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Transgenic Elephant Foot Yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) Expressing β-glucuronidase Reporter Gene

S. Kamala and T. Makeshkumar

Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram 695 017, Kerala, India
Corresponding author: T. Makeshkumar, e-mail: makeshctcri@gmail.com
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Abstract

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is infected by various diseases such as mosaic, collar rot and dry rot disease, which cause severe yield reduction. Disease resistance can be achieved in crop plants through transgenic strategies. The present study is focused on *Agrobacterium* mediated transformation of *A. paeoniifolius* using β-glucuronidase (GUS) reporter gene. Friable callus cultures were established from corm lateral buds of *A. paeoniifolius* on modified Murashige and Skoog (MS) medium supplemented with 0.5 mg l^{-1} each of 6-Benzyl aminopurine, 2,4-Dichloro phenoxy acetic acid and α- Naphthalene acetic acid. Friable callus and swollen petiole explants were infected with *Agrobacterium tumefaciens* strain AGLO harbouring the Ti vector pOYE 153 having *uidA* gene encoding GUS. The transformation event was confirmed by GUS histochemical analysis and later on the presence of *uidA* gene was determined by Polymerase Chain Reaction (PCR). The highest transformation efficiency of 36.37% was observed for callus when compared to swollen petiole explant. The presence of acetosyringone improved the transformation efficiency by 20 fold irrespective of the explant. The present study serves as an initial step towards the development of transgenic *A. paeoniifolius* for disease resistance.

Key words: β-glucuronidase, acetosyringone, elephant foot yam, friable callus, *in vitro*, transformation

Introduction

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is a tropical tuber crop belonging to the Araceae family. It is an economically important crop due to its production potential and popularity as a vegetable. The corms and pseudostems of elephant foot yam are popularly used in the preparation of indigenous ayurvedic medicines (Misra et al., 2002). The tubers are digestive, appetizer, anodyne, anti-inflammatory, astringent, haemostatic, rejuvenating and tonic in nature. They are used in the treatment of tumors, elephantiasis, inflammations, cough, asthma, constipation, fatigue and anaemia (Nair, 1993). In India, the plant is widely cultivated in Kerala, Tamil Nadu, Punjab, Assam, Bihar and West Bengal. The crop is mainly cultivated in the month of September-October, due to its high price and hence the crop is considered to be a cash crop due to high mean net profit. The plant is also cultivated as an intercrop with banana, turmeric and coconut (Ravi et al., 2009). In Kerala, this crop is cultivated in large areas in Ernakulam, Wayanad, Thrissur, Pathanamthitta and Kozhikode (Ravindran and George, 2008).

Elephant foot yam is subjected to a variety of infections such as mosaic disease caused by *Dasheen mosaic virus* (*DsMV*), collar rot caused by the fungus *Sclerotium rolfsii,* dry rot disease caused by *Fusarium solani* and *Rhizoctonia solani.* Among the diseases, mosaic disease caused by *DsMV* is the major yield constraint (Nehalkhan et al., 2006). Considering the importance of elephant foot yam and the diseases presenting serious constraints

to the production of this crop, resistance development becomes a high priority goal. As an initial step towards developing resistance in *A. paeoniifolius* through transgenic approach, an efficient callus multiplication as well as transformation protocol has to be established. The process of *in vitro* regeneration of *A. paeoniifolius* is far from being routine due to its recalcitrant nature (Mukherjee et al., 2009). Ban et al. (2009) developed resistance to soft rot disease in *A. konjac* using *Agrobacterium* mediated transformation. The *uidA* gene of *Escherichia coli* encoding β-glucuronidase (GUS) has been utilised in numerous plant species as a reporter gene to confirm transformation (Jefferson et al., 1987; Wijayanto and McHughen, 1999; Basu et al., 2004). Upon integration into genome, GUS leads to blue colouration upon simple biochemical analysis. Hence the objective of the present study was the establishment of *in vitro* callus cultures of *A. paeoniifolius* and their subsequent use as transformation explant source for *Agrobacterium* mediated transformation using GUS gene.

Materials and Methods

Plant material

The mother plants of *A. paeoniifolius* cv. Gajendra (Fig. 1a) were grown in open field of Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram, Kerala, India. Lateral buds of corms and petioles were used as explants.

