

# *Agrobacterium tumefaciens*-Mediated Transformation of Wild Tobacco Species *Nicotiana debneyi*, *Nicotiana clevelandii*, and *Nicotiana glutinosa*

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## Abstract

Studies on *Agrobacterium tumefaciens*-mediated transformation of wild tobaccos *Nicotiana debneyi*, *Nicotiana clevelandii*, and *Nicotiana glutinosa* were conducted. Leaf disks were infected and co-cultivated with *A. tumefaciens* strain EHA105 carrying the binary vector pBISN1 with an intron interrupted  $\beta$ -glucuronidase (GUS) reporter gene (*gusA*) and the neomycin phosphotransferase gene (*nptII*). Selection and regeneration of kanamycin resistant shoots were conducted on regeneration medium containing 8.88  $\mu$ M 6-benzylaminopurine (BAP), 0.57  $\mu$ M indole-3-acetic acid (IAA), 50 mg·L<sup>-1</sup> kanamycin and 250 mg·L<sup>-1</sup> timentin. Kanamycin resistant shoots were rooted Murashige and Skoog (MS) medium containing 100 mg·L<sup>-1</sup> kanamycin and 250 mg·L<sup>-1</sup> timentin. Using this protocol, kanamycin-resistant plants were obtained from all three wild tobaccos at frequencies of 75.6% for *N. debneyi*, 25.0% for *N. clevelandii*, and 2.8% for *N. glutinosa*. Transcripts of *nptII* and *gusA* were detected in kanamycin-resistant T<sub>0</sub> transformants (i.e., 2 for *N. glutinosa* and 5 for each of the *N. debneyi* and *N. clevelandii*) by the reverse transcript polymerase chain reaction (RT-PCR), and histochemical GUS assays confirmed expression of *gusA* in both T<sub>0</sub> plants and T<sub>1</sub> seedlings. The results indicate that the protocols are efficient for transformation of wild tobacco *N. debneyi* and *N. clevelandii*.

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## Keywords

Regeneration, Transformation, Transgenic Plant, *Nicotiana*

### 1. Introduction

Of over 75 *Nicotiana* species, *Nicotiana tabacum* (cultivated tobacco) and *N. benthamiana* (wild tobacco) are two major model species for genetic transformation due to their high susceptibility to *Agrobacterium tumefaciens* and high regeneration capacity of leaf explants through shoot organogenesis [1]-[4]. *N. tabacum* ( $2n = 4x = 48$ ) is an amphidiploid of interspecific hybrids and a desirable model plant for studying fundamental biological processes through functional genomics and biotechnology approaches [3]-[5]. Allotetraploid *N. benthamiana* ( $2n = 4x = 38$ ) is widely used for studying plant-microbe interactions and functional gene analyses through virus-induced gene silencing [6] [7]. Draft genome sequences for both *N. tabacum* and *N. benthamiana* have been released [5] [7]. In contrast, few studies have been conducted on other wild tobacco species.

*N. debneyi* ( $2n = 4x = 48$ ) is a desirable material to study hybridity and *N. debneyi*-derived resistance to black root rot disease in *Nicotiana* species [8]-[10]. *N. clevelandii* ( $2n = 4x = 48$ ) is susceptible to a large number of plant viruses and *N. glutinosa* ( $2n = 2x = 24$ ) shows resistance to a broad range of tobamoviruses [11], both species are desirable wild germplasm for studying genetic control of tobamoviruses. Reliable transformation protocols for *N. clevelandii*, *N. debneyi*, and *N. glutinosa*, which have not been reported, will facilitate studies on disease resistance in these wild species. We developed *A. tumefaciens*-mediated protocols for highly efficient transformation of both *N. tabacum* genotypes (e.g., “Xanthi” and “Samsun”) and *N. benthamiana* with transformation frequencies ranging from 50% to 90% when *nptII* was used as a selectable marker. In this study, the protocols were applied for transformation of *N. clevelandii*, *N. debneyi*, and *N. glutinosa*.

