Strain specific *Agrobacterium*-mediated genetic transformation of *Bacopa monnieri*

Sheetal Yadav ¹, Poojadevi Sharma ¹, Anshu Srivastava, Priti Desai, Neeta Shrivastava *

*B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, Thaltej, S.G. Highway, Ahmedabad 380054, Gujarat, India*

Received 13 June 2014; revised 9 October 2014; accepted 9 November 2014
Available online 6 December 2014

**KEYWORDS**

*Agrobacterium tumefaciens; Bacopa monnieri; β-Glucuronidase (GUS); Gene expression*

**Abstract**

*Agrobacterium*-mediated genetic transformation is the most preferred strategy utilized for plant genetic transformation. The present study was carried out to analyze the influence of three different strains of *Agrobacterium tumefaciens* on genetic transformation of *Bacopa monnieri* (L.) Pennell. In the present study, *B. monnieri* was genetically transformed with three different strains of *A. tumefaciens* viz. LBA4404, EHA105 and GV3101 harbouring expression vector pCAMBIA2301 containing β-glucuronidase (GUS) as a reporter gene. The putative transformants were analyzed by PCR method using transgene specific primers. Expression and presence of GUS reporter protein were analyzed by histochemical staining assay and quantitative analysis of GUS enzyme was done using fluorometric assay. No statistically significant difference in transformation efficiency was found for all the three strains. Interestingly, Gus expression was variable with LBA4404 plants showing highest GUS activity.

© 2014 Academy of Scientific Research & Technology. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

*Bacopa monnieri*, a well known medicinal plant of Indian system of medicine, has recently gained interest as a potential host system for expression of foreign proteins [1–3]. The plant has high regeneration response and a large number of pharmacological and clinical studies have indicated that this plant is non toxic for human consumption. These valuable features make *B. monnieri* a well suited plant to be explored as a model host plant for foreign protein production.

*Agrobacterium*-mediated genetic transformation is the most preferred method for genetic transformation in plants due to ease of implementation of method and cost effectiveness. Successful plant transformation needs a robust genetic transformation protocol which chiefly depends on host (plant) genome and *Agrobacterium* strain compatibility or interaction. Other factors like type of chromosomal backgrounds of the *Agrobacterium* strains, different opines and mechanism of transfer and integration of T-DNA, T-DNA copy number containing gene of interest (transgene) also influence this process of gene expression [4–8]. Choice of *Agrobacterium* strain...
used for the plant transformation process can dramatically alter transformation efficiency and or foreign protein expression, and therefore, is a critical factor to be analyzed during the process.

Considering the underexplored potential of *B. monnieri* to act as a suitable host system for foreign protein expression, we attempted to evaluate the genetic transformation susceptibility of *B. monnieri* to three different strains of *Agrobacterium tumefaciens* and effect of this interaction on level of foreign protein β-glucuronidase (GUS) expression. GUS gene was chosen as a transgene for the study on account of the fact that its expression in genetically manipulated plant can be visually detected with histochemical assay with high sensitivity.

2. Materials and methods

2.1. Transformation of *Agrobacterium tumefaciens* strains with vector pCAMBIA2301 and maintenance

Transformation of three different strains of *A. tumefaciens* viz. GV3101, LBA4404, (purchased from NCCB, Netherlands), and EHA105 [9–11] with binary plant expression vector pCAMBIA2301 was carried out using electroporation (Electroporation conditions: Voltage 2.4 kV, Capacitance 25 mF, Resistance 200 W; Electroporator- Biorad GenePulser Xcell). These cultures were maintained in the presence of pCAMBIA2301 selection antibiotic, i.e., kanamycin (50 mg/L) (Himedia, India) and strain specific selection antibiotics (50 mg/L) mentioned in Table 1. The pCAMBIA2301 vector contains GUS (coding sequence interrupted with intron sequence) as a reporter gene and neomycin phosphotransferase II (nptII) as a selective marker gene. Both genes are driven by the CaMV 35S promoter (Fig. 1A). All the three *Agrobacterium* strain cultures were grown in luria broth (Himedia, India) medium and agitated at 28 °C for 18 h at 200 rpm with required antibiotics.