Establishment of aseptic callus cultures

Lateral buds of field grown corms of *A. paeoniifolius* were surface sterilized with 0.1% (w/v) $HgCl_2$ for 1 min followed by 70% (v/v) ethanol for 1 min. The petiole explants were surface sterilized with 0.1% (w/v) Bavistin for 60 min followed by 70% (v/v) ethanol for 1 min and 0.1% $HgCl_2$ (w/v) for 7 min. Each treatment was followed by washing thrice with sterile distilled water. As an alternative, one step surface sterilization procedure by flaming the unopened leaves covered by cataphylls with absolute alcohol was done to get petiole explants. The method proved to be effective in getting contamination free cultures, thus avoiding long steps of surface sterilisation of petiole explants.

The explants were inoculated on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with various combinations of 6-Benzylaminopurine (BA), 2,4-Dichloro phenoxy acetic acid (2,4-D) and α-Naphthalene acetic acid (NAA). All components of the medium including 3% sucrose and hormones were mixed and adjusted to pH 5.6 and 0.7% plant agar was added for solidifying the media. The cultures were incubated under light intensity of 2500 lux and temperature of 25 ± 2°C with 16 h light/8 h dark cycle.

Bacterial strain and media

The culture of *Agrobacterium tumefaciens* strain AGLO harbouring the plant transformation plasmid *pOYE* 153 having *uidA* gene conferring GUS activity and *npt II* marker maintained in the Transgenic Laboratory of CTCRI was used for the transformation. *A. tumefaciens* was cultured in YEB medium (Beef extract - 3 g l^{-1}, casein hydrolysate - 5 g l^{-1}, bacto yeast extract - 1 g l^{-1}, sucrose - 5 g l^{-1}, pH 7.5) containing $MgSO_4$ (2 mM) and kanamycin (100 mg l^{-1}). The strain was allowed to grow in two sets of media one with acetosyringone (200 μM) and the other without acetosyringone at 28°C.

Preparation of *Agrobacterium* for transformation

The cultures of *A. tumefaciens* having pOYE 153 (OD_{600} = 1.0) were centrifuged at 5000 rpm for 10 min. and the pellet was washed twice in liquid MS followed by suspension in the same volume of liquid MS.

Agrobacterium mediated transformation

Friable callus as well as swollen petiole explants was co-cultured in a petridish containing *A. tumefaciens* culture and shaken for 30 min. and 60 min. respectively. The explants were dried on a filter paper. The explants were co-cultivated on callus induction (CI) medium with and without acetosyringone. After 4 days of co-cultivation at 22°C in the dark, the explants were washed thrice with sterile distilled water followed by liquid MS medium with ticarcillin (500 mg l^{-1}) twice. Then the explants were allowed to grow on selection medium (CI with 500 mg l^{-1} ticarcillin and 15 mg l^{-1} geneticin).

GUS assay and molecular analysis

GUS assay (Jefferson et al., 1987) was done to confirm the transformation event. GUS buffer was prepared with 50 mM Na_2HPO_4, 1 mM Na_2EDTA, 1 mM Fe^{+++}/ $Fe^{++}CN$ and 0.1% Triton X-100. The pH was adjusted

to 7.0 with NaOH, filter sterilised and kept in amber coloured bottles at 4°C. The stock solution of 25 mg ml^{-1} 5-bromo-4-chloro-3 indolyl-β-*D*-glucuronic acid (X-Gluc) was prepared in dimethyl sulfoxide and was stored at -20°C. The GUS assay buffer was prepared freshly by mixing 1 part of X-Gluc with 49 parts of GUS buffer. The explants were incubated overnight in GUS assay buffer at 37°C and were washed with 70% ethanol.

The putative transgenic callus and swollen petiole explants of *A. paeoniifolius* found free from *Agrobacterium* after four subsequent subcultures in ticarcillin containing selection medium were used for DNA isolation with Spectrum DNA isolation kit (Sigma). The DNA was amplified with GUS specific primers (GUS-F 5'-GGGCATTCAGTCTGGATC-3' and GUS-R 5'-GTGCGGATTCACCACTTG-3') by incubating at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min and a final extension of 72°C for 10 min. The reaction mixture for the amplification contained 5 ng μl^{-1} DNA, 1X PCR buffer, 0.5 mM dNTP mix, 0.5 pmol μl^{-1} each of forward and reverse primer, 0.05 U μl^{-1} Taq DNA Polymerase. The vector plasmid was used as positive control, whereas the DNA isolated from untransformed callus was used as negative control. Amplified PCR products were electrophoresed on 1.0% agarose gel with ethidium bromide and visualized under ultraviolet (UV) light. Transformation efficiency was determined by dividing the total number of PCR positive explants by the number of explants inoculated and then multiplied by 100.