### 2. Materials and Methods

#### 2.1. Plant Materials and Culture Media

Seeds of *N. clevelandii*, *N. debneyi*, and *N. glutinosa* were surface sterilized in 50% (V/V) Clorox (about 3% sodium hypochlorite) for 10 min in 1.5 ml-eppendorf tubes and were then rinsed five times with sterile distilled water. The seeds (50 - 100/dish) were cultured for 2 wk in a Petri dish (60 × 15 mm) on 10 ml Murashige and Skoog medium (MS) [12] containing 30% sucrose, and solidified with 0.6% (w/v) Bacto agar. Sterile seedlings (5/box) or the internode with a single auxiliary bud (1 per box for subculture) were cultured in a Magenta™ GA-7 box containing 50 ml MS medium for 6 to 8 wk to obtain leaf explants. The subculture was conducted every 8 wk. All cultures were grown at 25°C under a 16-h photoperiod of 45  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  from cool white fluorescent tubes.

Regeneration medium (RM) was MS salts plus Gamborg B5 vitamins [13], 4.44  $\mu\text{M}$  6-benzylaminopurine (BAP), 0.57  $\mu\text{M}$  indole-3-acetic acid (IAA), 3% (w/v) sucrose, and 0.6% (w/v) Bacto agar. The pH of all plant culture media was adjusted to 5.7 before autoclaving at 121°C for 20 minutes at 105 kPa. YEP medium contained 10  $\text{g}\cdot\text{L}^{-1}$  Bacto peptone, 5  $\text{g}\cdot\text{L}^{-1}$  NaCl, 10  $\text{g}\cdot\text{L}^{-1}$  yeast extract, 15  $\text{g}\cdot\text{L}^{-1}$  agar (for agar plates), and pH = 7.0. Acetosyringone and all antibiotic (*i.e.*, kanamycin, rifampicin, and timentin) were filter-sterilized through 0.22  $\mu\text{m}$  Millipore filters, and added to medium cooled to 50°C - 60°C after autoclaving.

#### 2.2. *Agrobacterium* and Construct *A. tumefaciens*

EHA105 strain [14] was used. The binary vector pBISN1 (kindly provided by Dr. S. Gelvin, Purdue University) contains the neomycin phosphotransferase II (*nptII*) gene conferring kanamycin resistance and a potato ST-LS1 intron interrupted  $\beta$ -glucuronidase (GUS) gene (*gusA*) [15]. The pBISN1 was introduced into EHA105 competent cells using the freeze-thaw method [16]. Selection of transformed EHA105 cells was conducted at 28°C in the dark on YEP medium containing 100  $\text{mg}\cdot\text{L}^{-1}$  kanamycin and 30  $\text{mg}\cdot\text{L}^{-1}$  rifampicin.

#### 2.3. Regeneration Experiments

Leaves were cut into about 0.6  $\text{cm}^2$  using scissors. Five explants were placed onto 30 ml RM in each Petri dish

(100 × 15 mm) with either adaxial side up or abaxial side up. The Petri dishes, three for each of the *N. cleavelandii*, *N. debneyi*, and *N. glutinosa*, were cultured in the dark for one wk prior to the culture under a 16-h photoperiod for 3 wk. The explants were then transferred to Magenta™ GA-7 boxes, 5 explants/box and each containing 50 ml RM, and cultured for 4 wk. The number of explants that produced at least one shoot ( $\geq 0.5$  cm in length) and the total number of shoots were recorded. The experiment was repeated three times. The percentage of explants that produced at least one shoot and the average number of shoots of regenerated explants were calculated.

## 2.4. Transformation Protocols

A single colony of EHA105:pBISN1 was grown in a 50-ml Corning tube containing 15 ml liquid YEP medium, 100 mg·L<sup>-1</sup> kanamycin and 30 mg·L<sup>-1</sup> rifampicin. The culture was grown at 28°C for 48 hours with constant shaking at 300 rpm. The cells were collected by a 5 min centrifugation at 2500 × g and suspended to an OD<sub>600</sub> of 0.5 in liquid co-cultivation medium (liquid RM plus 100 μM acetosyringone).

