	Neomycin phosphotransferase

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PCR	Polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
TDZ	Thidiazuron
WPM	Woody plant medium

Introduction

Pumpkin ash [*Fraxinus profunda* (Bush) Bush] is a hardwood tree species native to eastern North America. First described in the late nineteenth century (Bush 1897), it has a severely fragmented range and is found predominantly in areas with extensive yearly flooding (Harms 1990). Pumpkin ash is an important riparian species serving many ecological functions. The wood is highly valued in the lumber industry and also in the manufacture of tool and implement handles. Although the origin of pumpkin ash has been contested, it is generally accepted to be a hybrid of white ash (*F. americana*) and green ash (*F. pennsylvanica*) (Hardin and Beckman 1982; Miller 1955; Wallander 2008), the two most common and economically valuable North American ash species.

Traditionally, *Fraxinus* spp. have been known to be relatively pest and disease free, with only stressed or dying trees being attacked (Jesse et al. 2011). The emerald ash borer (EAB) is a relatively new threat to indigenous ash species. First detected in the United States in Detroit, MI in 2002, the EAB has the potential to spread throughout the native range of *Fraxinus* spp. (Dobesberger 2002; Haack et al. 2002). The EAB is a highly aggressive, lethal, wood-boring insect that kills trees through the feeding of developing larvae in the phloem, effectively girdling the tree, causing complete mortality of healthy trees in as little as 5–6 years (Knight et al. 2013). EAB has spread rapidly and indefatigably from its epicenter in Detroit, now being found in 18 states and parts of Canada (EAB 2013). Found throughout the Midwest and southern Canada, EAB has killed millions of trees resulting in the loss of billions of dollars (Cappaert et al. 2005; Sydnor et al. 2007). Host range studies concluded that the EAB was unable to grow and develop on any non-ash North American hardwood species (Anulewicz et al. 2008) intensifying the pressure on *Fraxinus*.

Reports on the susceptibility of native ash species show that white ash and green ash were highly vulnerable, but blue ash (*F. quadrangulata*) showed limited resistance or at least non-preference (Anulewicz et al. 2007; Pureswaran and Poland 2009; Tanis and McCullough 2012), while Manchurian ash (*F. mandshurica*), which is a common host in EAB's native range, showed significant resistance (Rebek et al. 2008). The close phylogenetic proximity of pumpkin ash to green and white ash, coupled with risk assessments indicating the potential threat of EAB if left unchecked (Dobesberger 2002; Wei et al. 2004), suggests that it is also vulnerable to the EAB.

A number of control measures have proven effective against the EAB; however, many only work to slow the spread of the insect and do not protect against attack. Chipping, de-barking, or heat treatment of infested logs was shown to be effective in destroying the EAB larvae if proper guidelines were followed (McCullough et al. 2007). Given that a large portion of the EAB life cycle takes place under the bark of a tree, chemical control can be difficult. Although common insecticides have proven effective in killing the EAB, they often need to be applied systemically (Herms et al. 2009; Petrice and Haack 2006; Smitley et al. 2010). These insecticide applications would need to be applied yearly and would not be practical at a large scale, as repeated applications could have unwanted impacts on the surrounding ecosystem (Kreutzweiser et al. 2007). Efforts have been made to identify a number of parasitoid enemies that show promise in mitigating EAB population growth (Liu et al. 2003, 2007; Wang et al. 2008; Wu et al. 2008; Yang et al. 2006; Zhang et al. 2005). These species, specific to EAB, have been found to kill almost 75 % of the EAB individuals in North American ash planted in China (Liu et al. 2007). Their efficacy, along with an EAB specific entomopathogenic fungi (Liu and Bauer 2006), can often vary seasonally, however, and can be unreliable.

Genetic transformation, although no panacea because of regulatory limitations (Sederoff 2007; Sedjo 2006; Strauss et al. 2009), unknown long-term physiological ramifications (Ahuja 2011), and environmental impacts (Fladung et al. 2010; Gamborg and Sandoe 2010), offers a powerful tool to combat EAB. Insect-resistant trees carrying a *Bacillus thuringiensis* gene have been described in *Populus* (Hu et al. 2001; Tian et al. 1993), and have been shown to be effective at controlling the target pest. Introgression of genes imparting resistance to EAB into *Fraxinus* spp. would be of great economic and ecological importance to landowners, forest and conservation managers, as well as to the lumber industry. The *B. thuringiensis* gene encoding the Cry8Da toxin, lethal to EAB (Londo and Bauer 2007), or potential resistance genes identified in Manchurian ash (Cipollini et al. 2011; Eyles et al. 2007; Whitehill et al. 2011) would be ideal candidates to be incorporated directly into the ash genome via genetic transformation, to impart systemic resistance. Previous reports have shown that genetic transformation of *Fraxinus* spp. was possible (Bates 1997; Du and Pijut 2009; Roome 1992). However, no transformation system exists for *F. profunda*. Therefore, the focus of this study was to develop an efficient, reliable,

