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Agrobacterium mediated Transformation of rice, var. Pusa Basmati-1

M. Anbazhagan, R. Rajendran, M. Kalpana V. Natarajan, R. Panneerselvam*
Department of Botany, Annamalai University, Annamalainagar-608 002, Chidambaram, Tamilnadu, India.

**Corresponding Author, Email: anbungm@yahoo.co.in*

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Abstract
The present study was aimed at the *Agrobacterium* mediated transformation of rice (*Oryza sativa* L.) variety Pusa Basmati-1 .*Agrobacterium tumefaciens* strain LBA 4404 harboring the binary vector (pIG121) that carries the genes for ß-glucuronidase and hygromycin phosphotransferase (hpt) was used for transformation. Co-cultured calli were undergone for GUS histochemical analysis of transformed calli and followed by molecular analysis by using PCR to confirm the presence of transgenes.

1. Introduction

Rice (*Oryza sativa* L.) belongs to the family Poaceae, is the second largest cereal crop in the world and providing a staple food for mankind in the developing as well as developed countries [1]. Rice is primarily a high energy or high calorie food. The protein content of milled rice is 6-7 % and its biological value is high. The fat content of rice is low 2-2.5 %. Rice is rich in B group vitamins. The by-products of rice are used as cattle and poultry feed and insulation materials. In India, rice occupies an area of about 44 million hectares with productions of 89 million tones [2].

The world wide projected demand of rice by 2020 is 880 million tones and that of India is around 200 million tones of paddy. Due to the increased rate of population the productivity level must be increased further by 35-40 % in order to retain self-sufficiency the years to come. Though rice has a food value and demand, it is susceptible to many pathogens like bacteria, fungi, various insects and pests and also to abiotic stresses like drought, salinity which lead to decrease in yield of rice [3]. To overcome these problems, with the advancement of biotechnology and r-DNA technology, there is a novel and powerful way to minimize the loss of productivity through transformation technique in paddy rather than traditional plant breeding methods.

The transgenic approach has opened up a plethora of opportunities to genetically manipulate plants across the species barrier thus making it possible to transfer any gene, be it of bacterial, animal or plant origin to the desired crop [4]. Significant advances in tissue culture and gene delivery techniques have allowed the incorporation of beneficial genes for specific agronomic traits into diverse crop plants. Genetic engineering of rice enables breeders to design new varieties by the introduction of desired alien genes into existing commercial lines. In the past few years, considerable progress has been made in optimizing and refining genetic transformation techniques in rice. Direct DNA uptake into rice explants was reported in japonica and in a few indica varieties [5-9]. The development of technologies for the culture, regeneration and plant transformation that led to the production of transgenic crops which have become key to many applications of plant biotechnology.

Agrobacterium mediated transformation of several dicotyledonous plants is a routine work. However, monocots are generally considered as recalcitrant to *Agrobacterium*. In general, the *Agrobacterium* mediated transformation was found very effective mainly because of single copy integration of T-DNA region,

high fertility of transgenics and transmission of transgenes in a Mendelian fashion. Hence, this technique has become the most preferred option to transform desired genes in rice varieties when compare to direct gene transfer techniques, in which, multi-copy integration, transgene silencing, reduced fertility and not genotype-independent in terms of efficiency are found [10-13]. As the former has more advantages than the latter technique, in this study, an attempt has been made on the *Agrobacterium* mediated gene transformation of pusa basumathi-1 rice variety. Hiei *et al*., [14] reported the unequivocal evidence of *Agrobacterium* based transformation in japonica rice. Later, the host range was extended to a few javanica and indica rice varieties [15–17]. However, the efficiency of *Agrobacterium* based transformation in rice is modulated by genotype, choice of tissue and choice of vector besides culture conditions [18]. In rice, different binary vectors have been used for achieving genetic transformation. Hiei *et al.,* [14], Rashid *et. al.,* [17], and Cheng *et. al.,* [19] used pIG121Hm vectors, which is a derivative of the most commonly used vectors, viz. pBI121 [20]. Through this study, *Agrobacterium* mediated transformation of an elite indica variety Pusa Basmati-1 has been reported and this is confirmed by molecular analysis.