Leaf disks were prepared by punching stacked leaves in a Petri dish (100 × 15 mm) using a sterile cork borer (10 mm in diameter). Newly prepared disks (60/species) were inoculated in EHA105:pBISN1 suspension cells for 1 - 2 min, blotted dry on sterile filter paper, and then placed onto a piece of sterile filter overlaid on 30 ml solidified co-cultivation medium (RM plus 100 μM acetosyringone) in a Petri dish (100 × 15 mm). Co-cultivation was carried out at 25°C in the dark for 4 d.

After co-cultivation, the explants were transferred to a 50 ml Corning tube and washed three times (1 min per time), twice in liquid RM and once in liquid RM supplemented with 500 mg·L<sup>-1</sup> timentin, to remove excess *Agrobacterium*. The washed explants were blotted dry on sterile filter paper and placed, 10 disks/dish, on 30 ml selection RM supplemented with 50 mg·L<sup>-1</sup> kanamycin and 250 mg·L<sup>-1</sup> timentin in a Petri dish (100 × 15 mm). Selection and regeneration were conducted in the dark for one wk followed by a 16-h photoperiod for the rest process. After 4-wk culture in Petri dishes, the explants (5/box) were transferred on 50 ml selection RM in a Magenta™ GA-7 box and cultured for 4 wk.

After 8-wk on selection RM, regenerated shoots (one or two per explants) were excised and transferred to 50 ml MS medium containing 100 mg·L<sup>-1</sup> kanamycin and 250 mg·L<sup>-1</sup> timentin in a Magenta™ GA-7 box for rooting at 25°C under a 16-h photoperiod for 3 wk. The number of leaf disks that produced kanamycin resistant plants was recorded. The transformation experiments were repeated three times.

Well rooted transformants were washed to remove agar, and individual plants were planted in 4-inch pots and zipped in a one-gallon size bag for one wk at 25°C under a 16-h photoperiod. The bags were then opened to acclimate the plants for one wk before they were transferred to the greenhouse. Seeds of each plant were harvested separately.

Surface-sterilized T<sub>1</sub> seeds were placed onto MS medium (without sucrose) containing 200 mg·L<sup>-1</sup> kanamycin. Seed germination was investigated after 3 wk culture at 25°C under a 16-h photoperiod.

## 2.5. Histochemical GUS Assay

The GUS assay in transformed and non-transformed tissues was determined histochemically according to Jefferson *et al.* [17] and was used as a quick screen of the putative transformants. All tissues (*i.e.* inoculated leaf explants, T<sub>0</sub> leaves and T<sub>1</sub> seedlings) were stained in 2 mM X-Gluc (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) overnight at 37°C. The GUS expression was documented by photography.

## 2.6. Reverse Transcript Polymerase Chain Reaction (RT-PCR)

Isolation of total RNA from young leaves of greenhouse-grown T<sub>0</sub> plants was performed using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). After DNase treatment, purified RNA (1 μg for each sample) was transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The resulting cDNA was used for PCR amplification of a 377 bp fragment of the coding region of *gusA* (primers: 5'-gatcctcgcattacccttacgc-3' and 5'-gtgagcgtcgcagaacattac-3') and a 600-bp fragment of *nptII* with the primers 5'-GAGGCTATTCGGCTATGACTG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3'. The PCR reaction conditions were 94°C for 2 min, 35 cycles of 94°C for 10 s, 58°C for 1.5 min and 72°C for 2 min, with a final 10 min extension at 72°C. PCR products were separated on 1.0% agarose gel containing ethidium bromide, visualized

and photographed under UV light.