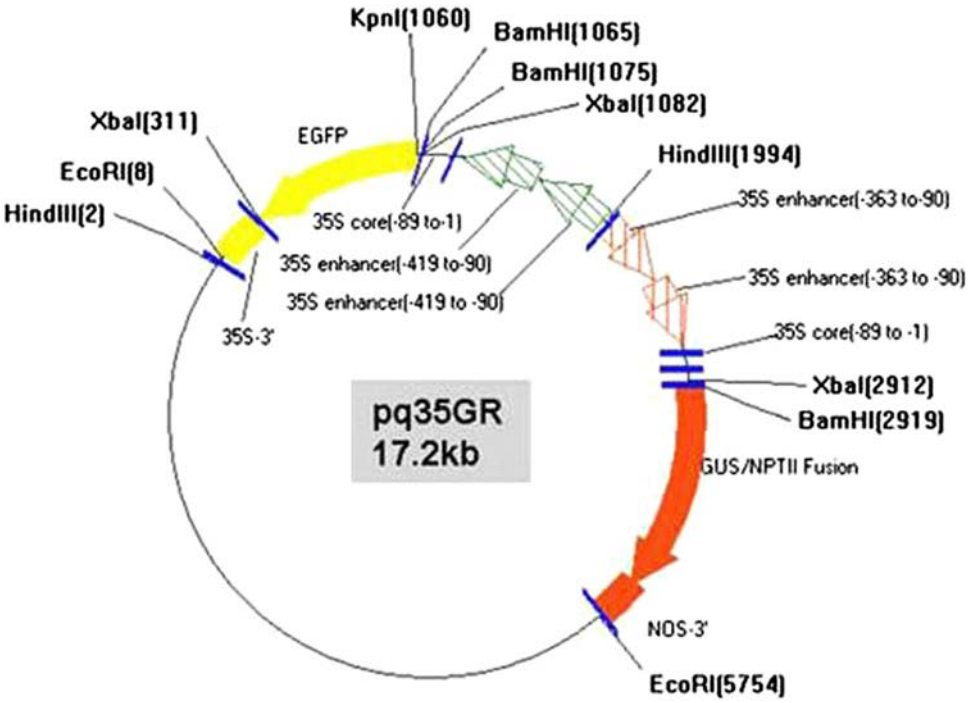

Fig. 1 pq35GR vector composed of two independent CaMV35S-driven enhancer repeats corresponding to an *nptII* and *gusA* fusion gene, and an *EGFP* gene (Li et al. 2004)

and optimized *Agrobacterium tumefaciens*-mediated genetic transformation system for pumpkin ash, to be used in future studies to develop *F. profunda* trees expressing specific genes imparting resistance to EAB.

Materials and methods

Explant source

Mature pumpkin ash seed was obtained from the National Seed Laboratory (Dry Branch, GA) and stored in sealed containers at 5 °C in the dark until used. Disinfestation and embryo extraction were achieved following our previously established protocol (Stevens and Pijut 2012). Freshly isolated aseptic embryos were cultured horizontally on a modified Murashige and Skoog (1962) basal medium (MS; *Phyto*Technology Laboratories, Shawnee Mission, KS; M499) with organics (100 mg L^{-1} myo-inositol, 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), 4.5 μM thidiazuron (TDZ), 50 mg L^{-1} adenine hemisulfate (AS), 10 % coconut water (CW), 3 % (w/v) sucrose, and 0.7 % (w/v) Bacto agar (Becton–Dickinson, Franklin Lakes, NJ; No. 214030) for 7 days to allow for germination (100 × 25 mm Petri plates; 50 mL medium). Hypocotyls were then excised and used for testing explant sensitivity to kanamycin and timentin, as well as for transformation.

