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ORIGINAL ARTICLE

Optimization of *Agrobacterium*-mediated genetic transformation of *Fraxinus nigra* and development of black ash for possible emerald ash borer resistance

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Abstract
Emerald ash borer (EAB; *Agrilus planipennis* Fairmaire) is the most devastating insect pest of North American ash species, including black ash (*Fraxinus nigra* Marsh.). As a first step in an effort to develop transgenic black ash plants resistant to EAB, we successfully established an efficient *Agrobacterium*-mediated transformation system for black ash hypocotyls. Kanamycin and timentin at 40 and 300 mg L^{-1}, respectively, were most effective to select transformed explants and control excess *Agrobacterium* growth. Using a plant transformation vector harboring the enhanced green fluorescent protein (*eGFP*) gene, the effects of *Agrobacterium* strain, bacterial density, and the concentration of Silwet L-77 on transformation efficiency were evaluated. The best result was obtained when *Agrobacterium* strain EHA105 was used at a density of $OD_{600} = 1.0$. Silwet L-77 failed to promote transformation frequency and showed an adverse effect at higher concentrations (> 0.015%). Using this optimized transformation system, transgenic black ash shoots expressing a synthetic *Bacillus thuringiensis* toxin gene (*cry8D2*) were regenerated. Although no morphological abnormality was observed, transgenic shoots showed severe growth restriction. Three independent transgenic lines were selected for further assessment. All selected lines contained two copies of the *cry8D2* gene, and the expression of the transgene was verified in transcript and protein levels. These transgenic shoots can be used for future bioassay to evaluate its efficacy against EAB.

Keywords Black ash · Emerald ash borer · *Fraxinus* · Genetic transformation

Introduction

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is an invasive wood-boring insect native to Asia (China, Japan, Korea, Mongolia, and eastern Russia) that is threatening North American native ash trees; first identified in Michigan in 2002. Although a tremendous effort has been made to contain the nascent infestation, EAB has spread rapidly through natural dispersal and human-assisted firewood movement, and as of August 2017, it was found in 31 states, and the Canadian provinces of Windsor and Quebec (http://www.emeraldashborer.info). Indeed, the EAB is the single most destructive insect invasion in North America, often being compared to the chestnut blight, a devastating fungal disease that destroyed 3.5 billion American chestnut trees in the early 1990s. It appears likely that approximately 9 billion ash trees are potentially at risk of extirpation nationally (Mercader et al. 2009; Muirhead et al. 2006).

Black ash (*Fraxinus nigra* Marsh.) is one of the highly susceptible ash species, and is native to northeastern North America and eastern Canada. In addition to the economic impact of black ash mortality caused by EAB to the timber industry (flooring, millwork, and crates), the loss of black ash could have devastating impacts ecologically resulting in dramatic shifts in riparian ecosystem structure and function, as black ash is the dominant species in wetland forests (Ellison et al. 2005; Telander et al. 2015). Black ash wood is also preferred by Native Americans for making

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splints for basketry. This is a significant component of cultural and historical identity as well as a good income source, and now this tradition is under threat (Herms and McCullough 2014; Willow 2011).

Currently, a number of management strategies have been implemented to combat EAB including insecticide options (Poland et al. 2016) and biocontrol using parasitoids (USDA–APHIS/ARS/FS 2017). Molecular approaches have also been studied with Manchurian ash (*F. mandshurica*), resistant Asian ash species sharing a co-evolutionary history with EAB, to find endogenous factors that may confer resistance (Bai et al. 2011; Cipollini et al. 2011; Whitehill et al. 2012). Genetic engineering, meanwhile, could be an excellent alternative to control EAB and conserve ash trees through the introduction and expression of an insecticidal δ-endotoxin gene (*cry* gene) from *Bacillus thuringiensis* (*Bt*) into the ash genome. Attempts have been made to develop *Bt*-transgenic plants, and insect-resistance was successfully established in various plant species, not only for agricultural crops but also for a few forest trees (Génissel et al. 2003; Harcourt et al. 2000; Lachance et al. 2007; Shin et al. 1994; Tang and Tian 2003). Among more than 750 different *Bt* genes, the Cry8Da toxin produced by *Bt* subsp. *galleriae* SDS-502 strain was tested and its efficacy was confirmed against the EAB (Bauer and Londoño 2011).

Agrobacterium-mediated genetic transformation is widely used as a tool for the introduction of foreign genes into plants. Many tree species, however, have proven to be recalcitrant to genetic modification showing relatively low transformation frequency and regeneration rate. Thus, the transformation conditions need to be optimized for a particular plant species. Previous reports have demonstrated that *Agrobacterium*-mediated transformation was successful for other *Fraxinus* spp. including white ash (*F. americana*) (Palla and Pijut 2015), green ash (*F. pennsylvanica*) (Du and Pijut 2009; Roome 1992), and pumpkin ash (*F. profunda*) (Stevens and Pijut 2014), but no transformation system has been developed for black ash. Therefore, the main goal of this research was to develop an efficient *Agrobacterium*-mediated transformation protocol for black ash. Our previously developed methods for in vitro regeneration of black ash were used as a basis for regeneration of putative transformants (Beasley and Pijut 2013; Lee and Pijut 2017a). Various factors affecting transformation efficiency were evaluated to optimize the procedure. We then used a synthetic, full-length *cry8D2* gene and its expression in transgenic shoots was confirmed by real-time polymerase chain reaction (qPCR) and Western blot analysis. We herein describe the first production of genetically modified *Bt*-ash lines that can be used for future bioassays to evaluate their efficacy against the EAB.

Materials and methods

Plant material

Mature black ash seeds were obtained from the National Tree Seed Centre (Natural Resources Canada, Fredericton, New Brunswick, Canada) and stored in sealed bags at 4 °C in the dark until used. Seeds were disinfested and stratified as described by Beasley and Pijut (2013), mature embryos were extracted and cultured horizontally on a modified Murashige and Skoog (1962) (MS; M499, *Phyto*Technology Laboratories, Shawnee Mission, KS) pre-culture medium 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), and supplemented with 13.3 µM 6-benzylaminopurine (BA), 4.5 µM thidiazuron (TDZ), 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water (C195, *Phyto*Technology Laboratories) in Petri plates (100 × 25 mm; 45 mL medium) to allow germination. Hypocotyls were excised from 7-day-old in vitro seedlings and used to determine explant sensitivity to the antibiotics and for transformation experiments. Unless noted otherwise, all media contained 3% (w/v) sucrose and 0.7% (w/v) Bacto agar (No. 214030; Becton Dickinson and Co., Sparks, MD) with the pH adjusted to 5.7 before autoclaving for 20 min at 121 °C. All cultures were maintained in a growth room at 24 ± 2 °C under a 16 h photoperiod (approximately 80 µmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps.

