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Development of an Efficient *Agrobacterium* Mediated Transformation Protocol for Sri Lankan Rice Variety - Bg 250

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ABSTRACT. *Experiments were conducted to establish an efficient and simple protocol for regeneration and Agrobacterium mediated genetic transformation of an agronomically important indica rice variety Bg 250. Callus induction was achieved on modified N_6B_5 medium supplemented with 2,4 –D (2.0 mg/L), BAP (1.0 mg/L) and NAA (1.0 mg/L). The maximum callusing frequency of 90% was observed after 21 days followed by 4 days of incubation on callus induction medium under dark. The highly prolific, nodular, compact yellowish large calli produced after 25 days were first checked for regeneration ability. Ninety five percent of regeneration frequency was observed with N_6B_5 medium supplemented with 3.0 mg/L BAP and 1.5 mg/L NAA. Therefore, embryonic calli induced after 25 days were used for genetic transformation in subsequent experiments. Agrobacterium tumefaciens strain GV 3101 was transformed with pCAMBIA 1303 binary vector which contains hygromycin marker and GUS reporter gene. The transformed colonies were selected on 50 mg/L kanamycin and 25 mg/L rifampicin and confirmed by colony PCR. The PCR positive colonies were used to transform Bg 250 rice calli. The maximum transformation efficiency of 20% was obtained using 500 mg/L cefotaxime as a bacteriostatic agent to inhibit growth of Agrobacterium. 100 μM acetosyringone in co-cultivation medium and co-cultivation for 3 days were the optimum conditions for maximum transformation. The expression of GUS gene revealed that the calli were successfully transformed.*

Key words: *Agrobacterium, Bg 250, Callus induction, Rice transformation.*

INTRODUCTION

Rice is the most important crop in Sri Lanka occupying 34% of the total cultivated area. There is an enormous need to improve the yield of local rice varieties in order to combat the food demands of increasing population. The most economical method of obtaining higher yield is increasing the productivity of local rice varieties rather than increasing the cultivated area. The improvement can possibly be achieved by genetic manipulation, which depends heavily on the use of *in vitro* techniques. Hence, the establishment of an effective *in vitro* plant regeneration system enables rapid production of fertile and genetically stable plants.

Plant tissue culture has become a useful tool to mass produce new transgenics. Callus induction is generally chosen for monocots such as rice. Khaleda and Al-Forkan (2006) reported that the mature seed scutellum is the best explant for callus induction and plant regeneration in rice. Identification and screening of useful cultivars for embryonic callus

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formation and subsequent *in vitro* plant regeneration are key steps in rice genetic improvement programs through application of biotechnology.

Although monocotyledonous plants were considered to be outside the *Agrobacterium tumefacience* host range (Raineri *et al*., 1990; Gould *et al*., 1991), successful transformation of rice by *Agrobacterium* was reported in the last decade. Chan *et al.,* 1992 and 1993 showed regeneration of *Agrobacterium*-transformed calli from root explants and immature embryos those were the first reported transgenic rice plants obtained by inoculating immature embryos with *Agrobacterium.*

Bg 250 is an improved Sri Lankan rice variety with high quality grains, resistance to leaf blast, bacterial leaf blight, thrips and brown plant-hopper. This is an ultra short duration variety that matures in about 80 days. Bg 250 is suitable for drought and flood-prone areas. In the former, it can escape from drought, if planted early; in the latter, it can escape from flood if planted after the flood has receded, yielding well before the subsequent dry period. However, from all these characters it gives low yielding. The productivity of this variety can be increased by genetic manipulation. The main objective of this study was to develop an efficient *Agrobacterium tumefaciens* mediated transformation protocol for Bg 250 Sri Lankan rice variety.

MATERIALS AND METHODS

Plant material

Seeds of Bg 250 were obtained from Rice Research and Developmental Institute, Bathalagoda, Ibbagamuwa, Sri Lanka.

Bacterial strain and plasmid

Agrobacterium tumefaciens stain GV3101 was used for co-cultivation in the transformation protocol. Binary vector pCAMBIA1303 which is harbouring the *udi*A gene encoding GUS (β-glucuronidase) and the gene conferring hygromycin resistance was used for transformation.

Callus induction

The mature seeds of Bg 250 were de-husked and surface sterilized by the protocol described by Rashid *et al*. (1996). The sterilized seeds were cultured on modified N_6B_5 callus induction medium: N_6 (Chu *et al*., 1975) macro- and micro-elements and B_5 vitamins (Gamborg *et al.*,1968) (N_6B_5 medium), 2,4-D (2 mg/L), BAP (1 mg/L), NAA (1 mg/L), 3% Sucrose and 0.8% agar at pH - 5.8. The cultured seeds were incubated for 14, 21 and 25 days in order to study the time required for maximum callusing.