2. Materials and Methods

2.1. Gene transformation procedure

Rice seeds of Pusa Basmati-1 variety were obtained from the MSSRF (M.S. Swaminathan Research Foundation) Chennai, India. Healthy seeds were selected and dehusked manually. They were surface-sterilized with 70 % ethanol for 2 min and this is followed by 0.1 % $HgCl_2$ containing 3 drops of teepol for 5 min. After three rinses with sterilized distilled water, they were placed on solid N6 medium [21] for callus induction. The N6 basal medium used for this studies containing standard salts and vitamins, 3 % sucrose (w/v), 0.8 % agar (Himedia Pvt. Ltd., India) and the plant growth regulator 2,4-D (2.5 mg/l). The pH of the medium was adjusted to 5.8 before adding agar and the medium was autoclaved at 15 psi for15 min at 121°C. The cultures were incubated at 25±2°C using a 16 h lights (50 µmol m^{-2} s^{-1}) and 8 h dark cycle. After 3 weeks, small, vigorously dividing calli of 2–4 mm in diameter were sub-cultured on the same fresh medium for 3 days, and then the healthy looking embryogenic calli were used for transformation. A plastic petri dish of 9 cm in diameter was used for induction and subculture of the calli, and the petri dish was sealed with surgical tape.

Agrobacterium tumefaciens strain LBA 4404 (pIG121) was cultured on liquid LB medium [22] containing kanamycin sulfate (50 mg/l), hygromycin-B (50 mg/l) and agar (15 g/l) for 3 days at 28°C in the dark. The bacteria were collected and suspended in AAM medium [14] containing acetosyringone (0, 15 and 40 mg/l). For *Agrobacterium* infection, the density of the bacteria was adjusted (OD600 = 0.02, 0.4 and 0.2) and the rice calli were immersed in a bacterial suspension for 2 min. Excess bacteria were removed by blotting the calli on filter paper. The calli (2–4mm in diameter) were transferred onto a single sterilized filter paper placed on a 9 cm diameter petri dish containing about 30 ml of solid N6AS medium. The N6AS medium was supplemented with glucose (5 g/l), L-cysteine (100 mg/l), and acetosyringone (0–40 mg/l). The plates were sealed with parafilm to prevent evaporation of the medium and subjected to 3 days of co-cultivation at 25±2°C in the dark. Calli were then washed twice in sterile water to remove *Agrobacterium*. The co-cultured calli were blotted dry on filter paper and plated on N6AS medium supplemented with hygromycin-B (50 mg/l). The plates were sealed with surgical tape and incubated at 25±2°C using a 16 h light (50 µmol m^{-2} s^{-1}) and 8 h dark cycle. Proliferating hygromycin resistant calli were transferred to the same fresh medium.

2.2. Assay for GUS gene expression

Histochemical assay was done to detect the transformation and integration of the reporter gene GUS in to the tissue containing target genome. After ten days, the *Agrobacteria* were eliminated and the co-cultured calli were incubated in X-Gluc solution at 37°C for overnight and ß-glucuronidase activity was determined microscopically. The tissue containing ß-glucuronidase turned blue in colour and this confirmed the transformation of the GUS gene [23].

2.3. PCR analysis of transformation

Genomic DNA was isolated from transformed and untransformed calli by the CTAB method [24]. PCR analyses were carried out by using two GUS primers namely, forward 5'GGA ATG GTA ATT ACC GAC C3' and reverse 5' ATA CCT GTT CAC CGA CGA CG3' for amplification of GUS gene transformants [25]. The reaction mixture (20 µl**)** of PCR consists of 1.0 µl DNA template, 2.0 µl 10x buffer, 1.0 µl (2.5mM) dNTPs, 2.0 µl (25 mM) $MgC1_2$, l.0 µl of each primer (F/R), 0.4 µl *Taq* DNA polymerase and ddH_2O 13 µl. Reaction procedures

were carried out at 94°C for 4 min and followed by 25 cycles at 94°C for 1 min, 56°C for 45 sec. and 72°C for 1 min. After the final cycle, the reactions were maintained at 72°C for 5 min before completion. Finally, PCR products were analyzed on 1% agarose gel with 0.5x TBE buffer.