Effect of kanamycin and timentin on hypocotyl explants

The effect of antibiotics on callus induction and shoot formation was investigated to determine the optimum concentration for selection of transformed explants. Seven-day-old hypocotyls were cultured horizontally on MS pre-culture medium with kanamycin (0, 10, 20, 30, 40, or 50 mg L^{-1}) or timentin (0, 100, 200, 300, 400, or 500 mg L^{-1}) in Petri plates (100 × 25 mm; 45 mL medium). Antibiotics were dissolved in sterile, deionized water and filter-sterilized (0.22 µm) and added to the medium after autoclaving. Hypocotyls were cultured for 3 weeks, transferred to fresh treatment medium, and the regeneration response for callus and shoot induction were recorded after 6 weeks of culture in vitro. Three replicates of 12 hypocotyls each were used for each treatment.

Transformation vectors and *Agrobacterium* culture

Based upon previous results in our lab, the pq35GR vector (Fig. 1a; Li et al. 2004) that contained a reporter gene

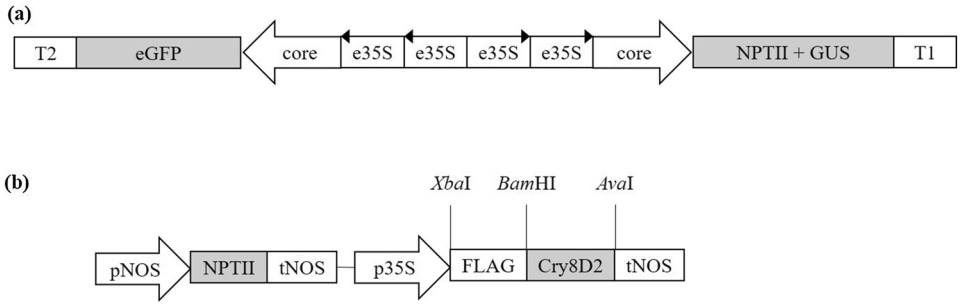

Fig. 1 Schematic diagram of T-DNA regions of pq35GR and pBI121-*cry8D2* constructs. **a** The pq35GR vector consisted of bi-directional CaMV 35S promoters containing two divergently arranged enhancer repeats, an *eGFP* gene, and an *NPTII* and *GUS* fusion gene (Li et al. 2004). eGFP, enhanced green fluorescent protein gene; core, 35S core promoter; e35S, enhancer fragment isolated from the CaMV 35S promoter; NPTII + GUS, neomycin phosphotransferase gene and β-glucuronidase gene; T1 and T2, terminator and polyadenylation signal sequences from the NOS gene and the 35S transcript, respectively; **b** A binary vector pBI121 was used for insertion of synthetic *cry8D2* gene driven by CaMV 35S promoter. pNOS and tNOS, promoter and terminator sequence from the NOS gene, respectively

encoding enhanced green fluorescent protein (eGFP) driven by the CaMV 35S promoter was utilized to test the various factors affecting transformation efficiency. The pq35GR vector was transformed into two *Agrobacterium tumefaciens* strains, EHA105 and GV3101, to test the effect of *Agrobacterium* strain on transformation efficiency. EHA105 strain harboring pq35GR vector was used for further experiments to test the effects of bacterial density (OD_{600} = 0.5, 1.0, or 1.5) and Silwet L-77 concentration (0, 0.0075, 0.015, or 0.03%).

Single *Agrobacterium* colonies harboring pq35GR vector were inoculated in 20 mL liquid YEP medium (10 g L^{-1} yeast extract, 10 g L^{-1} bactopeptone, and 5 g L^{-1} NaCl, at pH 7) in the presence of appropriate antibiotics (20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin), and cultured in the dark for 2 days at 28 °C. Once OD_{600} = 0.9–1.0 was reached, the cells were collected by centrifugation at 4500 rpm for 15 min, and the pellet was re-suspended in 20 mL liquid MS co-culture medium (13.3 µM BA, 4.5 µM TDZ, 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water) with the addition of 100 µM acetosyringone. To test the effect of bacterial density on transformation efficiency, *Agrobacterium* grown at stationary phase was harvested and re-suspended in 20 mL liquid MS co-culture medium to adjust a final OD_{600} = 0.5, 1.0, or 1.5. The suspension of *Agrobacterium* was incubated at 28 °C for another 2–3 h before being used for co-cultivation with hypocotyl explants. To test the effect of Silwet L-77 concentration on transformation efficiency, Silwet L-77 (0, 0.0075, 0.015, or 0.03%) was added to liquid MS co-culture medium before vacuum-infiltration.

The vector pBI221-8D2, containing a synthetic, full length Cry8Da protoxin was obtained from Phyllom LLC (Mountain View, CA). In this gene, the ratio between GC and AT contents was modified to increase the GC content from the naturally occurring *Bt cry8Da* sequence (personal communication from Phyllom). This vector was previously used in a particle-gun transformation system for turf grass (Phyllom). We were not successful in cloning the *cry8Da* gene into our pq35GR vector. We therefore utilized the pBI121 binary vector, excised out GUS, and inserted the *cry8Da2* from pBI221-8D2 downstream of the CaMV 35S promoter. A FLAG tag was added to the N-terminal region of *cry8D2* sequence using *Xba*I and *Bam*HI (Fig. 1b). The final expression cassette for Cry8D2 was transformed into *Agrobacterium* strain EHA105.

Agrobacterium-mediated transformation and regeneration of putative transformants

Hypocotyls excised from 7-day-old embryos were submerged in 20 mL liquid MS co-culture medium and sonicated for 90 s. Explants were then immersed in *Agrobacterium* suspension and vacuum-infiltrated (62.5 cm Hg) for 10 min. Explants were then blotted dry on sterile Whatman filter paper and placed horizontally on semi-solid MS co-culture medium for 2–3 days in the dark at 28 °C. After co-cultivation, the explants were rinsed three times in liquid MS co-culture medium to remove excess *Agrobacterium* prior to being cultured horizontally on MS selection medium (13.3 µM BA, 4.5 µM TDZ, 50 mg L^{-1} adenine hemisulfate, 10% (v/v) coconut water, 40 mg L^{-1} kanamycin, and 300 mg L^{-1} timentin) for 6 weeks. Once adventitious shoot buds were induced from putative transformed explants, the kanamycin-resistant explants were cultured on selection medium without TDZ in order to prevent shoot elongation inhibition. Once the insertion of *cry8D2* transgene was confirmed by PCR, the transgenic shoots were regularly subcultured to fresh medium without kanamycin every 3 weeks.