Transformation of *Agrobacterium* GV3101 and Colony PCR

A super virulent *Agrobacterium* GV3101was transformed with pCAMBIA 1303 plant binary vector by freeze thaw method. Transformed colonies were confirmed by performing a colony PCR with 35S CaMV promoter specific primers. The colony PCR was carried out according to the protocol described by Hettiarachchi (2003).

Agrobacterium mediated callus transformation and regeneration

Agrobacterium strain GV3101 carrying pCAMBIA1303 was grown to an optical density of 1.0 (O.D $_{600nm}$= 1.0) in Luria Bertani (LB) medium containing 50 mg/L kanamycin and 25 mg/L rifampicin. The culture was centrifuged at 3500 rpm for 10 min and the pellet was re-suspended in liquid N_6B_5 medium, 2% sucrose, 1% glucose with different concentrations of Acetosyringone: 50, 100, 150 µM (pH – 5.6). Twenty five day-old proliferated yellowish, nodular, compact embryogenic calli were used for transformation.

The calli were immersed in *Agrobacterium* cell suspension for 3, 5, 7 and 10 min, and blotted on a sterile filter paper. The infected calli were transferred onto N_6B_5 co-cultivation medium (N_6B_5 medium, 3% Maltose, 1% Glucose, 0.3% Phytagel, 50, 100, 150 µM Acetosyringone , pH–5.6) and incubated at 28°C for 1, 2, 3, 4 and 5 days in the dark in order to determine the best co-cultivation period. The co-cultivated calli were then washed 4-5 times with sterile distilled water and series of cefatoxime solutions with concentrations of 1 g/ L, 750 mg/L and 500 mg/L. The washed calli were blotted on a sterile tissue paper and transferred to N_6B_5 callus induction medium containing 0.3% Phytagel as the gelling agent and 250 mg/L, 500 mg/L, 750 mg/L cefotaxime. The calli were then transferred to N_6B_5 selection medium: N_6B_5 medium, 2,4-D (2 mg/L), BAP (1 mg/L), NAA (1 mg/L), 3% Sucrose, 0.4% Phytagel, 50 mg/L Hygromycin, 500 mg/L Cefotaxime, pH - 5.8 (tested with 250, 500 and 700 mg/L Cefotaxime) and incubated for two weeks under dark in order to select transformed calli. After two weeks, green colour calli were transferred to fresh shoot regeneration medium; N_6B_5 medium, BAP (3.0 mg/L), NAA (1.5 mg/L), 3% Maltose, Ascorbic acid (0.2 g/L), Sorbitol (0.1 g/L), Adenine sulphate (0.1 g/L), Cysteine (0.1 g/L), 0.4% phytagel , Hygromycin (50 mg/L), Cefotaxime (500 mg/L), pH - 5.8 (tested with 250, 500 and 700 mg/L of Cefotaxime) and incubated under light. (Before regenerating the transformed calli, the regeneration frequency was checked). The well developed calli with shoot primordia were sub-cultured on N_6B_5 shoot regeneration medium in jam jars and incubated at 28°C under continuous light.

Healthy shoots with defined stem were transferred to MS rooting medium: MS medium (Murashige and Skoog, 1962), 0.4% phytagel, 50 mg/L Hygromycin, 500 mg/L Cefotaxime, pH–5.8 (tested with 250, 500 and 700 mg/L Cefotaxime) and incubated at 28°C under continuous light. The plantlets with well developed root system were planted in 250 mL plastic pots containing autoclaved mud that was collected from rice fields. The plantlets were established in such a way that the clump of plantlets originated from each callus was separated carefully and planted in several pots.

Analysis of transformants

The two week old transformed calli were screened histochemically for verification of the expression of *uidA* gene. The assay for GUS gene was carried out for selected calli. The calli were submerged in fixation buffer and vacuum infiltrated for 4-5 min on ice and kept at room temperature for 10 min. The fixation buffer was removed and the material was washed twice with 50 mM sodium phosphate buffer to remove fixative buffer. The tissue samples were stained with 1.5 mM of X-gluc, 50 mM sodium phosphate and 0.1% Triton X-100 by vacuum infiltrating for 5-10 min. and then kept at 37°C overnight in the dark. The calli which showed blue patches were recorded as positive transformants.