2.2. In vitro shoot regeneration

The authenticated *B. monnieri* growing in botanical garden of the institute (B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, Ahmedabad, Gujarat, India) was used as mother plant to obtain the leaf explants for the in vitro shoot regeneration. Leaf explants were surface sterilized aseptically by tween 80 (Teepol, Reckitt Benckiser, India) treatment for 2 min at 37 °C and incubated for 3 h at 37 °C. Leaf explants were gently and manually agitated for 2 min. Excessive suspension culture was removed by soaking the explants on sterilized blotting papers and were finally placed on the MS medium for incubation for 48 h at 25 ± 2 °C culture conditions. After 2 days *Agrobacterium* infected explants were transferred on MS medium containing 500 mg/L cefotaxime (Injection vial, Alkem, India) for 12 days to prevent the excessive growth of *Agrobacterium* cells. Finally these explants were transferred on MS medium (mentioned in Section 2.2) containing kanamycin (15 mg/L) and cefotaxime (500 mg/L) antibiotics for selection of putative transformants and incubated for 28 days under same culture conditions [Fig. 1(B–F)]. (Kanamycin sensitivity was performed by culturing uninfected leaf explants on MS medium supplemented with 0, 3, 6, 9, 12, 15, 18 and 21 mg/L concentration of kanamycin.)

3. Confirmation of transformed plants

3.1. Histochemical assay for GUS

Putative transformants were confirmed using histochemical GUS assay with few modifications. Fresh leaves of putative transformed and non-transformed plants were put in 2 ml eppendorf tubes with 1 ml of histochemical reagent [5 mg; 1 mM of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc, PhytoTechnology Laboratories, USA) dissolved in 1.0 ml dimethyl formamide and final volume was made up to 10 ml with 50 mM NaPO4, pH 7.0] and incubated for 3 h at 37 °C [14]. The leaves were then washed with absolute alcohol to clear chlorophyll. Then treated leaves were observed under microscope at 100X magnification.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Strain</th>
<th>Chromosomal background</th>
<th>Tri-plasmid</th>
<th>Opine</th>
<th>Genome selection antibiotic (50 mg/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LBA4404</td>
<td>TiAch5</td>
<td>pAL4404</td>
<td>Octopine</td>
<td>Rifampicin (Himedia, India)</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>EHA105</td>
<td>C58</td>
<td>pTiBo542D T-DNA</td>
<td>Succinamopine</td>
<td>Rifampicin (Himedia, India)</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>GV3101</td>
<td>C58</td>
<td>pTiC58D T-DNA</td>
<td>Nopaline</td>
<td>Gentamycin (Himedia, India) and Rifampicin (Himedia, India)</td>
<td>9</td>
</tr>
</tbody>
</table>
3.2. PCR analysis of nptII gene and GUS gene

Putatively transformed and non-transformed plants were subjected to genomic DNA isolation by CTAB method [15,16]. These DNA samples were used for PCR amplification of nptII gene by using forward primer 5’CTTTTCTGGATTCATCGACT3’ and reverse primer 5’TACAGAAGAACTCGTGAA3’ (Synthesized by Sigma Aldrich, USA). The PCR reaction mixture contained 10 pmol of each primer (forward primer and reverse primer), 0.2 mM dNTPs, 1.5 mM MgCl2, 1X Taq buffer 1X, 2U (unit) of taq polymerase (All PCR reagents purchased from Fermentas, India) and 50–100 ng of template in total reaction mixture volume of 50 µl. The reaction conditions were set as follows; initial denaturation at 94 °C for 2 min, subsequent denaturation at 95 °C, 1 min, annealing at 55 °C for 40 s, elongation at 72 °C for 1 min, subsequent denaturation to elongation steps were repeated for 30 times (cycles) and final extension was carried out at 72 °C for 3 min. Similarly PCR amplification was performed for GUS gene with forward primer 5’CCGGCAATAACGATACGCCGTTG 3’ (Synthesized by Sigma Aldrich, USA). Reaction conditions were set as follows; initial denaturation at 95 °C for 5 min, subsequent denaturation at 95 °C for 15 s, annealing at 68 °C for 20 s, elongation at 72 °C for 1 min, subsequent denaturation to elongation steps were repeated 30 times and final extension was carried out at 72 °C for 3 min. The above cycle was repeated for 30 cycles. All PCR products were analyzed on 2% agarose gel (Invitrogen, India) containing 0.5 mg/ml ethidium bromide (Himedia, India) and visualized under UV-transilluminator and photographed using gel documentation system (Bio-Rad, USA).