Visualization of enhanced green fluorescent protein

The expression of GFP in the explants was monitored by using a microscope (Nikon Eclipse C*i*, Nikon Instruments Inc., Melville, NY) equipped with a GFP3 filter (excitation 470/40, emission 525/50 nm). Leaves from non-transformed control explants and putative transgenic explants were examined to compare the presence or absence of green fluorescence. Images were photographed with a digital camera, and GFP-positive explants were counted from the total transformed explants to evaluate transformation efficiency.

Molecular analysis of transgenic lines

DNA extraction and PCR analysis

Genomic DNA was isolated from the leaves of non-transformed control shoots and the regenerated putative transgenic shoots following the procedure described by Lefort and Douglas (1999). PCR analysis was performed to specifically amplify a 352-bp fragment of *npt*II, and a 430-bp fragment and 3.5-kb full-length of *cry8D2*. The pBI121-*cry8D2* plasmid was used as a positive control template. The PCR reaction mix was prepared in a total volume of 25 µL containing 1 µL genomic DNA (100–200 ng μL^{-1}), 12.5 µL One *Taq* Hot Start 2 × Master Mix (New England Biolabs, Ipswich, MA, USA), 1 µL each of 10 µM *npt*II_F and *npt*II_R or 10 µM cry_430_F and cry_430_R or 10 µM cry_Full_F and cry_Full_R primers (Table 1), and sterile, deionized water was added to make the final volume. All cycling programs consisted of an initial denaturation at 94 °C for 30 s, followed by 35 cycles of 94 °C for 15 s, 55 °C for 15 s, 68 °C for 30 s for *npt*II and *cry8D2* partial fragments, or 3 min for *cry8D2* full sequence, and a final extension at 68 °C for 5 min. Amplified products of the PCR were visualized under UV light after gel electrophoresis (1% w/v agarose plus 0.3 µg mL^{-1} ethidium bromide).

Table 1 Primer sequences

Primer	Sequence (5′–3′)
cry_430_F	GAT CGC TCC TTG CGA CTA AC
cry_430_R	GCG TTG CAT AAC CAT TTG TG
nptII_F	TGC TCC TGC CGA GAA AGT AT
nptII_R	AGC CAA CGC TAT GTC CTG AT
cry_Full_F	GCA CAA TCC CAC TAT CCT TCG
cry_Full_R	ATT GCC AAA TGT TTG AAC GA
cry_qRT_F	TGG GAC TAT CAC GTC CAA CA
cry_qRT_R	GCG TAG TTC GCG AGG TC
FnAG_qRT_F	CCT ATT ACT CTC TAC AAC TCG
FnAG_qRT_R	TGA GGA ATC TGA GCA GGC TTT C
eEFAα_F	ACC AGC AAG TCC CAG TTG AGA TG
eEFAα_R	TGA GCC AGG TTC AGC TTC CAA TG

RNA extraction and transgene expression analysis

Total RNA was isolated from leaves of non-transformed control shoots and the regenerated putative transgenic shoots using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Isolated RNA was treated with DNase I (Thermo Fisher Scientific, Grand Island, NY, USA) in order to remove genomic DNA, and the first-strand cDNA was synthesized from 1 µg total RNA using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and an oligo-dT primer. To analyze the relative expression of *cry8D2*, semi-quantitative reverse transcription (RT)-PCR and qPCR were conducted. The qPCR was performed as described by Lee and Pijut (2017b) with 20 µL reaction solution containing 1 µL cDNA, 1 µL each of 10 µM cry_qRT_F and cry_qRT_R or 10 µM eEFAα_F and eEFAα_R primers (Table 1), 10 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), and sterile, deionized water was added to make the final volume. The PCR reaction was as follows: 95 °C for 60 s, followed by 40 cycles of 95 °C for 10 s, 57 °C for 20 s, 72 °C for 30 s, and followed by a melting curve analysis from 65 to 95 °C performed with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Relative transcript levels for mRNAs were obtained using the comparative cycle threshold (C_t) method and normalized to translation elongation factor alpha (*eEFAα*) from black ash. Each reaction was repeated three times.

Determination of transgene copy number

To determine gene copy number of *cry8D2* in PCR-confirmed transgenic lines, qPCR method was used. Black ash *AGAMOUS* (*FnAG*) was selected as a single-copy endogenous reference gene (Lee and Pijut 2017b). Genomic DNA was extracted as described previously, and used as a template. A 143-bp fragment of *FnAG* and a 99-bp fragment of *cry8D2* were amplified using primers FnAG_qRT_F and FnAG_qRT_R or cry_qRT_F and cry_qRT_R, respectively (Table 1). Each reaction contained the following reagents in 20 µL:2 µL genomic DNA (100 ng μL^{-1}), 1 µL of each primer, 10 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad), and sterile, deionized water to make the final volume. The PCR reaction was as follows: 95 °C for 60 s, followed by 40 cycles of 95 °C for 10 s, 57 °C for 20 s, 72 °C for 30 s, and followed by a melting curve analysis from 65 to 95 °C performed with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Each reaction was repeated three times, and the relative standard curve of the serial dilution of genomic DNA was constructed for both the *cry8D2*

and reference gene. Gene copy number was estimated by comparing C*t* values of the *cry8D2* with *FnAG*, following the calculation reported by Weng et al. (2004).

Western blot analysis

Total protein was extracted from leaves of transgenic lines of which the *cry8D2* transcript expression was confirmed. Plant tissues were ground in liquid nitrogen and re-suspended in extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA pH 8, protein inhibitor cocktail, 1 mM PMSF, 2 mM DTT, 0.1% Triton X-100). The total protein from each transgenic line was separated on 8% SDS-PAGE gels (Laemmli 1970), transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific), and immunoblotted with a monoclonal anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA). The membrane probed with antibody was detected using the SuperSignal® West Pico Chemiluminescent Substrate™ according to the manufacturer's protocol (Thermo Fisher Scientific).

Statistical analysis

Data were analyzed using SAS® 9.3 software package (SAS® Institute Inc. 2011). The mean with standard error (± SE) was presented. The percent callus formation and shoot induction, and transformation efficiency were subjected to analysis of variance (ANOVA). Significant difference between treatments was tested by a Duncan's multiple comparison test ($p = 0.05$).