RESULTS AND DISCUSSION

Callus induction and regeneration

Transformation of rice using *Agrobacterium* mediated methods and subsequent regeneration are dependent on several factors. Among them, the choice of explant, hormonal composition of the medium used and nutritional supplements highly affects the callusing and regeneration (Lin *et al*., 1995; Katiyar *et al*., 1999). Hiei *et al*. (1994) reported that scutellum derived callus was the most amenable explant for *Agrobacterium* mediated transformation. In the present study, mature rice seeds were used to obtain scutellum derived calli and the maximum callus induction frequency was obtained on modified N_6B_5 medium containing 2 2,4 D (mg/L), BAP (1 mg/L) and NAA (1 mg/L). Some reports recommended 2,4 D (6 mg/L) (Aldemita and Hodges, 1996; Lin and Jhang, 2005) however most reports recommended 2,4 D (1.5 – 2.0 mg/L) for callus induction which is confirmed by our results too. To study the time required for obtaining embryogenic calli, mature seeds were incubated on the callus induction medium for 14, 21 and 25 days and the calli obtained were then sub cultured on the same but fresh medium for 4 days. Twenty one days followed by four days were found to be the best time period for maximum callusing and callus induction frequency was 90%. The resulting calli were yellowish, nodular, compact and highly prolific with 10.24 mm diameter (Fig. 1B).

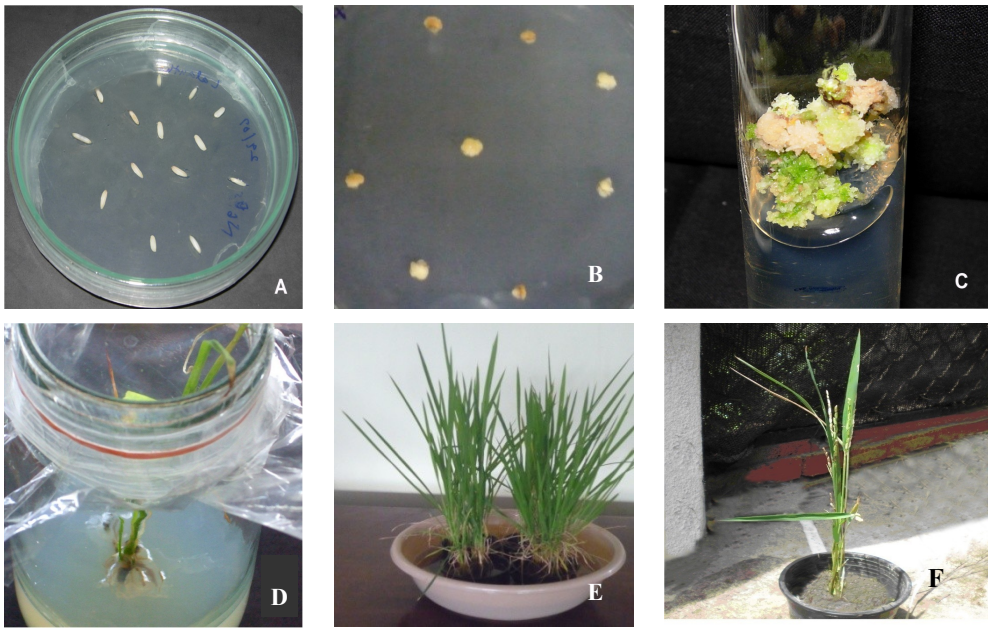

Fig. 1. Plant regeneration from scutellum derived callus in rice variety Bg 250

(A) De-husked, surface sterilized mature rice seeds on Callus Induction medium, (B) 21 days old scutellum derived calli on N_6B_5 callus induction medium, (C) greenish, compact and well proliferated calli on shoot regeneration medium, (D) plantlets in rooting medium, (E) acclimatization under growth room conditions and (F) regenerated mature rice plant.

The greatest difficulty one would encounter in rice tissue culture is the shoot regeneration or the organogenesis from the scutellum derived calli of rice seeds. Regeneration or organogenesis is highly dependent on its genotype. Therefore, before using the above calli in genetic transformation, it was necessary to confirm its regeneration ability. The best regeneration frequency of 95% was observed with N_6B_5 medium supplemented with 3.0 mg/ L BAP and 1.5 mg/L NAA. In order to study the regeneration efficiency, 25 days old calli were treated under dark and light for two weeks. The calli incubated under light turned to green after 7 days but regeneration frequency was very poor compared to the dark treated ones. The dark incubated calli took two weeks to turn green. Green shoot buds with well developed leaf like structures were obtained after 7 to 10 days (Fig. 1C).

