Agrobacterium-mediated Transformation of *Buddleia* Species

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Abstract. Two *Buddleia* cultivars, *B. davidii* ‘Potters Purple’ and *Buddleia* ‘Lochinch’, were transformed using *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pBI121 carrying the neomycin phosphotransferase gene and β -glucuronidase gene (*uidA*). Transgenic plants were recovered from the *Agrobacterium*-infected leaf tissues through organogenesis in the selection medium (woody plant medium containing 250 mg·L⁻¹ cefotaxime plus 500 mg·L⁻¹ carbenicillin plus 40 mg·L⁻¹ kanamycin). The rate of shoot regeneration from transformed leaf tissues increased from 5.7% to 32% through extending cocultivation time from 3 to 9 days. Integration of marker genes was verified with polymerase chain reaction (PCR) and Southern blot analysis. Southern blot analysis confirmed that one to three copies of transgenes were integrated into the *buddleia* genome. This transformation system could be used for improvement of *buddleia* or other related species. Chemical names used: 6-benzyladenine (BA), naphthalene acetic acid (NAA), acetosyringone (AS), 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium (X-Glu), cefotaxime (Cef), carbenicillin (Carb), kanamycin (Km).

The genus *Buddleia* consists of more than 100 species, of which many are woody shrubs providing colorful and fragrant flowers for landscaping and gardening (Dirr, 1998; Hogan, 2003). *Buddleia* plants have a wide adaptability to various environmental conditions such as high soil pH and cold hardiness and can be grown in most states of the United States (Dirr, 1998; Hogan, 2003). *Buddleia davidii*, known as butterfly bush, is a perennial landscape plant that can also be used as an annual bedding plant. Butterfly bush was imported to North America from China in the early 1900s. It is characterized as a fast-growing deciduous shrub with a long flowering period and is well received by many gardeners. However, this species has such concerns as limited flower color (most reds and blues are muddled), excessive growth (1 to 5 m tall, lanky, and wide-spreading), and is a natural virus host (e.g., cucumber mosaic virus, alfalfa mosaic or tomato ringspot virus) (Podaras, 2005). Recently, invasiveness of this species has become an issue because *Buddleia davidii* is a prolific seed producer and its seeds can be dispersed by wind and water and may remain dormant in the soil for many years (Sheppard et al., 2006).

A colorful, compact, and sterile *Buddleia davidii* cultivar would be highly desirable for landscape purposes. Attempts to improve *Buddleia davidii* using conventional breeding methods have resulted in limited success. Different ploidy levels among species in the genus *Buddleia* may impede interspecific crosses; therefore, elite traits cannot be efficiently integrated into newly developed plants (Rose et al., 2000; Tobutt, 1993).

Transgenic plants have been obtained in many woody species (Petri and Burgos, 2005; Poupin and Arce-Johnson, 2005). However, transformation of *Buddleia* species has not been documented to date. The objective of this study was to develop a gene transformation protocol for further improvement of *Buddleia* species.

Materials and Methods

Plant material. Two cultivars, *Buddleia davidii* ‘Potters Purple’ and *Buddleia* ‘Lochinch’, were used in this study. In vitro cultures of the cultivars were subcultured every 4 weeks in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2.67 μ M benzyladenine (BA), 2% sucrose, and 0.35% phytagel (Sigma Chemical Co., St. Louis, MO) and adjusted to pH 5.8 before autoclaving. These cultures were maintained at 25 °C under cool-white light at 36 μ mol·m⁻²·s⁻¹ with a 16-h photoperiod.

Plant transformation. An efficient shoot regeneration system of these two *Buddleia* cultivars was developed by Dai and Castillo (2007). Previous experiments determined that kanamycin at 40 mg·L⁻¹ completely inhibited shoot regeneration from leaf tissues. Therefore, a series of media used for the *buddleia* transformation were made based on the result of the previous experiments:

1. Shoot regeneration medium (SRM): For ‘Potters Purple’: woody plant medium (WPM) (Lloyd and McCown, 1980) + 0.35% phytagel + 5 μ M BA + 5 μ M indole acetic acid (IBA); for ‘Lochinch’: WPM + 0.35% phytagel + 20 μ M BA + 4 μ M IBA.
2. Cocultivation medium (CCM): SRM + 200 μ M acetosyringone (AS).
3. Selection medium (SLM): SRM + CCK₄₀ [250 mg·L⁻¹ cefotaxime (Cef) + 500 mg·L⁻¹ carbenicillin (Carb) + 40 mg·L⁻¹ kanamycin] (antibiotics were filter-sterilized and added into the autoclaved medium when the medium temperature cooled down to $\approx$ 50 °C).