Results and discussion

Effect of kanamycin and timentin on hypocotyl explants

To determine the optimal concentration of antibiotics for selection of putative transgenic lines, hypocotyls were exposed to various concentrations of kanamycin and timentin. Kanamycin effectively inhibited callus formation and shoot bud induction as the concentration was increased, and showed significant inhibition at 30 mg L^{-1} or higher concentration (Table 2). Other ash studies reported that 20 mg L^{-1} kanamycin significantly inhibited organogenesis of green and pumpkin ash (Du and Pijut 2009; Stevens and Pijut 2014). Palla and Pijut (2015) found a similar result that 30 mg L^{-1} kanamycin was lethal to white ash shoot organogenesis. Timentin had little influence on black ash hypocotyl regeneration, showing a shoot induction frequency ranging from 40 to 53.3% (data not shown). We determined 40 mg L^{-1} kanamycin and 300 mg L^{-1} timentin to be the optimal concentration for screening transgenic shoots in subsequent transformation experiments, in order to minimize the potential for escape of non-transformed explants and to ensure only transformed tissues would be regenerated.

Table 2 Effect of kanamycin concentration on percent callus formation and adventitious shoot regeneration of black ash hypocotyls

Kanamycin (mg L^{-1})	Callus formation (%)[a]	Shoot bud induction (%)[a]
0	55.6 ± 2.8a	58.3 ± 8.3a
10	38.9 ± 2.8b	36.1 ± 2.8a
20	16.7 ± 4.8c	2.8 ± 2.8b
30	2.8 ± 2.8d	5.6 ± 2.8b
40	0d	0b
50	0d	0b

[a]Frequency of callus formation and shoot bud induction were evaluated from 6-week-old explants. Values represent means ± SE; followed by the same letter in the same column were not significantly different by the Duncan's multiple comparison test ($p < 0.05$)

Optimization of conditions for efficient transformation of black ash hypocotyls

The transformation conditions such as *Agrobacterium* strain, bacterial density, and the concentration of Silwet L-77 were optimized. Leaves regenerated from transformed hypocotyls were collected after 6 weeks, and the expression of GFP was monitored to aid in calculation of transformation efficiency (Fig. 2).

The effect of two commonly used *Agrobacterium* strains, EHA105 and GV3101, were tested. Two-day cultures of *Agrobacterium* were harvested when the OD_{600} value reached between 0.7 and 0.8. The rate of GFP positive explants was significantly higher when *Agrobacterium* strain EHA105 was used than using GV3101; it was approximately 2.7-fold higher (Fig. 3a). The choice of appropriate *Agrobacterium* strain for a particular plant species is one of the most important factors for successful plant transformation, as different strains show various virulence on host plants, determining the rate of T-DNA transfer (Han et al. 2000; Ko et al. 2003; Song et al. 2011). For European plum (*Prunus domestica* L.), *Agrobacterium* strains LBA4404 and EHA105 overall exhibited higher rates of transformation than GV3101 (Song et al. 2011). Han et al. (2000) found that EHA105 was superior to C58 and LBA4404 for some recalcitrant cottonwood hybrids, although C58 was the most efficient for *Populus nigra* transformation (Confalonieri et al. 1994). Soybean (*Glycine max* L.) cotyledon explants transformed with strains EHA105 and GV3101 showed low or complete inhibition of embryogenic response and no GUS expression, while those transformed with strain KYRT1 showed the

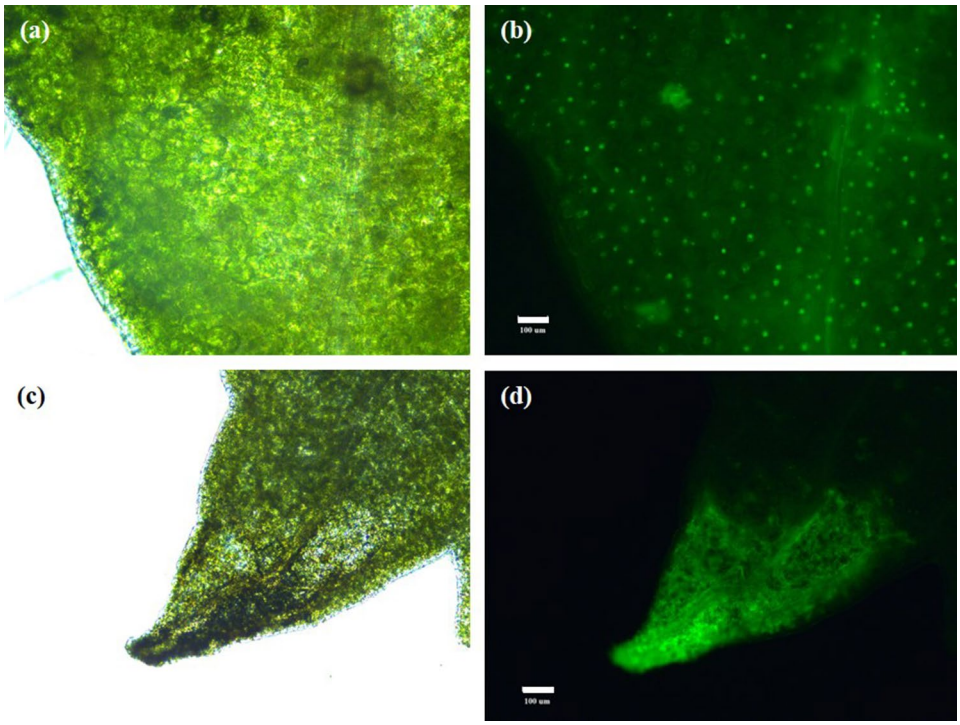

Fig. 2 Visualization of green fluorescent protein (GFP) from transformed leaves at week six under bright field image **a** and **c** and through GFP excitation filter (470/40 nm) **b** and **d** (bar 100 µm)