Agrobacterium vector and culture

The pq35GR vector, in *Agrobacterium* strain EHA105, used in this study was created by Li et al. (2004) and was also used for transformation studies with green ash (*F. pennsylvanica*) (Du and Pijut 2009). The pq35GR vector (Fig. 1) was composed 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 an enhanced green fluorescent protein (*EGFP*). *Agrobacterium* colonies were grown in the dark for 2 days 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 on a rotary shaker (125 rpm). One hour prior to co-cultivation with hypocotyls, the *Agrobacterium* suspension was centrifuged for 15 min at 3,000 rpm and the pellet re-suspended in 20 mL liquid MS medium containing 100 μM acetosyringone.

Hypocotyl sensitivity to kanamycin and timentin

To determine the optimum concentration for selection of transformed tissue and controlling excess *Agrobacterium* growth, 7-day-old hypocotyls were cultured horizontally on MS medium with organics supplemented with 22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 3 % (w/v) sucrose, 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, and solidified with 0.7 % (w/v) Bacto agar, pH 5.7 (100 × 25 mm Petri plates; 50 mL medium). Antibiotics were dissolved in sterile deionized water and filter-sterilized (0.22 μm) prior to being added to cooled autoclaved medium. Regeneration response of callus and shoot formation frequency and mean number of shoots were recorded after 6 weeks on initial induction medium plus 8 weeks on shoot elongation medium [MS medium plus Gamborg B5 vitamins (Gamborg et al. 1968) and 0.2 g L^{-1} glycine (MSB5G) supplemented with 10 μM BA, 10 μM TDZ, and kanamycin or timentin concentration corresponding to adventitious shoot formation medium from which shoots were regenerated; pH 5.7]. Three replicates of 12 hypocotyls each were cultured for each treatment. All plant materials were incubated under a 16 h photoperiod provided by cool-white fluorescent lamps (80 μmol m^{-2} s^{-1}) at 24 ± 2 °C, and all shoot cultures were subcultured every 4 weeks.

Statistical analysis

Data were analyzed with an analysis of variance (ANOVA) performed with R statistical software (R Development Core Team 2006). A Tukey's HSD comparison test at the 5 % level of probability was run on means shown to be significant by ANOVA.

Agrobacterium inoculation and plantlet regeneration

Hypocotyls were excised from 3- to 7-day-old embryos growing on MS pre-culture medium (22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 3 % (w/v) sucrose, pH 5.7, and 0.7 % (w/v) Bacto agar), submerged in 20 mL liquid MS pre-culture medium, and then sonicated for 90 s. After sonication, hypocotyls were vacuum infiltrated (62.5 cm Hg) in 20 mL *Agrobacterium* suspension (OD_{600} = 0.6–1) for 10 min. Explants were then blotted dry on autoclaved filter paper and placed on MS co-culture medium (22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 3 % (w/v) sucrose, pH 5.7, and 0.7 % (w/v) Bacto agar) for 2–4 days in the dark at 27 °C. Hypocotyls were rinsed four times in liquid MS pre-culture medium to remove excess *Agrobacterium* prior to being cultured horizontally on selection medium (MS medium plus 22.2 or 13.3 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 3 % (w/v) sucrose, 400 mg L^{-1} timentin, 20 mg L^{-1} kanamycin, pH 5.7, and 0.7 % (w/v) Bacto agar). After 6 weeks, putatively transgenic shoots resistant to kanamycin were transferred to shoot elongation medium plus 400 mg L^{-1} timentin and 20 mg L^{-1} kanamycin for 8 weeks, at which time they were evaluated for the number of resistant shoots formed. Elongating shoots were transferred to shoot elongation medium without kanamycin and a 3 mL liquid shoot elongation medium overlay was added to increase proliferation rate. Adventitious roots were initiated on kanamycin-resistant shoots (3–4 cm in length) after exposure to woody plant medium (Lloyd and McCown 1981) (WPM; *Phyto*Technology Laboratories, L154) supplemented with 4.9 μM indole-3-butyric acid (IBA) in the dark for 10 days prior to being cultured under a 16 h photoperiod (80 μmol m^{-2} s^{-1}) at 24 ± 2 °C. Rooted microshoots were acclimatized to ambient conditions according to Stevens and Pijut (2012).

Visualization of enhanced green fluorescent protein

Leaves from control (non-transgenic plant) and putative transgenic plants were examined under a fluorescence stereomicroscope (Leica MZFLIII) equipped with a 470/40 nm excitation filter (GFP-Plant) and a 525/50 nm barrier filter to detect the presence of the EGFP. The light source was provided by a 100 W mercury bulb. An interference filter was not used to block chlorophyll autofluorescence. Presence or absence of green fluorescence was compared between the control and putative transgenic shoots, hereafter referred to as independent lines, using Spot™ imaging software.