Genetic transformation of rice callus

Agrobacterium tumefaciens strain GV 3101 was transformed with pCAMBIA 1303 binary vector according to the protocol described by Holsters *et al.* (1978). Transformed colonies were selected on LB medium supplemented with 50 mg/L kanamycin and 25 mg/L rifampicin and then confirmed by colony PCR (Fig. 2). The transformed colonies resulting 123 bp fragments from 35S CaMV promoter specific primers were selected as positive colonies and they were used for transformation of rice calli.

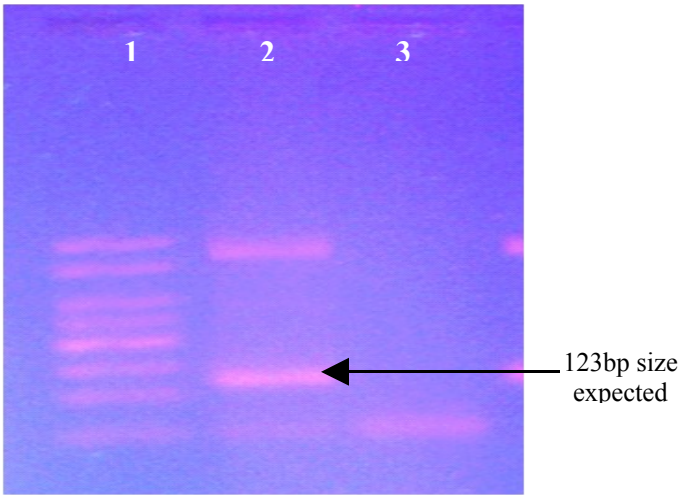

Fig. 2. Confirmation of the presence of CaMV 35S promoter region in A*grobacterium tumefaciens* strain GV3101 transformed with binary vector pCAMBIA1303.

Lane No 1: 50 bp ladder – Sizes ranging from 50, 100, 150, 200, 250, 300, 350, 400 bp , Lane No 2: Colony PCR product of *Agrobacterium tumefaciens* strain GV3101 transformed colony, and Lane No 3: Negative control

The temperature of incubation, duration of co-cultivation with *Agrobacterium*, concentration and the composition of bacteriostatic agent and duration of selection and concentration of antibiotic selection marker are the other factors reported which affect *Agrobacterium* mediated transformation of rice (Pollock *et al.,* 1983; Dillen *et al*., 1997; Okkels and Pederson, 1998; Katiyar *et al*., 1999). To find the optimum conditions for co-cultivation, different concentration of acetosyringone and the duration of co-cultivation were tested. The

frequency of hygromycine resistant calli obtained following each variation in co-cultivation conditions was taken as the transformation frequency. The presence of 100 μM acetosyringine in co-cultivation medium and co cultivation for 3 days were found to be the most suitable for optimum transformation.

Regeneration and analysis of transformants

Compared to the non transformed calli regeneration frequency of 95%, transformed calli showed very low regeneration frequency (30%). Kumar *et al*. (2005) reported a transformation efficiency of 4.6% - 5.5% and 6.4% - 7.3% for two recalcitrant elite *Indica* rice cultivars, which were lower transformation frequencies than these results. Formations of embryogenic calli were enhanced with the use of high percentages of gelling agent (phytagel) and maltose as a carbon source (Kumar *et al*., 2005). The modified regeneration medium substituting agar with phytagel increased the regeneration frequency up to 73% and therefore all media used after transformation was substituted with phytagel. A bacteriostatic agent, cefotaxime was used to prevent *Agrobacterium* growth after co-cultivation. It has been reported that the use of high concentration of bacteriostatic agents may reduce the regenerability of the calli as they structurally resemble auxins. Further, in combination with other callus inducing hormones such as 2,4 D may cause loss in regeneration potential (Lin *et al*., 1995; Okkels and Pederson, 1998). The green colour areas appeared on the middle of the calluses after 3 weeks (Fig. 3 B) and when transferred to regeneration medium later developed into tiny shoots (Fig. 3 C). *In vitro* regenerated shoots with defined stems were cultured on hormone free MS rooting medium for vigorous root development before transferring them into soil (Fig. 3D). After 6 days, a well developed root system was observed in the plantlets. The plantlets transferred to soil (Fig. 3 E) survived under the normal environmental conditions and grew to maturity. In order to confirm the presence of the transgene, few calli were subjected to histochemical staining. The CaMV 35S promoter used to drive the reporter gene (GUS) was found to be constitutively expressed in rice. Appearance of blue colour following overnight incubation at 37^0 C with GUS assay revealed the presence of the transgene and stable transgenic nature of the plants (Fig. 4). In this study stable genetic transformation by *Agrobacterium* mediated transformation protocol of Bg 250 was demonstrated.