### 3. Results and Discussion

#### 3.1. Shoot Regeneration Capacity

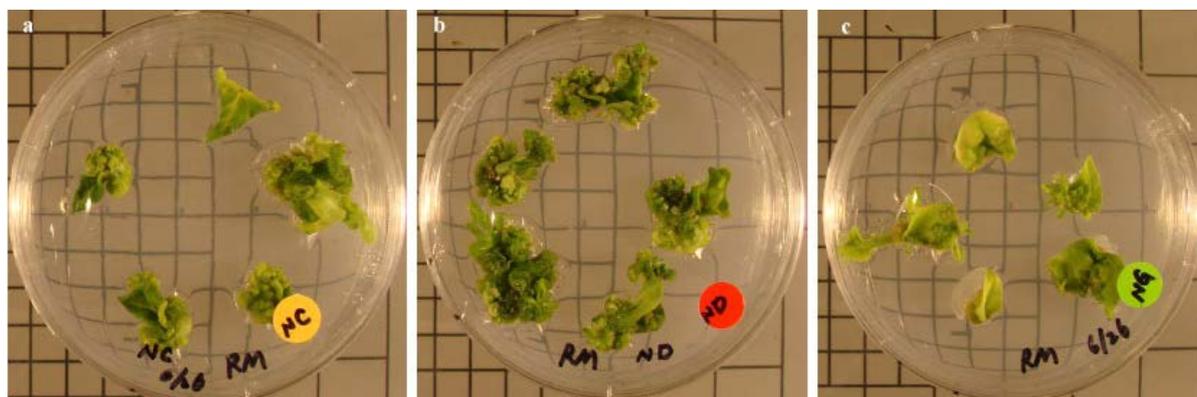
The RM (8.88  $\mu\text{M}$  BAP plus 0.57  $\mu\text{M}$  IAA) used in this study is different from the commonly used one [4.44  $\mu\text{M}$  BAP plus 0.54  $\mu\text{M}$   $\alpha$ -naphthalene acetic acid (NAA)] [1] [2], it was highly efficient for shoot production from leaf explants (100% leaf explants with over 10 shoots/explants) of *N. tabacum* genotypes (e.g., “Xanthi” and “Samsun”) and *N. benthamiana*. When it was used for *Nicotiana debneyi*, *N. clevelandii*, and *N. glutinosa*, multiple shoots mainly from the cut edges were produced after 8 wk at frequencies of 66.7% for *N. glutinosa*, 95.6% for *N. clevelandii*, and 100% for *N. debneyi*. The average number of shoots of regenerated explants was 3.1 for *N. glutinosa*, 5.2 for *N. clevelandii*, and 6.5 for *N. debneyi*. Apparently, *N. debneyi* and *N. clevelandii* are more efficient than *N. glutinosa* in shoot regeneration from leaf explants (**Figure 1**). These results suggest that optimal regeneration medium is species/genotype-dependent. Genetically, *N. debneyi* is believed to be a parent of *N. benthamiana* (*N. suaveolens*  $\times$  *N. debneyi*). Since the RM is the optimal regeneration medium for *N. benthamiana*, high genetic similarities to *N. benthamiana* could contribute to efficient shoot regeneration of *N. debneyi* on the RM.

The explant orientations on RM (*i.e.*, adaxial side up or abaxial side up) did not result in significant difference in shoot production, and therefore, the orientations were not a consideration in our transformation experiments.