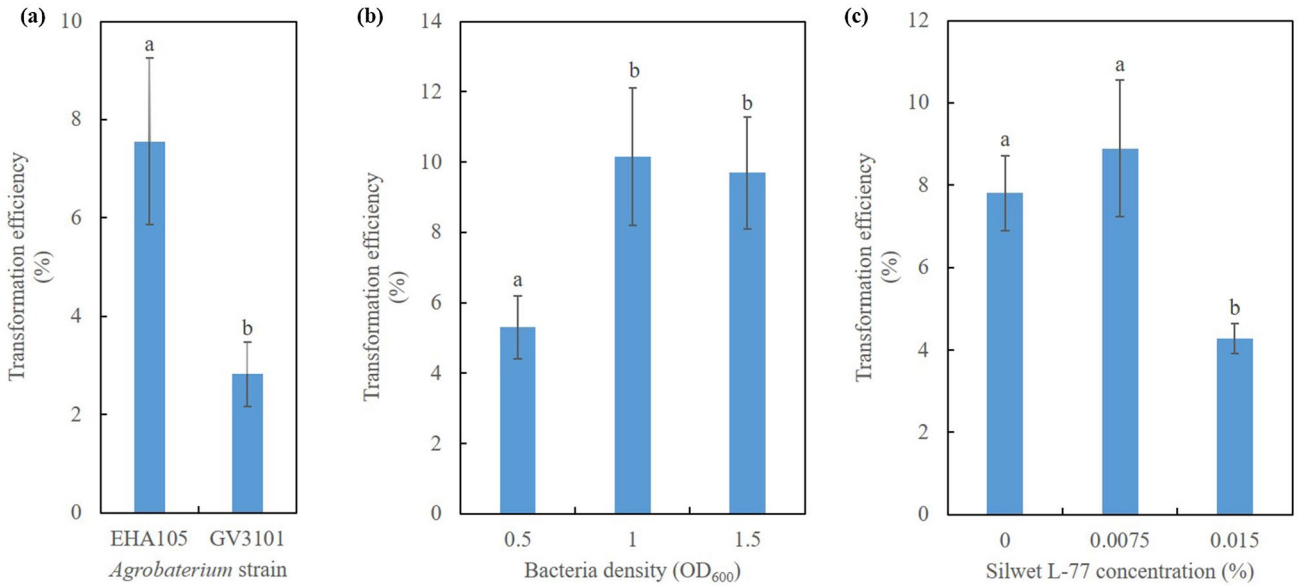

Fig. 3 *Agrobacterium*-mediated transformation efficiency in black ash hypocotyls. Effects of **a** *Agrobacterium* strain, **b** bacteria density, and **c** Silwet L-77 concentration on transformation. Bacterial cultures were harvested at $OD_{600}=0.7–0.8$ to compare the effect of *Agrobacterium* strain. EHA105 strain ($OD_{600}=0.9–1.0$) was used to test the effects of bacterial density and Silwet L-77 concentration. Values are means ± SE for 40–52 explants per treatment, replicated three times. Different letter indicates a significant difference based on a Duncan's multiple comparison test ($p<0.05$)

highest frequency of embryogenesis and GUS expression (Ko et al. 2003). According to our results, we used strain EHA105 for subsequent transformation experiments for black ash.

The influence of bacterial density on transformation efficiency was investigated. The highest frequency of transformation was obtained with a final bacterial density of $OD_{600}=1.0$ (Fig. 3b), while the lowest transformation

efficiency was observed when $OD_{600} = 0.5$ was used. Although the transformation efficiency was not statistically different between $OD_{600} = 1.0$ and $OD_{600} = 1.5$, *Agrobacterium* overgrowth followed by necrosis was observed for explants cultured in *Agrobacterium* with $OD_{600} = 1.5$. Transformation of peanut (*Arachis hypogaea*) petioles was optimized at an $OD_{600} = 0.6$, and exposure to higher concentrations ($OD_{600} = 0.8$ and 1.0) of bacterial suspension caused contamination of the explants (Liu et al. 2016). The highest transformation efficiency for hybrid aspen (*Populus tremula*×*P. tremuloides*) was obtained with a final bacterial density of $OD_{600} = 1.0$, while the transformation yield dropped by half for the $OD_{600} = 0.5$ and 2.0 (Takata and Eriksson 2012). Although higher density of bacteria should be used for recalcitrant species to ensure increasing bacterial attachment, it may cause hypersensitive physiological response of explants, resulting in decrease of regeneration potential and unsuccessful recovery of transformed cells (Sreeramanan et al. 2008; Yong et al. 2006).

The effect of the surfactant Silwet L-77 concentration was also investigated. Silwet L-77 is often used for *Agrobacterium*-mediated transformation, especially in *Arabidopsis* transformation, replacing the vacuum-infiltration step of the floral dip method. Silwet L-77 allows a bacterial solution to penetrate into plant tissues such as flowers and leaves, enhancing the transformation efficiency. However, necrosis of explants was observed when a high concentration of Silwet L-77 was used (Li et al. 2009), as higher concentrations over 0.02% (v/v) may be toxic (Zhang et al. 2006). Thus, a proper concentration of Silwet L-77 should be determined. In our study, the control group of zero Silwet L-77 showed similar statistical transformation efficiency with 0.0075% Silwet L-77 treatment (Fig. 3c). But, with 0.015% Silwet L-77, the transformation efficiency showed a significant decrease (Fig. 3c), and necrosis was observed for most hypocotyls transformed in medium containing 0.03% Silwet L-77 (data not shown). The concentration of Silwet L-77 was optimized at 0.005 and 0.02% for *Arabidopsis* seedling cotyledons and flowers, respectively (Li et al. 2009; Zhang et al. 2006). For hybrid aspen leaf explants, Silwet L-77 concentration up to 0.015% was positively correlated with transformation efficiency, but the viability of explants was decreased with 0.03% Silwet L-77 (Takata and Eriksson 2012). Our data suggested that the surfactant Silwet L-77 was not necessary for black ash transformation using hypocotyls, as long as sonication and vacuum-infiltration were applied. Sonication-assisted *Agrobacterium* transformation (SAAT) has been widely applied for various plants to enhance transformation efficiency (Alam et al. 2017; Beranová et al. 2008; Chu et al. 2016), including other ash species (Du and Pijut 2009; Palla and Pijut 2015; Stevens and Pijut 2014). Brief periods of SAAT causes a large number of microwounds throughout the plant tissue, allowing *Agrobacterium* easy access into the target plant cells, even in meristematic tissue buried under several layers (Trick and Finer 1997). Combined with sonication, vacuum-infiltration has been used to increase the rate of T-DNA delivery by enhancing *Agrobacterium* penetration into plant tissue (de Oliveira et al. 2009; Liu et al. 2005; Subramanyam et al. 2011).

Therefore, an efficient transformation procedure consisting of 90-s sonication plus 10-min vacuum-infiltration using *Agrobacterium* strain EHA105 at $OD_{600} = 1.0$ without the addition of Silwet L-77, was developed for black ash hypocotyl explants.