Therefore, this protocol can be modified in order to regenerate and recover transgenic rice plants of any rice variety. Modifications can be made at any stage i.e. before and after transformation with *Agrobacterium* (i.e pre-incubation treatment of calli, selection of transformed calli and shoot regeneration stages). Therefore the protocol used in the present study can be applied to rice callus induction and regeneration for breeding, transformation and other biotechnological applications.

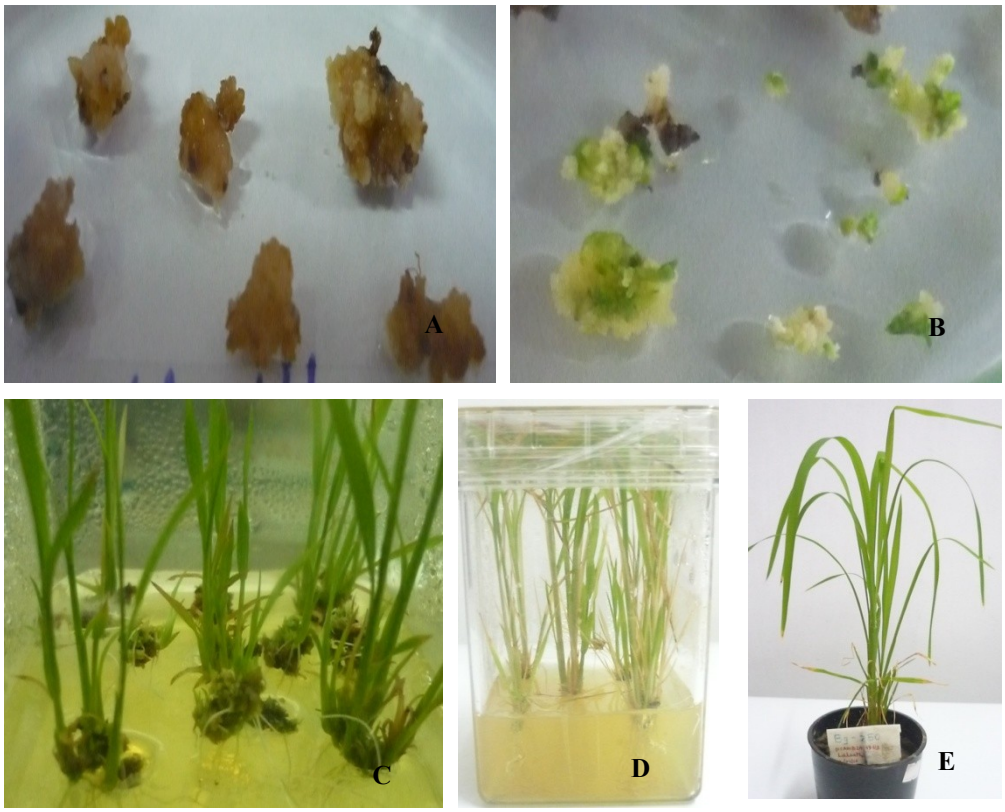

Fig. 3. **Regeneration of transgenic Bg 250 from scutellum derived rice calli after *Agrobacterium* mediated transformation. (A) Scutellum derived calli on co-cultivation medium (B) hygromicine resistant proliferating calli on shoot regeneration medium, (C) regenerated shoots on shoot regeneration medium, (D) plantlets on rooting medium, and (E) acclimatization of Hygromicine resistant transformed Bg 250 rice plantlet.**

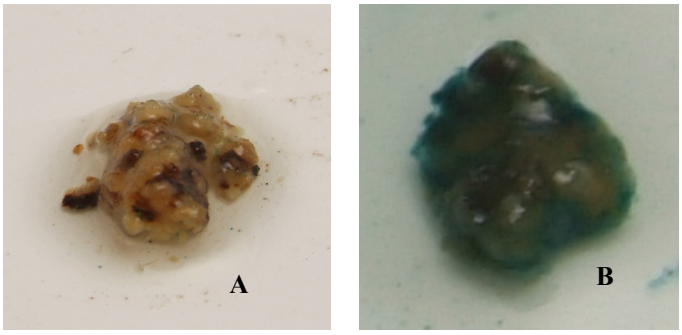

Fig. 4. **Expression of GUS gene in two week old transformed rice calli when stained with X-gluc. (A) Untransformed callus and (B) transformed callus**

CONCLUSIONS

The calli obtained from mature rice embryos were be good explants for efficient *in vitro* plant regeneration. This study showed that, it is possible to obtain transgenic rice plants from scutellum derived calli of Bg 250 variety under *in vitro* manipulation. The method described was simple, in expensive and does not require any advanced equipment.

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