DNA isolation and analysis of transgenic plants

Leaves of eight, out of a total of thirty, independent lines were randomly chosen for genomic DNA extraction as well as one control non-transgenic plant using a modified cetyltrimethylammonium bromide method (Lefort and Douglass 1999). Plasmid DNA extracted from *Agrobacterium* was used as the positive control and sterile distilled water was used as a negative control. Polymerase chain reaction (PCR) was designed to specifically amplify *nptII*, GUS, and EGFP. PCR products were visualized under ultraviolet light after gel electrophoresis [1 % agarose (w/v) plus 0.5 μg mL^{-1} ethidium bromide]. Primers (forward *nptII*-F 5′-TGCTCCTGCCGAGAAAGTAT-3′ and reverse *nptII*-R 5′-AATATCACGGGTAGCCAAGC-3′) amplified the 364 bp PCR fragment for the *nptII* gene. Primers (forward GUS-F 5′-TGCTGTCGGCTTTAACCTCT-3′ and reverse GUS–R 5′-GGCACAGCACATCAAAGAG A-3′) amplified the 332 bp PCR fragment for GUS. Primers (forward EGFP-F 5′-TCCCCCGGGCCATGGGATCG ATGC-3′ and reverse EGFP-R 5′-CGAGCTCTTACTTGT ACAGCTCGTCCA-3′) amplified the 720 bp PCR fragment for the EGFP.

Detection of *nptII*, GUS, and EGFP was achieved with a PCR reaction containing 2.5 μL 10× PCR buffer (Life Technologies, Grand Island, NY), 50 mM Mg^{2+} (0.8 μL

GUS only; 1 μL EGFP only), 1 μL 50 mM dNTP, 1 μL of 10 μM *nptII*-F and *nptII*-R, GUS-F and GUS-R, or EGFP-F and EGFP-R primers, 3 μL 14.7–57.5 ng μL^{-1} DNA template, 0.2 μL 5 U μL^{-1} Taq polymerase (0.25 μL EGFP only), and sterile distilled H_2O to a final volume of 25 μL. The *nptII* PCR reaction was as follows: 94 °C denaturing for 2 min followed by 40 cycles of 94 °C for 45 s, 56 °C annealing for 45 s, 72 °C for 2 min, and finally 72 °C extension for 10 min. The GUS PCR reaction was identical except it was run for 45 cycles and the annealing temperature was 55 °C. The EGFP reaction cycle was as follows 95 °C for 4 min followed by 40 cycles of 95 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min, and finally 72 °C for 7 min.

RNA isolation and reverse transcription-PCR (RT-PCR) analysis

RNA was isolated from leaves of four genomic DNA PCR-confirmed transgenic lines and one control non-transgenic plant following the recommended protocol for the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Isolated RNA was treated with DNase to remove all DNA. Up to 5 μg RNA was used as a template for reverse transcription to generate first-strand cDNA using Agilent AccuScript first-strand cDNA synthesis kit (Agilent Technologies, Santa Clara, CA) following the manufacturers recommended protocol. The first-strand cDNA was then used as the template to amplify a specific 720 bp EGFP fragment following the aforementioned protocol for genomic DNA EGFP amplification. To validate RT-PCR results, primers were designed to amplify the *26S* housekeeping rRNA gene. Primers (Forward 26S-F 5′-GTCCTAAGATGA GTCCAA-3′ and reverse 26S-R 5′-GGTAACTTTTCTGA CACCTC-3′) amplified the 160 bp PCR product of the *26S* gene. Detection of the 26S PCR product was achieved with a 25 μL PCR reaction containing 2.5 μL 10× PCR buffer (Life Technologies), 1 μL 50 mM Mg^{2+}, 1 μL 50 mM dNTP, 1 μL each of 10 μM 26S-F and 26S-R 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 and sterile deionized water were used as negative controls, cDNA from a non-transformed plant served as a positive control, and DNase-treated RNA that had not been reverse transcribed served to detect DNA contamination. The 26S PCR reaction was as follows: 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 PCR products were visualized under ultraviolet light after gel electrophoresis (1 % agarose (w/v) plus 0.5 μg mL^{-1} ethidium bromide).