3.3. Fluorometric GUS assay

Expression of GUS in B. monnieri plants transformed with three Agrobacterium strains was evaluated by fluorometric GUS assay [14]. After 4th subculture, fresh leaves (fresh weight = 3 g) of transformed and non-transformed plants were lyophilized (dry weight-300 mg) and homogenized by a
pestle and mortar with extraction buffer containing 50 mM NaPO₄, pH 7; 10 mM EDTA (Himedia, India), pH 8; 0.2% Triton X-100 (Sigma, USA) and 10 mM β-mercaptoethanol (Himedia, India). The homogenate was centrifuged to pellet out debris and supernatant was collected. Assay buffer was prepared by mixing extraction buffer with 1 mM MUG (4-methylumbelliferyl-β-glucoronide, Phytotechnology Laboratory, USA) and incubated at 37 °C for 15 min. 200 µl of supernatant was added in 2 ml of assay buffer. The assay was terminated by adding 200 µl of this reaction mixture into 1.8 ml of 0.2 M Na₂CO₃, (Merck, India) (stop buffer) after every 15 min. Assay was performed in triplicates for each strain sample at each time interval point. Finally, all samples were analyzed in a spectrofluorometer SL 174 (Elico, India). Fluorescence was recorded at an excitation wavelength of 365 ± 5 nm and an emission of 455 ± 5 nm. GUS activity was calculated as micromoles of 4-MU (MP Biomedicals Inc., France) formed mg/protein/min as described by Jefferson et al. in 1987 [14]. Total protein concentration of each test sample was determined by Bradford assay and the absorbance was measured at 595 nm using spectrophotometer (UV-2450, Shimadzu, Japan) [17].

Table 2  Transformation efficiency of transformed plants of *B. monnieri* with three different strains of *A. tumefaciens* viz. LBA4404, EHA105, GV3101.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Agrobacterium strain</th>
<th>Total No. of explants cocultivated with Agrobacterium</th>
<th>Explants showing response on selection medium</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>LBA4404</td>
<td>15 × 3 = 45</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>EHA105</td>
<td>15 × 3 = 45</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>GV3101</td>
<td>15 × 3 = 45</td>
<td>12</td>
<td>11</td>
</tr>
</tbody>
</table>

Figure 2  (A) PCR analysis of *NptII* gene: PCR amplification of *nptII* gene in putative transformants of *B. monnieri* plants. NC = negative control; NT = non-transformed; putative transformants of *B. monnieri* L1–L5 = transformed with LBA 4404 strain; E1–E5 = transformed with EHA 105 strain; G1–G5 = transformed with GV3101 strain; P = positive control (isolated plasmid DNA from bacterial cells); M = Marker. (B) PCR analysis *GUS* gene: PCR amplification of *GUS* gene in putative transformants of *B. monnieri*. NC = negative control; NT = non-transformed; putative transformants of *B. monnieri* L1–L5 = transformed with LBA 4404 strain; E1–E5 = transformed with EHA 105 strain; G1–G5 = transformed with GV3101 strain; PC = positive control (isolated plasmid DNA from bacterial cells); M = Marker.
4. Results