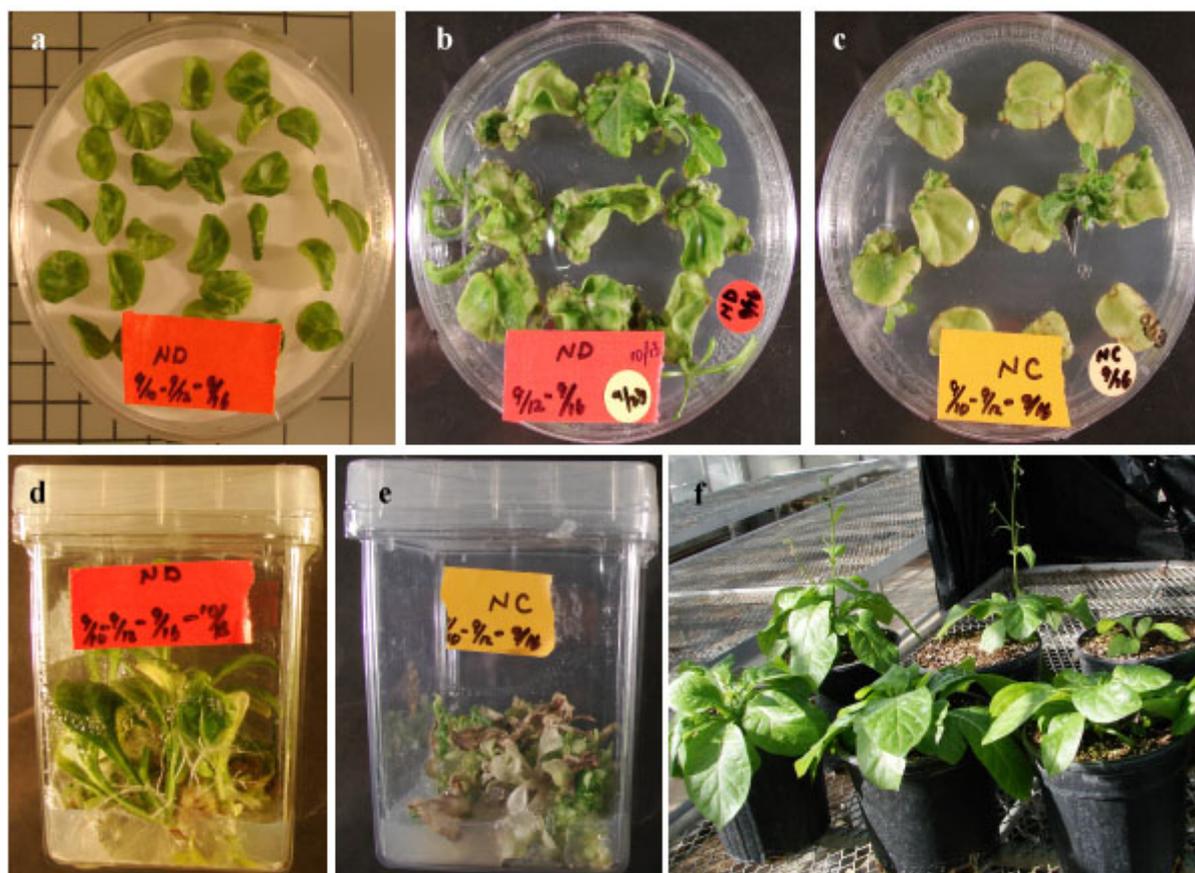
#### 3.2. Transformation

The pBISN1 containing a potato ST-LS1 intron interrupted *gusA* to inhibit GUS expression in *Agrobacterium* cells was routinely used to optimize our transformation protocols for different plant species [18]-[22]. In this study, transient GUS expression assay was conducted after 4-d co-cultivation. Under our co-cultivation conditions, all leaf disks tested for each of *N. glutinosa*, *N. clevelandii*, and *N. debneyi* showed strong blue staining in cutting edges and weak blue foci in non-wounded surfaces. The result indicates EHA105 is efficient in delivering the transfer DNA (T-DNA) of the pBISN1 to these species. In addition, by using RM as co-cultivation medium and a piece of filter paper to prevent overgrowth of EHA105, the 4-d co-cultivation did not cause any necrosis in leaf explants (**Figure 2(a)**). With the washes after co-cultivation, 250  $\text{mg}\cdot\text{L}^{-1}$  timentin was able to eliminate EHA105, and no EHA105 overgrowth was observed during the entire selection process.

After 4-wk selection, kanamycin resistant clusters of callus/shoot appeared mainly along the cut edges of the explants (**Figure 2(b)**, **Figure 2(c)**). In the Magenta™ GA-7 boxes containing selection RM, multiple shoots were observed from some explants and rooted plants were also observed on the explants of *N. debneyi* (**Figure 2(d)**, **Figure 2(e)**). All rooted plants survived and were morphologically normal after they were transplanted to soil (**Figure 2(f)**). Transgenic plants from separate explants were considered to be independent transgenic events. With this transformation system, transformation frequencies based on the percentage of explants that produced at least one kanamycin resistant plants were 75.6% (136/180) for *N. debneyi*, 25.0% (45/180) for *N. clevelandii*,



**Figure 1.** Adventitious shoot regeneration on leaf explants of *N. clevelandii* (a), *N. debneyi* (b), and *N. glutinosa* (c) after 4 wk culture with adaxial side up on MS medium with 4.44  $\mu\text{M}$  BAP and 0.54  $\mu\text{M}$  NAA.