Table 1 Effect of kanamycin on callus formation, adventitious shoot formation, and number of adventitious shoots regenerated from *Fraxinus profunda* hypocotyls

Kanamycin (mg L^{-1})	Callus formation[a] (%)	Shoot formation[a] (%)	Mean no. shoots[a]
0	91.7 ± 4.7a	41.7 ± 8.3ab	0.8 ± 0.2a
5	83.3 ± 6.3a	47.2 ± 8.4a	1.2 ± 0.3a
10	36.1 ± 8.1b	22.2 ± 7.0b	0.2 ± 0.1b
15	5.6 ± 5.6c	0c	0b
20	0c	0c	0b
30	0c	0c	0b
40	0c	0c	0b
50	0c	0c	0b

Hypocotyls were cultured on MS medium supplemented with 22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, and 10 % CW for 6 weeks, then transferred to a MSB5G medium supplemented with 10 μM BA and 10 μM TDZ with corresponding concentrations of kanamycin. Data were collected after 8 weeks on MSB5G medium

[a] Values represent mean ± SE for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Tukey's multiple comparison test ($P \leq 0.05$)

Results and discussion

Hypocotyl sensitivity to kanamycin and timentin

Pumpkin ash hypocotyls were highly sensitive to elevated concentrations of kanamycin. As kanamycin concentrations increased, organogenesis was significantly impaired, and concentrations of 20 mg L^{-1} or higher inhibited all adventitious shoot and callus formation (Table 1). We determined 20 mg L^{-1} to be the optimal concentration to screen for transgenic plant material as only tissue expressing the *nptII* gene could tolerate this concentration. These results were similar to kanamycin concentrations found effective in other woody plant transformation systems to select for transformed tissue (Roome 1992; Bates 1997; Wenck et al. 1999; Pérez-Clemente et al. 2004; Yevtushenko and Misra 2010). Specifically, Du and Pijut (2009) found that 20 mg L^{-1} kanamycin was lethal and sufficient to inhibit shoot organogenesis of non-transformed *F. pennsylvanica* hypocotyls, supporting our results.

Conversely, pumpkin ash hypocotyls were insensitive to timentin, as increasing timentin concentration had little influence on organogenesis frequency. Shoot organogenesis ranged from 44 to 72 % depending on timentin concentration (0–600 mg L^{-1}), and the highest regeneration frequency (72 %) was recorded in the presence of 400 mg L^{-1} timentin (data not shown). Adventitious shoots produced at this concentration had no noticeable morphological abnormalities (Fig. 2). Therefore

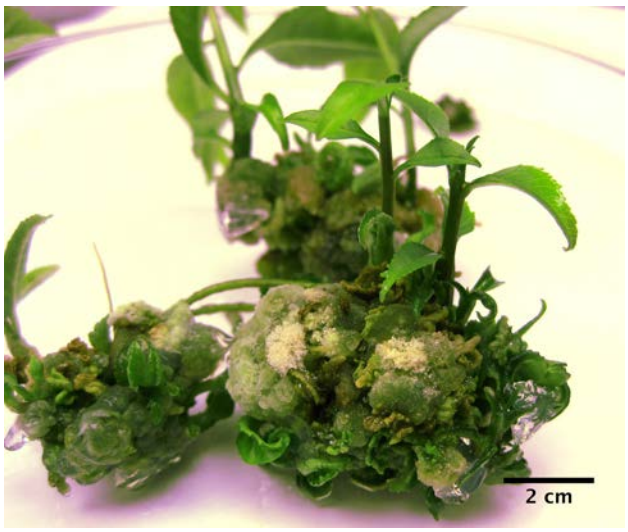

Fig. 2 Adventitious shoots of pumpkin ash displaying normal morphology. Shoots regenerated on hypocotyls after 6 weeks on MS medium supplemented with 22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 3 % (w/v) sucrose, and 400 mg L^{-1} timentin, then 4 weeks on MSB5G medium with 10 μM BA, 10 μM TDZ, and 400 mg L^{-1} timentin

400 mg L^{-1} timentin was used to control excess *Agrobacterium* growth, similar to concentrations in the *F. pennsylvanica* transformation system (Du and Pijut 2009).