Putative transformants were selected on kanamycin selection medium and transformation efficiency was calculated for plants transformed with three different strains. It was found to be $64.4 \pm 1.53\%$ for LBA4404, $71.1 \pm 1.53\%$ for EHA105 and $77.78 \pm 0.58\%$ for GV3101 (Table 2). ANOVA test ($p$-value $\leq 0.05$) revealed no significant difference in transformation efficiency of B. monnieri transformed with all the three strains of A. tumefaciens. Purified plant genomic DNA samples of putatively transformed and non-transformed B. monnieri were subjected to PCR analysis of nptII gene [Fig. 2(A)] and GUS gene [Fig. 2(B)] using gene specific primers. Expected amplicon sizes of 190 bp and 385 bp of the nptII gene and GUS gene were respectively observed on agarose gel from transformed plants only. The size of amplicons was determined by comparison with 50 bp DNA ladder on gel. All randomly selected transformants were PCR positive for both genes. PCR analysis proved that the plants were positively transformed with nptII and GUS transgene. GUS histochemical analysis revealed strong blue coloration in all transformed tissues with visually different expression pattern. On the basis of visual analysis transformed plants infected with LBA4404 strain had relatively larger sized stained zones (dark blue color) in comparison to plants transformed with EHA105 and GV3101 strains [Fig. 3(A)]. Specific GUS activity was quantified in terms of umole 4-MU/min/mg of total protein present in the extract. The highest GUS activity (6.01 umol 4-MU/min/mg) was found in plants transformed with LBA4404 strain when compared to other strains EHA105, GV3101 [Fig. 3(B); Table 3].

5. Discussion

The data from the study suggest that B. monnieri plant was readily transformed with all the three different Agrobacterium strains. Thus, B. monnieri is amenable to genetic manipulation without any Agrobacterium incompatibility issues with these three strains. Further, plants transformed with these strains showed variation in the expression level of GUS foreign protein. Our results are in agreement with many previous studies which reported variability in GUS protein expression of plants upon transformation with different strains of Agrobacterium [18,19].

B. monnieri has the potential to become viable host system for foreign protein production. Based on this possible use, few research studies have carried out Agrobacterium mediated transformation of B. monnieri using GUS as transgene to assess the feasibility of the model system [1–3]. However, the particular aspect of strain cultivar compatibility is yet to be addressed. In our study, variability in GUS activity was observed between the plants transformed with different Agrobacterium strains in concord with many previous studies [18,19]. Many research studies have been performed to evaluate underlying mechanism behind this phenomenon. Genomic and proteomic analysis, during the process of Agrobacterium–plant interaction, has revealed many important genes and protein candidates which could be playing an important role in influencing transformation efficiency and foreign protein expression in plants [20–22]. Multiple T-DNA integrations can increase the transformation efficiency.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Transformed plants with different strains</th>
<th>GUS enzyme activity (µmol 4-MU/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LBA4404</td>
<td>6.01</td>
</tr>
<tr>
<td>2</td>
<td>EHA105</td>
<td>3.03</td>
</tr>
<tr>
<td>3</td>
<td>GV3101</td>
<td>2.43</td>
</tr>
<tr>
<td>4</td>
<td>Non transformed</td>
<td>0.50</td>
</tr>
</tbody>
</table>
but reduces the expression of foreign proteins because of transgene silencing. Alternatively, single T-DNA integration can result in better level of expression of proteins in plants at the cost of transformation efficiency [23]. Again some studies suggest that copy number effect may have no influence on foreign gene expression [24]. Other processes like position effect in the host genome or other complex configurations of the integrated T-DNA may also have important role in dictating the eventual expression of level of foreign protein obtained in transgenic plant [25,26].

Based on GUS protein expression analysis, our study indicates that LBA4404 is a better choice compared to EHA105 and GV3101 for genetic transformation of B. monnieri. Thus, the choice of Agrobacterium strain chosen for B. monnieri plant transformation could be crucial in dictating the expression of foreign proteins in the plant. Therefore, this parameter should be thoroughly analyzed when carrying out genetic transformation of B. monnieri for the expression of any foreign protein in future such studies.

Acknowledgments

We are greatly thankful to Korea University, Korea; for gifting EHA105, Purdue University, Indiana; for gifting GV3101, and CAMBIA organization, Australia; for gifting pCAMBIA2301. We are also thankful to the National Research Centre for Medicinal and Aromatic Plants (NRC-MAP), Boriavi, Anand, Gujarat (India) for microscopic photographs of GUS stained leaves of transformed B. monnieri. Authors Sheetal Yadav and Poojadevi Sharma acknowledge Nirma University for accepting them as Ph.D. Student.

References