Agrobacterium strain EHA105 (Hood et al., 1993), carrying pBI121 (Clontech, Palo Alto, CA) containing the *npII* gene encoding neomycin phosphotransferase and the *uidA* gene coding for β -glucuronidase (GUS) (Fig. 1), was grown overnight in Luria-Bertani medium with 100 mg·L⁻¹ kanamycin at 28 °C in a shaker at 150 rpm. Cells were collected by centrifugation at 6000 rpm for 15 min, resuspended to 1.0 o.d. at Abs⁶⁰⁰ in WPM medium supplemented with 20 μ M AS without kanamycin, and incubated at 28 °C in a shaker at 150 rpm for 2 h. In vitro leaves were cut through the main vein once ($\approx$ 0.5 $\times$ 0.5 cm) and submerged in a bacterial culture solution for 30 min at 28 °C. Leaf explants were then removed from the bacterial culture solution, blotted on sterilized paper towels, and transferred to the CCM medium in petri dishes (100 mm $\times$ 15 mm, 25 mL medium) for cocultivation in the dark at room temperature. The effect of cocultivation time on transformation efficiency was tested. After cocultivation for 3, 6, and 9 d, leaf explants were washed twice with sterile deionized and distilled water (ddH₂O) and once with sterile ddH₂O plus 250 mg·L⁻¹ Cef and 500 mg·L⁻¹ Carb, and then blotted with sterile paper towels. These *Agrobacterium*-infected leaf explants were inoculated on the SLM for selection in the dark. Cultures were subcultured in fresh SLM and moved to a culture room under the regular culture conditions previously described. Each treatment (cocultivation time) had at least three SLM plates with 10 explants per plate. The control plates were SLM without kanamycin. The experiment was repeated three times.

Shoots regenerated/recovered from SLM were proliferated in MS medium supplemented with 2.67 μ M BA and CCK₄₀. Proliferated shoots (greater than 2.0 cm) were rooted based on the method of Dai and Castillo (2007). Rooted plants were transferred to Sunshine Mix #1 (Fisons Western Corp., Vancouver, Canada) and grown in the greenhouse under a 16–8-h photoperiod with the temperature adjusted to 25 °C in the daytime and 15 °C at night.

Histochemical β -glucuronidase assay. Leaves from the recovered plants were subjected to GUS screening as described by Jefferson (1987). In brief, in vitro young and greenhouse-grown leaves were submerged in

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Fig. 1. Schematic representation of the T-DNA portion of pBI121 plasmid (Clontech, Palo Alto, CA). RB and LB = T-DNA right and left borders; NP = nopaline synthase promoter; NT = nopaline synthase terminator; *nptII* = neomycin phosphotransferase gene; *uidA* = β -glucuronidase gene; 35S-P = CaMV 35S promoter from cauliflower mosaic virus. The vector was introduced into the disarmed *Agrobacterium tumefaciens* EHA 105.

a GUS staining solution containing 200 μ L ddH₂O and 200 μ L X-Glu solution (2 mg·mL⁻¹; Gold Biotechnology, Inc., St. Louis, MO). After incubating at 37 °C overnight, stained leaves were gradually bleached with 70% to 100% ethanol. GUS staining was observed and photographed under a microscope.