**Figure 2.** *Agrobacterium tumefaciens*-mediated transformation of *N. clelandii* (a, b, d, f) and *N. debneyi* (c, e). (a) Co-cultivation of leaf explants for 4 d in the dark. (b-e) Selection and regeneration of  $T_0$  transformants on regeneration medium (MS + 4.44  $\mu\text{M}$  BAP + 0.54  $\mu\text{M}$  NAA) containing 50  $\text{mg}\cdot\text{L}^{-1}$  kanamycin and 250  $\text{mg}\cdot\text{L}^{-1}$  timentin for 4 wk (b, c) and 8 wk (d, e), respectively. (f) Rooted  $T_0$  plants growing in the greenhouse for 6 wk.

and only 2.8% (5/180) for *N. glutinosa*. The results indicate that the protocols described enable efficient transformation of both *N. debneyi* and *N. clelandii* and have potential application for transformation of other wild *Nicotiana* species. The lower transformation frequency (2.8%) of *N. glutinosa* is due to its lower regeneration ability.

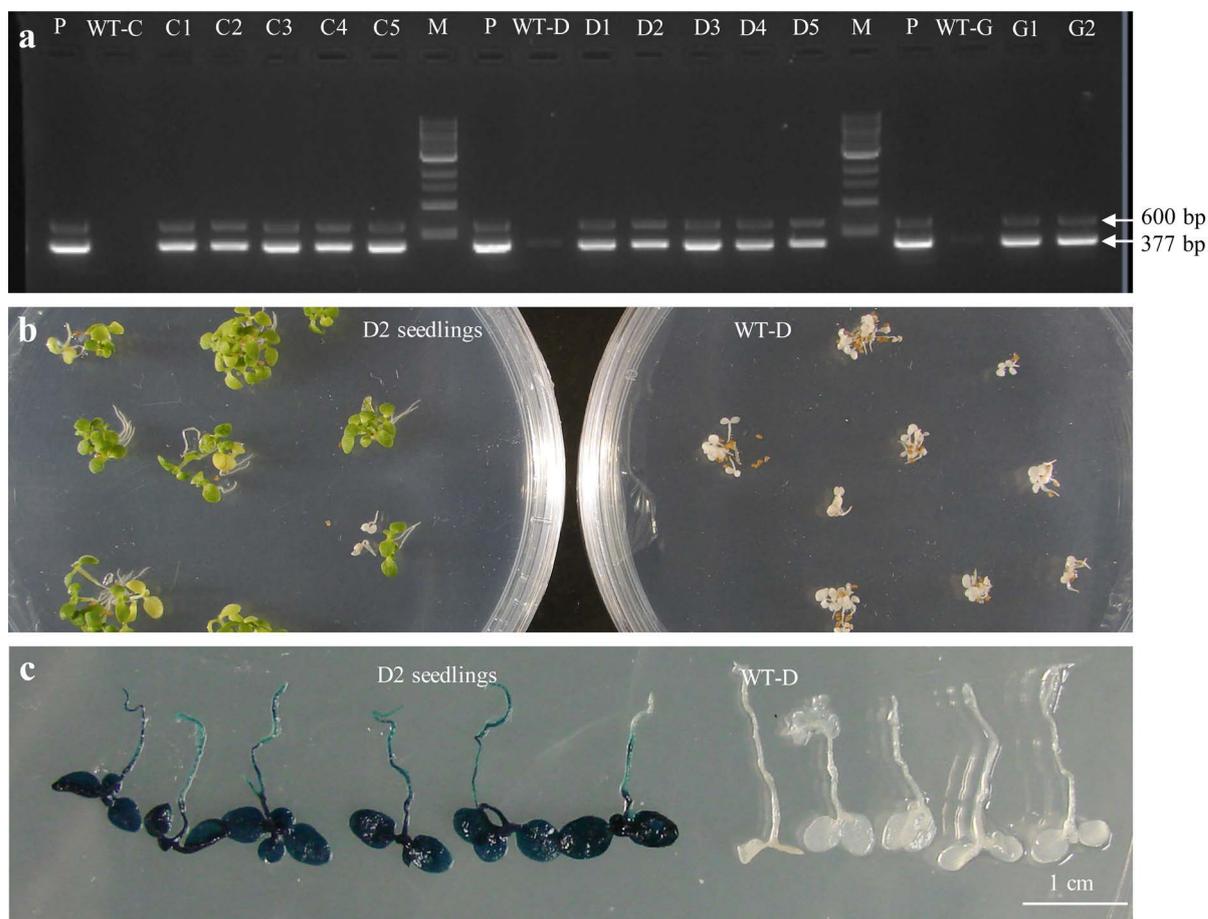
### 3.3. Expression of Transgenes

$T_0$  transformants, 2 for *N. glutinosa* and 5 for each of the *N. debneyi* and *N. clelandii*, were analyzed using RT-PCR. The fragments of *gusA* (377 bp) and *nptII* (600 bp) were present in all 12 transformants and were absent in nontransgenic plants (Figure 3(a)). The results confirmed transcription of both the *gusA* and *nptII*.

Inheritance of the *gusA* and *nptII* was investigated in  $T_1$  seedlings of one transgenic event for each of *N. debneyi*, *N. clelandii* and *N. glutinosa*. On MS (without sucrose) supplemented with 200  $\text{mg}\cdot\text{L}^{-1}$  kanamycin, both transgenic and nontransgenic seeds germinated after one wk; however, two wk later all seeds from nontransgenic plants turned white, in contrast, the majority seedlings from transgenic plants remained green and kept growing (Figure 3(b)). In histochemical GUS assays, blue staining was present in all kanamycin resistant seedlings but was absent in nontransgenic seedlings (Figure 3(c)). The results indicate inheritance of both the *nptII* and *gusA* in transgenic seedlings.