Transformation and plantlet regeneration

Preliminary trials indicated transformation was greatly improved by germinating pumpkin ash embryos on co-cultivation medium [MS medium supplemented with 22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 3 % (w/v) sucrose, and 0.7 % (w/v) Bacto agar] compared to other media. Hypocotyls excised from embryos that were not germinated on this medium or were pre-cultured for <7 days prior to exposure to *Agrobacterium* often became necrotic or overgrown by bacterial growth, and died shortly after inoculation. A short pre-culture period prior to inoculation has been shown to alleviate this hypersensitivity (Du and Pijut 2009; Toki et al. 2006), but has also been shown to inhibit transformation (Mondal et al. 2001). In our early trials, the combination of germination and pre-culture with a 3 day co-cultivation period was ideal for *Agrobacterium* infection. Co-cultivation longer than 3 days resulted in prolific *Agrobacterium* growth resulting in necrosis of the hypocotyls.

Pumpkin ash hypocotyls were successfully transformed and thirty independent kanamycin-resistant lines were recovered following our pumpkin ash adventitious shoot regeneration protocol (Stevens and Pijut 2012). After 6 weeks on selection and adventitious shoot regeneration medium (MS medium plus 13.3 or 22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 400 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin), and 8 weeks on shoot elongation medium (MS medium plus 10 μM BA, 10 μM TDZ, 400 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin), adventitious shoots formed that elongated normally at kanamycin concentrations shown to be lethal to non-transgenic plants (Fig. 3a; Table 1). Although resistant to kanamycin, its inclusion in the shoot elongation medium hindered rapid shoot proliferation and elongation. This was overcome by the addition of a liquid shoot elongation medium overlay, which was found to increase the overall growth rate of transformed shoots. These results were also found in reports in white ash axillary shoot and seedling growth (Preece et al. 1991; van Sambeek et al. 2001).

Early trials using 13.3 μM BA in the adventitious shoot regeneration medium led to relatively low transformation efficiency, 2.5 % (four shoots from 162 inoculated hypocotyls). This efficiency was more than doubled when the BA concentration was increased. A total of 30 independent shoot lines were recovered from kanamycin-resistant callus out of 554 inoculated hypocotyls, a 5.4 % efficiency when regenerated using 22.2 μM BA. The relation between BA concentration and transformation efficiency was similar to melon (*Colocynthis citrullus* L.) explants exposed to 22.2 μM BA during co-cultivation which had a much higher regeneration frequency than the control (zero BA) (Ntui et al. 2010). Our results suggested a positive effect from BA on *Agrobacterium* infection and regeneration frequency in *F. profunda.* This effect, however, was most likely a species-specific effect, as studies have shown high efficiency transformation was possible with high (44.4 μM BA), low (0.2 μM BA), or no exogenous cytokinin applications (Gutiérrez-E et al. 1997; Ozawa 2009; Yevtushenko and Misra 2010). Following our previously published protocol (Stevens and Pijut 2012), kanamycin-resistant microshoots were successfully rooted and acclimatized to ambient conditions (Fig. 3b, c).

Confirmation of transgene incorporation

Putatively transgenic shoots growing on a lethal concentration of kanamycin were confirmed to be transgenic by taking three approaches. First, genomic DNA from leaves of eight putatively transgenic shoots was tested using PCR for integration of the *nptII*, *gusA*, and *EGFP* genes. The PCR amplification revealed DNA fragments of 364 bp for *nptII*, 332 bp for GUS, and 720 bp for EGFP, the predicted band size for all three insertion events, and banding was not present in the negative controls (Fig. 4). These PCR results confirmed the presence of introduced genes into the eight pumpkin ash lines tested.

Elongating shoots that displayed resistance to kanamycin were further analyzed to detect the presence of EGFP.