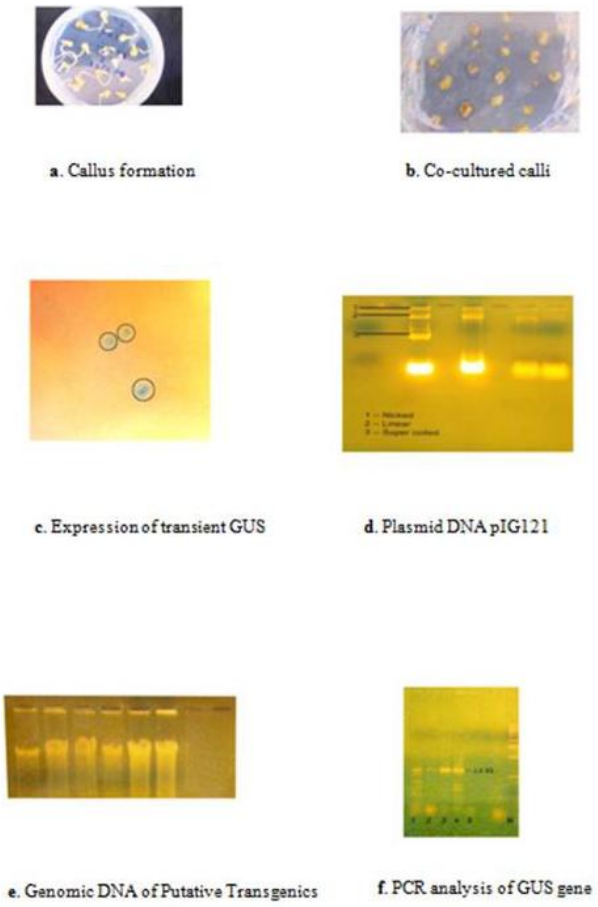
3. Results and Discussion

The calli derived from scutella of paddy were used for gene transformation. Various factors which may influence the transformation efficiency viz., co-cultivation duration of both *Agrobacterium* and calli, and the use of liquid AAM medium during co-cultivation were optimized to develop an efficient transformation protocol. After 21 days of culturing on N6 solid medium, embryonic calli were derived for transformation (Fig.1a) and then they were incubated and co-culturedwith *A. tumifaciencs* strain LBA 4404 Gus on N6AS solid medium for 3 days. The composition of co-cultivation medium is an important factor that affects the efficiency of transformation and active cell division of calli. The simple modified N6 medium, containing acetosyringone (AS) and hydroxy acetosyringone is suitable for co-cultivation [16]. The highest GUS activity was observed in third day of co-cultivation and this duration was optimized for further studies.

A. tumefaciens strain LBA 4404 (pIG121) and scutella-derived calli were co-cultivated for 3 days and after third day, the calli were washed with sterile water containing cefotaxime (250 mg/l) to remove *Agrobacterium* and then the calli were transferred to the selection medium containing hygromycin. In general, in the selection medium, small friable pieces of calli were started appearing from the sides of mother calli (Fig.1b).The transformed callus with hygromycin phosphotransferase (*hpt*) can only inactivate the hygromycin present in the N6 selection medium. The untransformed calli cannot survive in the medium containing hygromycin and the calli turned into brown colour and died because of the toxicity of the active hygromycin that inhibit the protein synthesis of calli. Randomly selected calli were used for GUS assay by using X-gluc as substrate and integration of genes were observed as blue spots. Multiple integrations were observed in different callus (Fig.1c).

It is clear that numerous factors are important in the *Agrobacterium* mediated transformation of rice. The type and stage of tissue to be infected, the kind of vector, the genotype of rice, various conditions in tissue culture and culturing environment may affect the transformation. The aim of the present investigation was to establish an efficient transformation technique for an elite indica rice variety Pusa Basmati-1. Various factors that influence the gene delivery efficiency were optimized. One important factor was the use of only embryogenic calli which could be selected visually. The presence of acetosyringone (AS) was found to be essential for transformation. Sub-culturing of calli for 3 days prior to co-cultivation was reported by Hiei *et. al.,* [14] and it was found to be very useful for the transformation technique. Similar observations have also been made by Toki [15] and others [9, 26-28].

Fig. 1. *Agrobacterium* mediated transformation of rice, var. Pusa Basmati-1.



In the transformation technique, the integrated GUS gene codes for ß-glucoronic enzyme. The substrate X-glucA is cleaved by GUS at the ß-glucoronic bond between glucoronic acid and the 5-bromo 4, chloro-3indosyl part of X-glucA through hydrolysis. The cleavage of X-glucA results in the precipitation of water insoluble blue and this blue colour indicates the presence of GUS gene [20]. In this study also, the transformation is confirmed by the same method. Further, Genomic DNA was

isolated from the transformed calli of this study and undergone PCR analysis for the confirmation of the presence of GUS gene by GUS forward and reverse primers (Fig.1d-f). All putative transgenic lines, exhibited the predicted GUS gene.

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