Results and Discussion

Induction and multiplication of callus cultures

Initially, lateral bud explants sprouted (Fig. 1b), while the petiole explants enlarged enormously (Fig. 1c). Whitish, yellowish white or pink friable callus (Fig. 1d) was initiated within 15-30 days of inoculation from lateral bud and petiole on modified MS medium supplemented with 0.5 mg l^{-1} each of BA, 2,4-D and NAA (CI medium). The days to callusing and the callusing % differed significantly depending on the explant type (Table 1). MS medium with BA, 2,4-D or NAA alone was not effective in inducing callus. The effectiveness of using two or more growth regulators in callus induction has been supported by previous studies on other plant species like *A. campanulatus* var. hortensis Backer (Irawati et al., 1986), *Gerbera jamesonii* (Aswath and Choudhary, 2002) and *A. albus* (Hu and Liu, 2008).

Transformation, GUS assay and molecular analysis

Transformed callus as well as swollen petiole explants was selected based on its survival on antibiotic selection medium. *Agrobacterium* has been successfully utilised here for genetic transformation and has been reported in many monocotyledon species like rice (Hiei et al., 1994), wheat (Cheng et al., 1997; Sahrawat et al., 2003), maize (Frame et al., 2002) and rye (Popelka and Altpeter, 2003). GUS assay proved to be effective in confirming the transformation event within a short period after transformation (Table 2). The GUS assay of transformed explants (swollen petiole and callus) showed positive blue

Table 1. Effect of growth regulators on callus induction in *A. paeoniifolius*

Growth regulators (mg l^{-1})			Days to callus induction		Callusing (%)	
BA	NAA	2,4-D	Lateral bud	Petiole	Lateral Bud	Petiole
0.0	0.0	0.0	0	0	0	0
0.5	0.0	0.0	0	0	0	0
0.0	0.5	0.0	0	0	0	0
0.0	0.0	0.5	25.43 ± 0.24	0	56	0
0.5	0.5	0.0	21.52 ± 0.19	29.00 ± 0.32	72	40
0.5	0.0	0.5	25.46 ± 0.24	0	52	0
0.0	0.5	0.5	25.84 ± 0.15	0	52	0
0.5	0.5	0.5	13.91 ± 0.18	15.56 ± 0.27	88	72

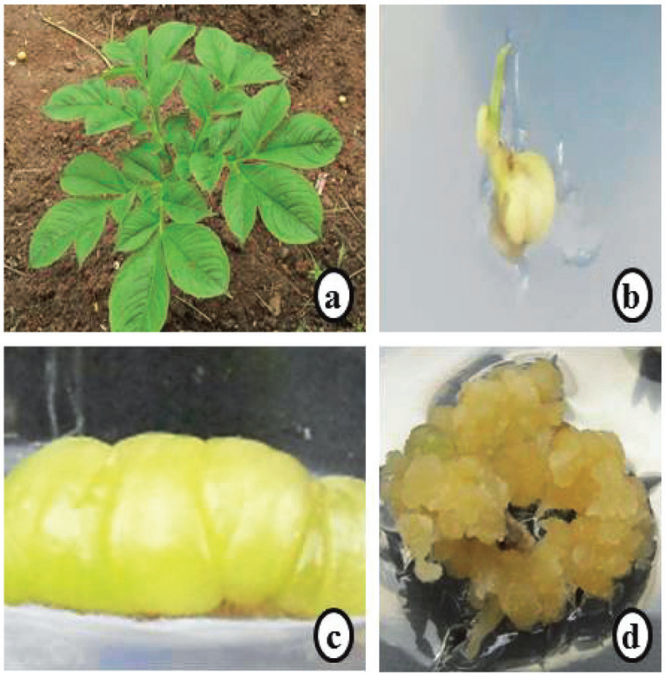

Fig.1. a. *A. paeoniifolius*; b. Sprouted lateral bud explant; c. Swollen petiole; d. Friable callus

colour irrespective of the type of explants. For effective GUS sector observation and photography, TS View Version 7.3.1.7. was utilised (Figs. 2a and 2b). Comparative analysis of transformation efficiency as influenced by explant type and acetosyringone is given in Table 2. The presence of acetosyringone was found to increase the transformation efficiency by 20 fold. Early reports also support the fact of transformation enhancing effect of acetosyringone (Sheikholeslam and Weeks, 1987; Suma et al., 2008). The callus was found to be more suitable for transformation due to its ease of regeneration as well as high transformation efficiency of 36.67%. Ban et al. (2009) also used callus cultures of *A. konjac* for *Agrobacterium* mediated transformation for soft rot disease resistance development. After 4 weeks of incubation in selection medium, the transformed callus showed good growth, while the untransformed underwent browning without any further growth (Fig. 2c). PCR amplification of transgenic callus with GUS specific