Polymerase chain reactions. Genomic DNA was extracted from young leaves of putative transformed and nontransformed buddleia plants based on the method of Lodhi et al. (1994). Reactions of polymerase chain reaction (PCR) were carried out in 25 μ L volume containing 200 μ M dNTPs, 1 μ M of each oligonucleotide primer, 2.5 units *Taq* DNA Polymerase (Promega, Madison, WI), and 25 ng DNA. The reaction conditions were: 1 cycle at 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 s and then one cycle at 72 °C for 7 min. Amplified DNA fragments (10 μ L of reaction) were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. The primers used for screening transgenes were: *nptII* reverse: 5'-GCAGGCATCGCCATGGGTCACGACGA-3' and *nptII* forward: 5'-GCCCTGAATGAAGTGCAGGACGAGGC-3'; and *uidA* reverse: 5'-CCCGGAATAACATACGGCGTG-3' and *uidA* forward: 5'-CCTGTAGAAACCCCAACCCGTG-3'; which produced 410-bp and 365-bp products, respectively.

Southern blot analysis. Approximately 25 to 35 μ g of genomic DNA was digested in a 50 μ L reaction with 1 μ L *Hind*III restriction enzyme at 37 °C for 2.5 h, electrophoresed on a 0.8% TAE (Tris-acetate ethylenediamine tetra-acetic acid) agarose gel, and blotted to a positively charged Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, U.K.). Similarly, digested DNA from untransformed buddleia plants was used as a negative control, whereas 25 to 40 ng of pBI121 plasmid DNA was used as a positive control. The blot was probed with randomly primed ³²P-labeled *nptII* PCR product (4 μ L ddH₂O, 1 μ L 6-mer oligo primers, 1.5 μ L 5 mM dNTPs, 1.5 μ L 10 $\times$ Klenow buffer, 1 μ L Klenow polymerase, and 5 μ L dCTP ³²P). Prehybridization was at 65 °C for 6 h in the hybridization solution [1% bovine serum albumin Fraction V (Sigma Chemical Co.), 0.5 M NaH₂PO₄ (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 mM ethylenediamine tetra-acetic acid]. The denatured DNA probe was added directly to the blot in the prehybridization mixture,

hybridized at 65 °C for 16 h, and then washed with 2 $\times$ SSC (200 μ M sodium chloride and 200 μ M sodium citrate) for 35 min followed with 0.5 $\times$ SSC + 0.1% SDS, and 0.1 $\times$ SSC + 0.1% SDS for 10 min each at 65 °C on a shaker. The blot was exposed to x-ray film (Kodak, New York, NY) at -80 °C for 72 h and developed per the manufacturer's instructions.

Statistical analysis. Data from all experiments were subject to analysis of variation and mean comparison using the GLM procedure of SAS software Version 9.1 (SAS Institute, 2004).

Results and Discussion

Effect of cocultivation time on shoot recovery. In vitro shoots were regenerated from leaf explants infected with *Agrobacterium* EHA105 carrying pBI121 after 8 weeks' culture (two subcultures) in the selection medium (Table 1; Fig. 2A). No shoot was regenerated from noninfected leaf explants. Cocultivation time significantly affected regeneration rate. An average of 32% of leaf explants produced shoots from SLM medium containing 40 mg·L⁻¹ kanamycin after 9 d cocultivation, whereas only 5.7% and 10.6% of explants developed shoots after 3 and 6 d cocultivation, respectively. However, leaf quality was crucial for a long (greater than 6 d) cocultivation. Several other experiments

showed that cocultivation with EHA105 more than 6 d caused severe damage to leaf tissues, resulting in no shoot regeneration. However, mature leaves (3 to 4 weeks old in vitro) showed tolerance to an extended cocultivation time and were easier to recover from the infection of *Agrobacterium* than young leaves (1 to 2 weeks old), allowing the plant cells to initiate regeneration more easily. Regenerated shoots from the selection medium were continuously subcultured three to four times in medium containing CCK₄₀ and then maintained in antibiotic-free medium. No *Agrobacterium* was detected from grinded tissues of transgenic plants inoculated on the antibiotic-free culture medium, indicating that all bacteria had been killed by antibiotics (cefotaxime and carbenicillin) after three to four consecutive subcultures. Confirmed transgenic plants were grown in the greenhouse (Fig. 2B).

Histochemical β -glucuronidase assay. GUS staining showed blue in the leaves collected from both in vitro and greenhouse-grown plants (Fig. 3), indicating that the *uidA* gene expressed in the leaf tissues. It was observed that blue staining can only be detected in in vitro young leaves for some lines. However, in a few other recovered lines, greenhouse-grown leaves showed much darker blue staining than in vitro leaves, showing a differential expression of *uidA* gene under different conditions.