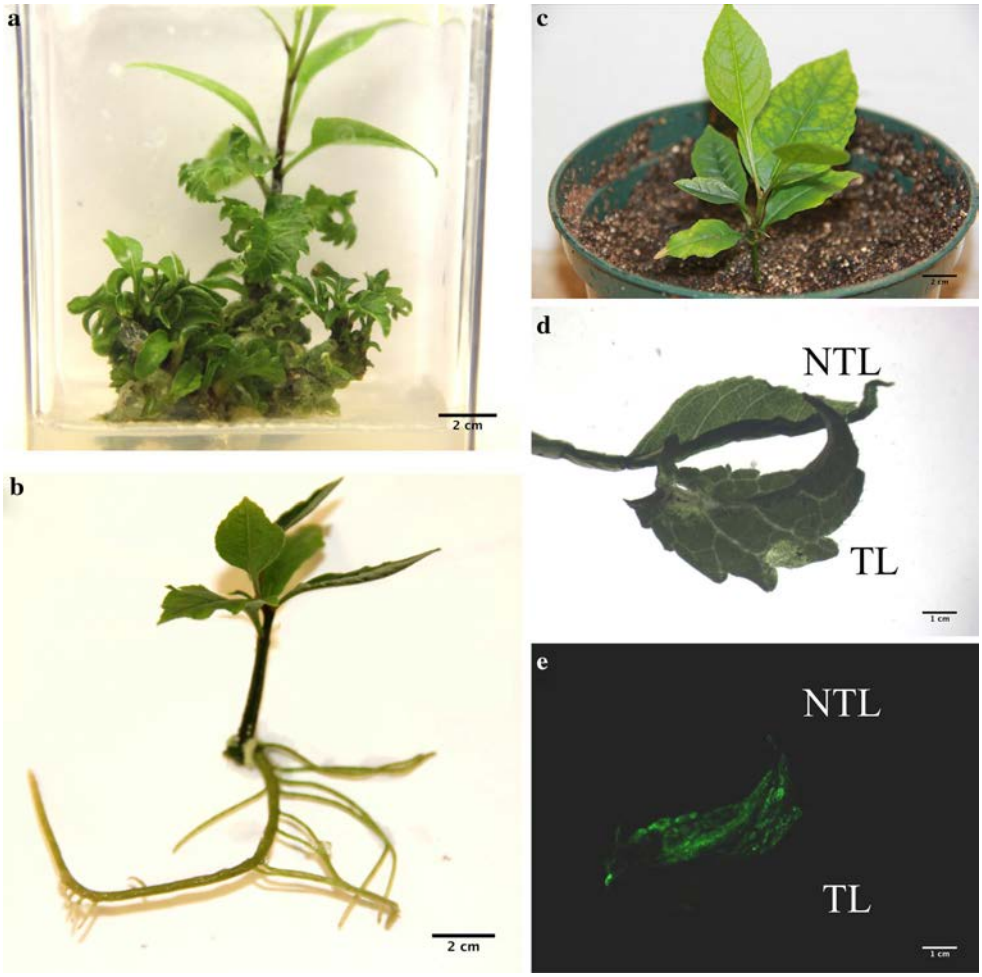

Fig. 3 *Agrobacterium*-mediated transformation and regeneration of pumpkin ash (*F. profunda*) hypocotyls and EGFP visualization of transformants. **a** Kanamycin-resistant adventitious shoots after 6 weeks on selection medium (MS plus 22.2 μM BA, 4.5 μM TDZ, 50 mg L^{-1} AS, 10 % CW, 400 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin), then 8 weeks on elongation medium (MSB5G plus 10 μM BA, 10 μM TDZ, 400 mg L^{-1} timentin, and 20 mg L^{-1} kanamycin). **b** Adventitious root formation on transgenic pumpkin ash microshoot after 10 days dark and 3 weeks light on WPM containing 4.9 μM IBA and 400 mg L^{-1} timentin. **c** Transgenic pumpkin ash plantlet acclimatized to ambient conditions. (**d** and **e**) EGFP visualization of control non-transformed leaves (NTL) and transformed leaves (TL) under normal light (**d**) and through GFP excitation filter (470/40 nm) (**e**)

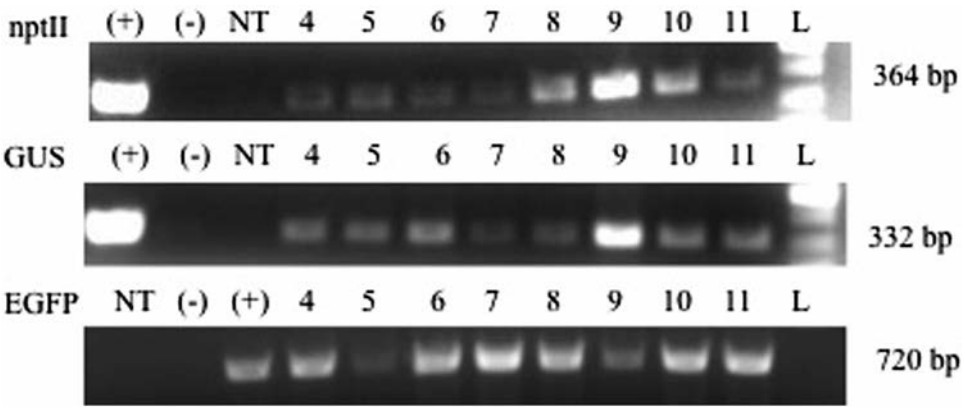

Fig. 4 PCR analysis of pumpkin ash (PA) genomic DNA from transgenic and non-transgenic leaves. Primers were used to specifically amplify a 364 bp *nptII* gene, the 332 bp GUS, and the 720 bp EGFP. (L) 100 bp ladder (1 kb for EGFP) (+) positive control, pq35GR plasmid DNA (−) water control (NT) non-transgenic PA DNA, and (lanes 4–11) PA transgenic lines