Regeneration of transgenic black ash shoots expressing *cry8D2*

Using our optimized transformation system, we developed transgenic black ash expressing the insecticidal *Bt-cry* gene. In the present study, we used the *cry8D2* gene, a synthetic version of the natural *cry8Da* gene produced by *Bt* SDS-502 that showed a narrow toxicity spectrum to *Anomala cuprea* (Coleopteran) (Asano et al. 2003), including EAB (Bauer and Londoño 2011). It has been documented that the expression levels were low when wild-type native *cry* genes were transformed into plants, since some features typical of native *cry* genes such as AT-rich nucleotide sequences, poor coding capacity, and cryptic polyadenylation signals reduced transcript stability in plants and resulted in rapid degradation of mRNA encoding the *cry* gene (Diehn et al. 1996; Murray et al. 1991). Thus, our *cry8Da* gene was manipulated for plant usage with increasing GC content. The Cry8D2 expression cassette *35S::FLAG-cry8D2* (Fig. 1b) was transformed into black ash hypocotyls, and transformants were selected on MS medium containing 40 mg L^{-1} kanamycin (Fig. 4a). A total of 117 kanamycin-resistant hypocotyls survived from a total of 3350 inoculated explants, giving a transformation frequency of 3.5%. Among the 117 putative transgenic lines, only 23 explants successfully regenerated adventitious shoots (Fig. 4b). PCR analysis confirmed the presence of the Cry8D2 expression cassette from 21 individual lines (Fig. 4c). However, transgenic shoots generally showed severe growth restriction in comparison to non-transformed shoots, and some PCR-positive transgenic shoots deteriorated followed by necrosis during continuous subculture (data not shown). Regarding the slow growth rate, negative effects of kanamycin were reported on shoot proliferation and elongation of transgenic pumpkin and white ash, even when the transgenic shoots showed kanamycin-resistance conferred by expression of the *npt*II gene (Palla and Pijut 2015; Stevens and Pijut 2014). However, a normal rate of shoot elongation was recovered when the transgenic white ash shoots, of which the presence of transgenes had been confirmed by PCR, were cultured on elongation

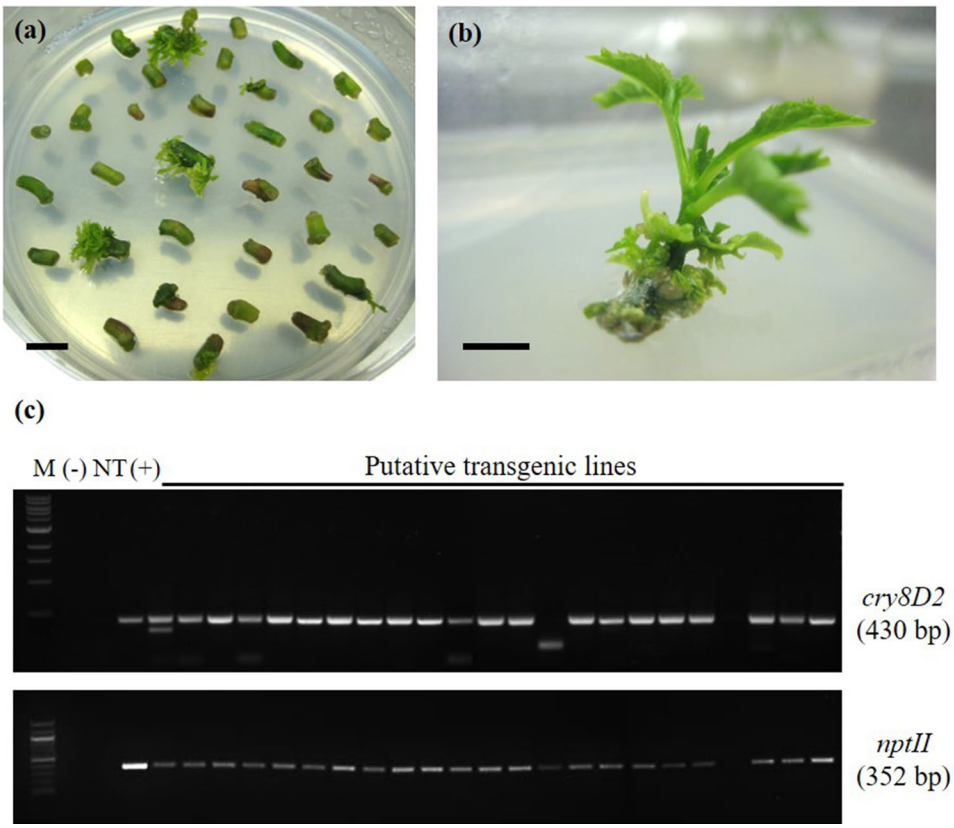

Fig. 4 *Agrobacterium*-mediated transformation and regeneration of transgenic black ash expressing *cry8D2*. **a** In vitro selection of transformed hypocotyls on medium containing 40 mg L^{-1} kanamycin (bar 1 cm); **b** Adventitious shoot regenerated from putative transgenic hypocotyl (bar 1 cm); **c** Detection of a 430-bp fragment of *cry8D2* transgene and a 352-bp fragment of npt*II* via PCR from the leaves of putative transgenic shoots. *M* DNA ladder (1-kb for *cry8D2* and 100-bp for *npt*II), (−) water control, *NT* negative control of non-transformed black ash DNA, (+), positive vector control

medium without kanamycin after two to three subcultures (Palla and Pijut 2015). In addition to removal of kanamycin from the medium, application of a liquid medium overlay has been shown to increase the overall growth rate of transgenic pumpkin ash shoots (Stevens and Pijut 2014). Neither of these steps made any difference on transgenic black ash shoot growth, but initiated excess callus formation. Similarly, liquid medium overlay failed to promote transgenic white ash shoot growth (Palla and Pijut 2015), although it enhanced elongation of non-transformed white ash axillary shoots (van Sambeek et al. 2001).