Table 2. Effect of explants and acetosyringone on transformation efficiency in *A. paeoniifolius*

Explants	Acetosyringone (μM)	Number of explants inoculated (~0.5 cm^2)	Number of GUS positive explants	Number of blue sectors per explant	Number of PCR positive explants	Transformation efficiency (%)
Callus	0	30	5	1-10	4	13.33
	200	30	14	5-25	11	36.67
Petiole	0	30	2	1-5	1	03.33
	200	30	9	1-15	7	23.33

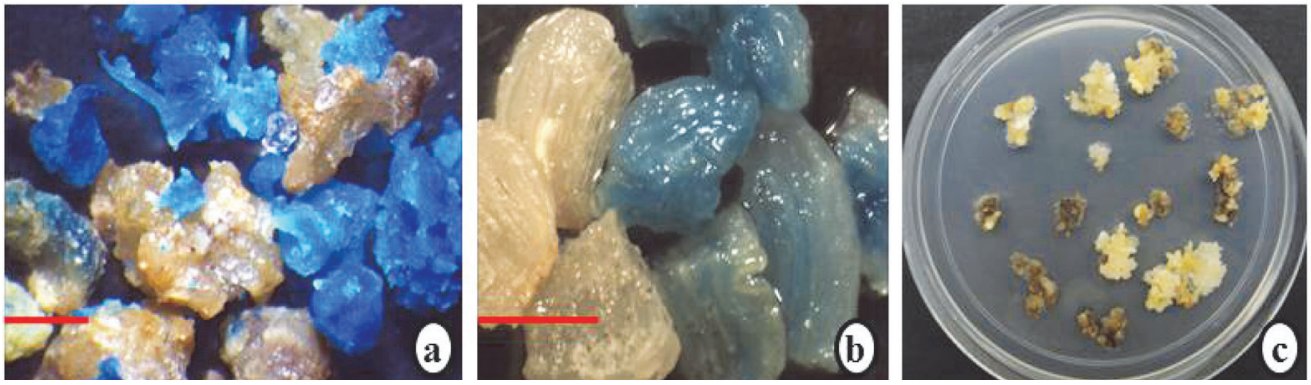

Fig.2. a. Transformed and untransformed callus as distinguished by GUS assay; b. Transformed swollen petiole showing blue colouration against untransformed explant on histochemical analysis; c. Transformed callus showing growth on selection medium Bars = 2mm

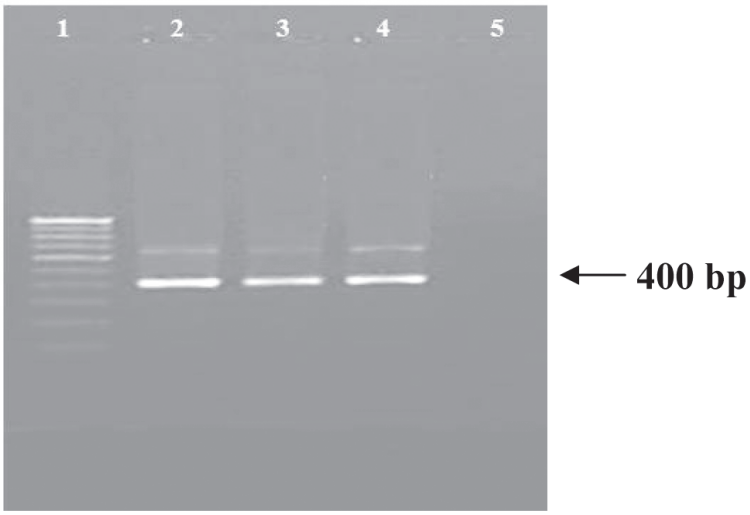

Fig.3. PCR amplification of GUS encoding gene in transgenic *A. paeoniifolius* explants. Lane 1: 100 bp ladder; Lane 2: Positive control; Lane 3: Transformed callus; Lane 4: Transformed petiole; Lane 5: Negative control

primers, which survived in the selection medium gave a band at 400 bp, while the non transformed control callus failed to give amplification (Fig. 3).

An *in vitro* callus multiplication as well as transformation protocol for *A. paeoniifolius* has been established. This paper is first of its type to report on *Agrobacterium* mediated transformation in *A. paeoniifolius*. It is expected that the present study may serve as an initial step towards the development of virus resistant variety and for other genetic modification with useful traits in *A. paeoniifolius* using transgenic approach. GUS gene proved to be an effective reporter gene in *A. paeoniifolius. In vitro* propagation of *A. paeoniifolius* as well as disease resistance development and their field release would greatly increase the availability of quality planting material.

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