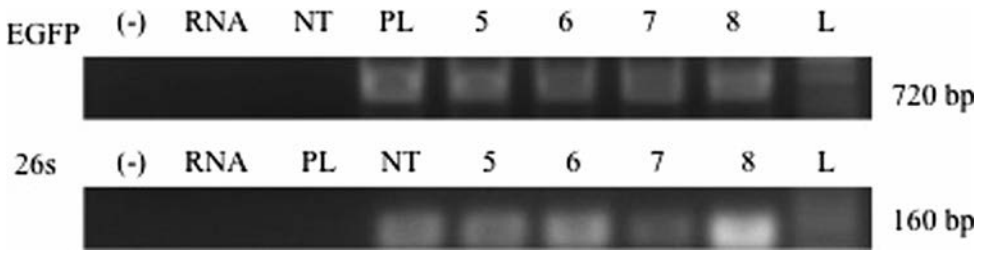

Fig. 5 RT-PCR analysis of pumpkin ash (PA) first-strand cDNA from transgenic and control non-transgenic leaves. Primers were used to specifically amplify the 720 bp EGFP and the 160 bp 26S. (L) 100 bp ladder (−) water control (RNA) non-reverse transcribed RNA (NT) non-transgenic PA cDNA (PL) pq35GR plasmid DNA, and (lanes 5–8) PA transgenic cDNA

Using a fluorescence stereomicroscope equipped with a 470/40 nm excitation filter, leaves from putative transgenic plants and control non-transgenic plants were compared in vivo. Green fluorescent spots confirming the presence of EGFP in pumpkin ash leaves were observed. These events in transgenic leaves could be clearly distinguished from the control where no green areas were detected (Fig. 3d, e).

The final method by which our transgenic lines were confirmed was using RT-PCR to detect EGFP expression. RNA from four of our genomic PCR-confirmed lines and one non-transformed plant was isolated, and first-strand cDNA was synthesized. DNA from the pq35GR vector was used as a control as well as DNased RNA that had not been reverse transcribed. Using the first-strand cDNA as a template, an EGFP 720 bp fragment was amplified for all transgenic lines and the plasmid DNA, but not for the negative controls (Fig. 5). These results suggest a high probability that EGFP was incorporated and being expressed in pumpkin ash, and RNA samples were free of *Agrobacterium* plasmid and genomic DNA contamination. To further validate the RT-PCR results, a PCR was performed using the first-strand cDNA as a template to amplify the 160 bp 26S fragment. Banding was evident for transgenic lines and the non-transformed plant, but not for the plasmid control (Fig. 5). There was no observable *Agrobacterium* growing in elongating microshoot cultures even in the absence of timentin. These results provided the most robust evidence that our regenerated pumpkin ash shoots were transgenic and free of *Agrobacterium*.

In summary, pumpkin ash hypocotyls were successfully genetically transformed using *Agrobacterium* strain EHA105 containing the pq35GR vector. Transgenic plantlets were successfully regenerated, and were corroborated as transgenic through three independent approaches. The successful development of genetically modified *Fraxinus* spp. plantlets has only been documented once prior to this study. Transformation success and efficiency benefited greatly from germinating embryos on pre-culture medium, and using 7-day-old hypocotyls co-cultured for 3 days on an elevated concentration of BA (22.2 μM). Kanamycin (20 mg L^{-1}) was found to be optimum for selection of transformed tissue, while 400 mg L^{-1} timentin was sufficient to control excess *Agrobacterium* growth without inhibiting organogenesis. Transgenic shoots were successfully rooted and acclimatized to ambient conditions. This transformation and regeneration protocol provides an integral framework for future genetic improvement of *F. profunda* while complementing traditional tree breeding for the eventual development of pumpkin ash that is resistant to the EAB. Current studies are underway to insert the *B. thuringiensis* Cry8Da protein into pumpkin ash, to impart resistance to EAB.

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