Some plant transformation studies reported that overexpression of a *Bt-cry* gene showed adverse effects such as significantly delayed plant growth and development (Acharjee et al. 2010; Chakrabarti et al. 2006; Khatodia et al. 2014; Rawat et al. 2011; Sachs et al. 1998) and phenotypic abnormality (Singh et al. 2016). Such detrimental effects in *Bt* transgenic plants might be because of the high-level of expression of Cry toxic protein. Chakrabarti et al. (2006) speculated that high-levels of Cry9aA2 protein accumulation (approximately 10% of the total soluble protein) in tobacco leaves caused the delay in plant development. Acharjee et al. (2010) reported significant reduction in growth rate and seed production of transgenic chickpea (*Cicer arietinum* L.) lines expressing high-levels of Cry2Aa protein. Similarly, transgenic rice expressing Cry1Ac and Cry2A protein at high-level (> 1% of total soluble protein) showed stunted growth and sterility, while such defects were not observed when another insecticidal gene (snow drop lectin; *gna*) was expressed at higher levels (up to 2% of total soluble protein) (Gahakwa et al. 2000). However, transgenic tobacco overexpressing *Bt-cry2Aa2* showed normal growth even with higher accumulation of *Bt* insecticidal protein in leaves (45.3% of the total soluble protein) (de Cosa et al. 2001), suggesting that other factors should be considered affecting variation in transgenic plants such as insertional-mutagenesis caused by random-integration of T-DNA, or somaclonal variation (Larkin and Scowcroft 1981; Shu et al. 2002; van Lijsebettens et al. 1991).

Three independent lines (T38, T40-4, and T41-6) were selected that showed stable viability (Fig. 5a) for further assessment. PCR analysis was conducted to confirm that the intact *cry8D2* gene (full length of 3.5-kb) was integrated into the genome of these three selected transgenic lines (Fig. 5b). Rearranged and/or truncated transgene fragments were often found in other plant transformations (Kohli et al. 2003; Makarevitch et al. 2003; Pawlowski and Somers 1998; Weng et al. 2004), so the integration of the intact transgene should be confirmed to ensure the expression of the functional transgene. We then confirmed the expression of *cry8D2* mRNA driven by CaMV 35S promoter. Although CaMV 35S promoter has been widely used for transgene

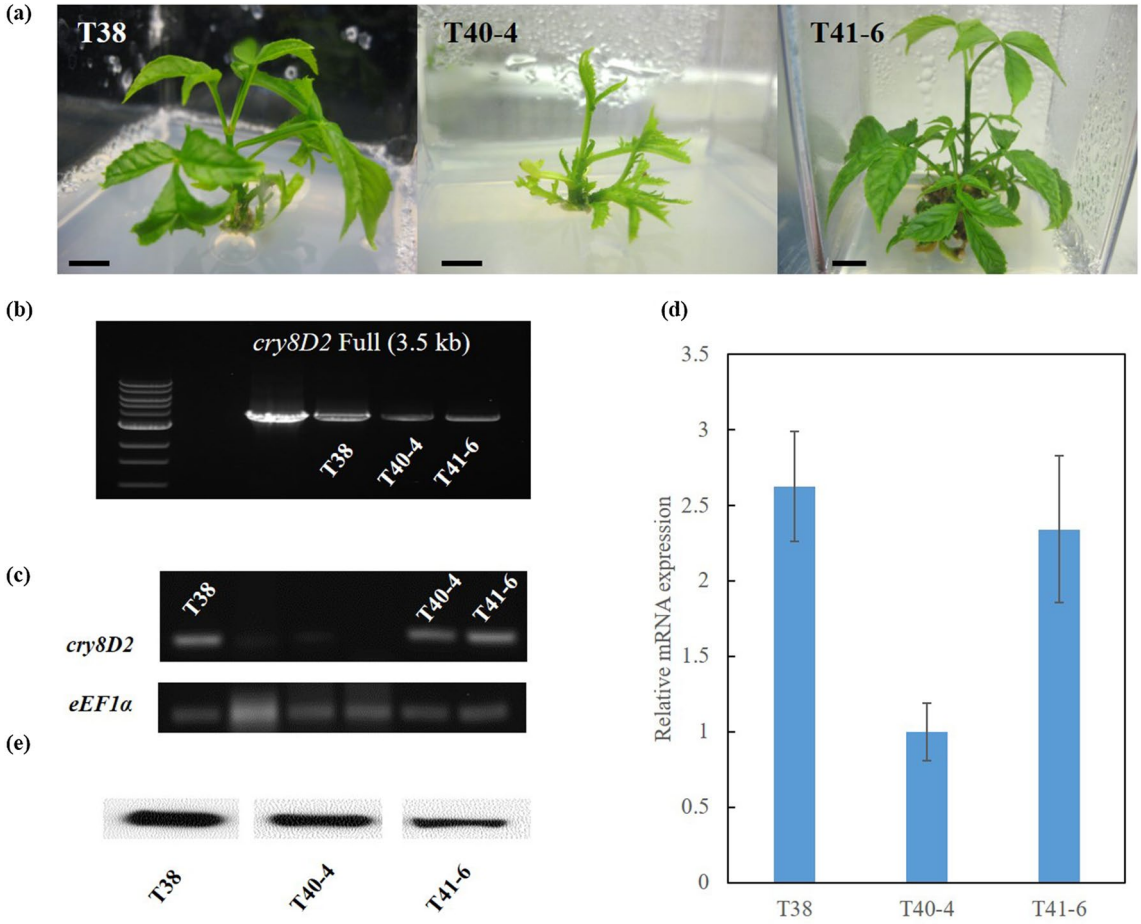

Fig. 5 Confirmation of the insertion of a full-length *cry8D2* transgene, *cry8D2* transcript expression, and Cry8D2 protein accumulation in transgenic black ash shoots. **a** Three independent transgenic black ash shoots harboring *cry8D2* gene (bar 1 cm); **b** Detection of a 3.5-kb full-length *cry8D2* transgene via PCR from the leaves of transgenic shoots. Lane 1 1-kb DNA ladder, lane 2 negative control of non-transformed black ash DNA, lane 3 positive vector control, lanes 4–6 three independent transgenic lines, **c** semi-quantitative RT-PCR analysis and **d** real time qPCR for detecting *cry8D2* transcript expression in the leaves of transgenic shoots. The translation elongation factor alpha (*eEF1α*) gene was used as a control to normalize the expression level. Each reaction was repeated three times. Error bars represent the standard deviation; **e** Western blot analysis to detect Cry8D2 protein expression in the leaves of transgenic shoots. A monoclonal anti-FLAG antibody was used. The Cry8D2 molecular weight observed in the Western blots was about 130 kDa

