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ORIGINAL ARTICLE

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

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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.

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

* Corresponding author. Tel.: +91 79 27439375; fax: +91 79 27450449.
E-mail address: neetashrivastava_perd@yahoo.co.in (N. Shrivastava).
[1] These authors are registered Ph.D. student of Nirma University, Ahmedabad, Gujarat, India.
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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 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, Netherland), 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 (*npt*II) 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 followed by 0.1% mercury chloride (Merck, India) treatment for 2 min. Explants were further washed with sterile water to remove remaining sterilants present on the surface of leaf explants. *In vitro* culture of *B. monnieri* was established by a previously standardized protocol developed in our laboratory using leaves as explants [12]. Explants were cultured on Murashige and Skoog (MS) medium [13] supplemented with 2 μM of 6-benzyladenine (Merck, India), 3% w/v sucrose (Fisher Scientific, India) and 0.2% (w/v) gelrite (Duchefa Biochemie, The Netherland). These cultures were maintained and incubated at temperature 25 ± 2 °C under suitable culture conditions (16 h light/8 h dark) and routinely subcultured after every 28 days. For *A. tumefaciens* mediated plant transformation, shoots were excised and its leaves were directly used as explants for the purpose.

2.3. Genetic transformation of B. monnieri

Leaf explants (8 × 4 mm) were cut from both the ends and inoculated on MS basal medium supplemented with 3% sucrose and 0.2% (w/v) gelrite for preconditioning for 48 h. Suspension culture of all the three strains of *A. tumefaciens* (OD 0.5–0.6) was used for co-cultivation of pre-conditioned leaves. Bacterial culture was centrifuged at 4000 rpm at 4 °C for 15 min and pellet was resuspended in basal MS medium. Briefly, 6 ml of above suspension culture was poured in sterilized petriplates and 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 1 Characteristics of different strains of *Agrobacterium tumefaciens*.

Sr. No.	Strain	Chromosomal background	Ti-plasmid	Opine	Genome selection antibiotic (50 mg/L)	References
1	LBA4404	TiAch5	pAL4404	Octopine	Rifampicin (Himedia, India)	10
2	EHA105	C58	pTiBo542D T-DNA	Succinamopine	Rifampicin (Himedia, India)	11
3	GV3101	C58	pTiC58D T-DNA	Nopaline	Gentamycin (Himedia, India) and Rifampicin (Himedia, India)	9