## 4. Conclusion

We developed protocols for transformation of *N. debneyi*, *N. clelandii* and *N. glutinosa*. Briefly, leaf explants were soaked in *A. tumefaciens* cells and co-cultivated in the dark for 4 d. Selection and regeneration of trans-



**Figure 3.** Expression of marker genes *gusA* and *nptII* in transgenic plants. (a) RT-PCR analysis of *gusA* (377 bp) and *nptII* (600 bp) in  $T_0$  transgenic plants of *N. clevelandii* (C1-C5), *N. debneyi* (D1-D5), and *N. glutinosa* (G1 and G2). WT-C: non-transgenic *N. clevelandii*. WT-D: nontransgenic *N. debneyi*. WT-G: nontransgenic *N. glutinosa*. P: positive control (plasmid pBISN1). M: 1 kb DNA ladder. (b) Germination of  $T_1$  transgenic *N. debneyi* (D2 line) and nontransgenic *N. debneyi* (WT-D) seeds after 3 wk culture on MS medium (without sucrose) containing  $200 \text{ mg}\cdot\text{L}^{-1}$  kanamycin. (c) GUS expression in kanamycin-resistant *N. debneyi* seedlings (D2 line) and nontransgenic *N. debneyi* (WT-D) seedlings.

formants were conducted on regeneration medium containing  $50 \text{ mg}\cdot\text{L}^{-1}$  kanamycin and  $250 \text{ mg}\cdot\text{L}^{-1}$  timentin for 6 - 8 wk. Rooting of the transformants was carried out on MS medium containing  $100 \text{ mg}\cdot\text{L}^{-1}$  kanamycin and  $250 \text{ mg}\cdot\text{L}^{-1}$  timentin for 2 - 3 wk. Production of kanamycin resistant plants from inoculated explants was obtained at frequencies of 75.6% for *N. debneyi*, 25.0% for *N. clevelandii*, and 2.8% for *N. glutinosa*. The protocols described have potential application for transformation of other *Nicotiana* species.

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