expression in plants, transgene silencing caused by hypermethylation in the transgene itself or promoter regions has been reported under the control of CaMV 35S promoter (Gambino et al. 2010; Okumura et al. 2016). Consistent transcriptional silencing was observed in transgenic gentian (*Gentiana triflora* × *G. scabra*) that was induced by cytosine methylation exclusively in the CaMV 35S promoter region, regardless of the copy number and the insertion location of T-DNA (Mishiba et al. 2005). In the present study, we also found that some transgenic lines showed gene silencing or very weak expression of *cry8D2* transgene (Fig. 5c; lane 2–4). However, all three selected lines (T38, T40-4, and T41-6) presented a strong *cry8D2* transgene expression, as measured by semi-quantitative RT-PCR (Fig. 5c). The relative transcript expression levels varied among three lines: approximately 2.5-fold higher expression in T38 and T41-6 compared to T40-4 (Fig. 5d). Western blot analysis using total protein extracts from leaves presented the expression of Cry8D2 protein with approximately 130 kDa, although the expression levels were different among the lines (Fig. 5e). T38 expressed the highest level of Cry8D2 protein production, while T40-4 produced more protein than T41-6, that was not consistent with the transcript expression levels (Fig. 5d, e). A similar inconsistent pattern between the level of mRNA and the amount of protein was reported in one transgenic line of hybrid poplar expressing a *cry3Aa* gene, possibly caused by a higher mRNA turn-over or a lower rate of protein synthesis (Génissel et al. 2003).

To determine the copy number of the *cry8D2* transgene in the genome of the transgenic black ash lines, qPCR-based method was used. The relative standard curves for both the single-copy endogenous reference gene

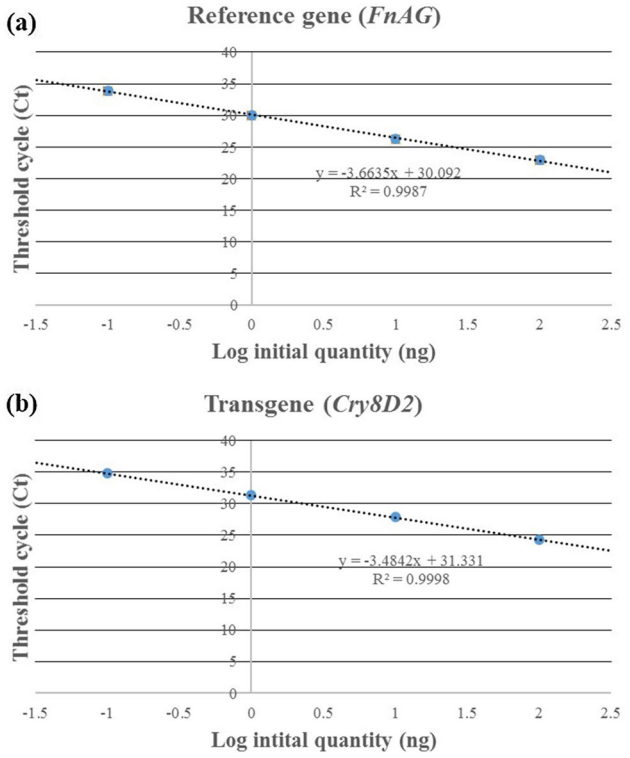

Fig. 6 The relative standard curve of **a** *FnAG*, a single-copy endogenous reference gene and **b** *cry8D2* transgene, obtained by plotting the threshold cycle (C_T) value versus the log of each initial quantity of genomic DNA

Table 3 Estimated copy number of *cry8D2* transgene in transgenic black ash shoots determined by quantitative PCR (qPCR)-based method

Sample	C_T (*FnAG*)	C_T (*cry8D2*)	2′ (X_0/R_0)	Estimated copy number
T38	20.26 ± 00.2	22.92 ± 0.07	2.00 ± 0.16	2
T40-4	19.12 ± 0.10	21.31 ± 0.20	2.02 ± 0.37	2
T41-6	22.92 ± 0.06	24.31 ± 0.02	2.02 ± 0.29	2

Values are mean ± SD. *X0/R0* was calculated by using equation $X0/R0 = 10[(C_{T,X} - I_X)/S_X] - [(C_{T,R} - I_R)/S_R]$. From standard curves of *FnAG* and *cry8D2*, the values of slope (S) and intercept (I) were obtained. Each qPCR reaction was repeated three times

(*FnAG*) and *cry8D2* transgene were constructed using the serial dilution of genomic DNA (Fig. 6). Based on the values of slope (S) and intercept (I) from the curves, the following equations for *FnAG* and *cry8D2* were obtained: $Y_{FnAG} = -3.6635X + 30.092$ and $Y_{cry8D2} = -3.4842X + 31.331$, respectively. The coefficients of determination (R^2) were 0.9987 and 0.9998 for *FnAG* and *cry8D2*, respectively, indicating good reproducibility. Using these equations, the copy number of the *cry8D2* transgene was estimated, and the results revealed that all the transgenic lines contained two copies of the transgene (Table 3). In plant transformation studies, single- or low-copy insertions are desirable, as it generally yields stable transgene expression. However, random integration of transgenes via *Agrobacterium*-mediated transformation often produce multiple-copy insertions in various plant species (Abou-Alaiwi et al. 2012; Dong et al. 2001; Wang and Pijut 2014; Weng et al. 2004), that could result in transgene silencing through transcriptional and/or post-transcriptional gene silencing (TGS and PTGS), which were associated with DNA methylation in the promoter and the coding region, respectively (Fagard and Vaucheret 2000; Matzke et al. 1994). Higher degrees of cytosine methylation within the GFP encoding region and CaMV 35S promoter region were found in transgenic sweet orange (*Citrus sinensis* L.) with multiple T-DNA copies when compared to the single copy transgenic plant (Fan et al. 2011). PTGS was observed in transgenic eastern white pine (*Pinus strobus* L.) with more than three copies of GFP transgene, whereas not in transgenic lines with one copy of T-DNA (Tang et al. 2007). In the present study, no gene silencing was observed in all three transgenic lines with two copies of *cry8D2* transgene (Fig. 5c).

Conclusions

In summary, an efficient *Agrobacterium*-mediated transformation system for black ash hypocotyls was established, and successfully applied to develop transgenic black ash shoots expressing the *Bt* insecticidal gene (*cry8D2*) for possible EAB resistance. Although transgenic *Bt*-black ash shoots showed growth restriction, strong expressions of mRNA and Cry8D2 toxin protein were confirmed. Studies to facilitate transgenic shoot elongation followed by adventitious root induction need to be established. Ultimately, a bioassay with EAB-adult and -larvae should be conducted to evaluate the efficacy of transgenic *Bt*-black ash shoots.

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Author contributions JHL and PMP conceived and designed the research. JHL conducted the experiments and analyzed the data. JHL and PMP wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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