Table 1. The effect of cocultivation time on shoot recovery from putative transformants of two *Buddleia* cultivars.

Cocultivation time (CCT) (days)	Shoot recovery rate (%) ^z		
	Potters Purple	Lochinch	Avg.
3	6.1	5.3	5.7
6	9.4	11.8	10.6
9	31.7	32.3	32
Least significant difference	7.0		
SOV ^y	df	MS	F
CCT	2	1,849.875	7.93**
Cultivar	1	0.00056	0.00 NS
Cultivar $\times$ CCT	2	12.9872	0.06 NS

^zThe shoot recovery rate is expressed as the percentage of leaves infected with *Agrobacterium* strain EHA105 forming shoots after 8 weeks (two subcultures) in vitro culture.

^ySOV = source of variance; MS = mean square; F = F value.

**Significant at $P \leq 0.01$.

NS = nonsignificant.

Fig. 2. (A) Shoots were regenerated from leaf explants infected with *Agrobacterium* EHA105 in woody plant medium containing 5 μ M 6-benzyladenine, 5 μ M naphthalene acetic acid, and CCK₄₀. (B) Transgenic *Buddleia* plants were grown in the greenhouse.

Polymerase chain reactions. GUS-positive regenerated lines were proliferated to have enough leaf tissues for genomic DNA extraction and subjected to PCR verification. The expected fragments of *uidA* (356 bp) and *nptII* (410 bp) genes were successfully amplified from these plants using the specific primers (Fig. 4).

Southern blot analysis. Genomic DNA was digested with *Hind*III. Gel electrophoresis showed that DNA was well digested and separated. The digested DNA was hybridized with the ³²P-labeled fragment of the *nptII* gene probe prepared from PCR product. The result showed that the transgenic lines exhibited one to three distinctive restriction fragments (Fig. 5), confirming that the *nptII* gene was integrated into the *Buddleia* genome.

In this study, genetic transformation of two *Buddleia* cultivars has been confirmed. Many factors such as genotype, *Agrobacterium* strain, conditions of infection, and cocultivation affect transformation frequency. The *Buddleia* cultivars used in this study are very amenable to plant regeneration from leaf tissues (Dai and Castillo, 2007). Thus, higher

transformation efficiency may be achieved if some improvements can be made during the *Agrobacterium* infection and transformant selection. For example, preconditioning explants, application of vacuum technique during the infection process, using different *Agrobacterium* strains, and extension of the cocultivation time may increase the T-DNA delivery efficiency. The large F value of cocultivation time in this study indicated that the time of cocultivation was one of the most important factors attributing to a significant increase of shoot recovery from the selection medium, which is in agreement with other research (Cardoza and Stewart, 2003; Forreiter et al., 1997).

Many transgenic plants such as most monocot species have been initiated using embryogenic tissues from seedlings such as immature embryos, hypocotyls, and cotyledons. This limits application of gene transformation for improving vegetatively propagated species, especially for elite cultivar improvement, because of the trait segregation of seed-propagated plants. This is the first article reporting genetic transformation of *Buddleia* species, which was achieved by using leaf tissues from a mature plant. Therefore, this

method can be used to improve target trait(s) of an elite plant cultivar without changing any other genetic makeup, providing a useful tool for developing new cultivars of this and other species.

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Fig. 3. β -glucuronidase staining of a leaf from transformed (A) and nontransformed (B) *Buddleia* plants. The blue color in A is the result of active beta-glucuronidase activity.

Fig. 4. Polymerase chain reaction amplification of *nptII* (A) and *uidA* (B) genes in transformed buddleia plants. Lanes M are DNA ladders. Lane P is positive control of plasmid pBI121. Lane N is negative control of untransformed buddleia. Lanes 1 to 4 are transformed lines.

Fig. 5. Confirmation of transgene (*nptII*) in transformed *Buddleia*. Lanes N, P, and 1 to 2 are digested DNA with *Hind*III from nontransgenic control (N), plasmid pBI121 (P), and transgenic lines (1 and 2), respectively. The digested DNA blot was probed with randomly primed ³²P-labeled *nptII* polymerase chain reaction product.