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Full Length Research Paper

Transient expression of β-glucuronidase gene in indica and japonica rice (*Oryza sativa* L.) callus cultures after different stages of co-bombardment

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Rapidly growing embryogenic calli of different ages derived from the scutellum of mature seed embryos of indica rice (Rasi) and mature seeds of japonica rice (Taipei 309) was biolistically transformed. Plasmid pUCGUS containing the *uidA* gene encoding β-glucuronidase was used to optimize transformation conditions using various combinations of helium pressure, target distance and gap distance. Plasmid pHX4 carrying hygromycin phosphotransferase (*hph*) gene and pUCGUS was used for co bombardment. Resistant calli were selected in the presence of hygromycin B. Successful co-transfer of DNAs to cells was monitored by analyzing transient *gus* expression 24 h after bombardment and 24 days after selection. Maximum level of *gus* expression was observed if calli were selected for transformation after 44 days in maintenance medium. Maximum callus transformation frequency of 37.5 was observed for Taipei 309 as compared to 24.2 for indica rice cv. Rasi.

Key words: Particle bombardment, rice, embryogenic calli, *gus* assay, hygromycin selection.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most versatile and important cereal crops of Poaceae family cultivated for more than 10,000 years. Currently this crop supports more than 50% of the World population. Recent advances in molecular biology and genetic engineering have provided tools that can increase the efficiency of existing breeding methods and allow unconventional approaches to rice improvement. The genotype-independent biolistic particle delivery system developed for rice (Christou et al., 1991) has shown reproducibility of results in different laboratories overcoming constraints related to other methods and remains the method of choice for introducing useful genes into rice crop (Christou,1997).

Embryogenic calli and regenerable embryogenic suspension cultures established from mature seeds of japonica cultivars (Christou et al., 1991; Cao et al.,1992; Li et al.,1993; Duan et al.,1996;), indica cultivars (Li et al.,1993; Sivamani et al., 1996; Zhang et al.,1996; Jain et al., 1996; Ghosh Biswas et al., 1998), elite cultivars belong to Central America and West Africa (Valdez et al.,1998), U.S. rice lines (Jiang et al ., 2000) and Australian rice cultivars (Abedinia et al., 2004) have been successfully used as target tissue to develop transgenic plants expressing various traits. Earlier, optimization of various parameters for the introduction of - glucuronidase gene into embryogenic callus cultures of indica rice variety Basmati 370 (Minhas et al., 1996) and suspension cells of Pusa Basmati 1 (Jain et al., 1996) have been reported. The objectives of the present study were to optimize the parameters of Biolistic™ PDS-1000/He driven particle delivery system for co-bombardment of two plasmid constructs into embryogenic callus cultures of rice and to analyze transient expression of *gus* gene after 24 h of transformation and 24 days of hygromycin selection.

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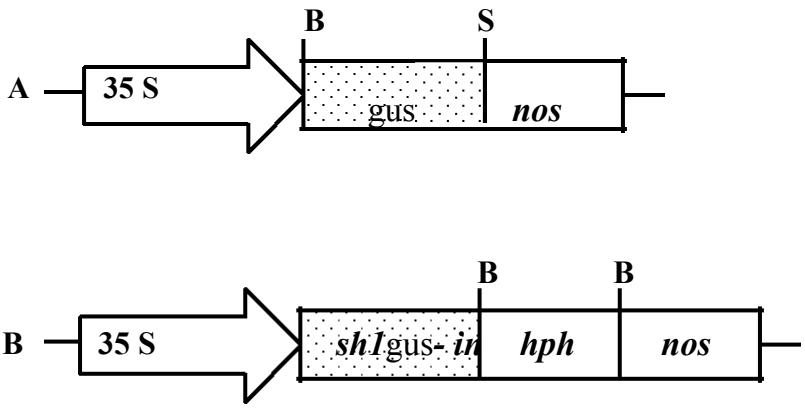

Figure 1. Schematic diagram of plasmid constructs **(A**) pUCGUS and (**B**) pHx4 used for co-bombardment. **35 S**, 35 S RNA promoter from cauliflower mosaic virus; **Sh1-in**, Shrunken locus intron; **B**, Bam H1 restriction site; ***hph***, hygromycin phosphotransferase gene encoding resistance to hygromycin; *nos*, the polyadenylation signal from nopaline synthase gene; ***gus*****,** β-glucuronidase gene.

MATERIALS AND METHODS

Plant material and *in vitro* callus induction

Mature seeds of indica (Rasi) and japonica rice (Taipei 309) were collected from TamilNadu Agricultural University, Coimbatore, India and Division of Plant Breeding IRRI, Philippines respectively. Dehusked seeds were surface sterilized in 70% (v/v) ethanol for 30 s, followed by 0.1% (w/v) mercuric chloride for 4 min. Seeds were thoroughly washed in sterile distilled water. For callus induction, indica seeds were plated on Petri dishes (6 cm diameter) containing LS (Linsmaier and Skoog, 1965) supplemented with 11.3 M 2,4-D (2,4-dichlorophenoxyacetic acid), 30 g/L sucrose and 2.5 g/L phytagel (pH 5.8). Mature seeds of Taipei 309 were cultured on NB medium (Li et al., 1993) containing 9.0 M 2,4-D. After 4 weeks of culturing at 26°C, friable loosely arranged embryogenic calli were separated and sub cultured onto fresh medium for proliferation.

Plasmids

The following plasmids were used for transformation experiments: pUCGUS is a 6.1 kb construct containing the *uidA* gene encoding β-glucuronidase and pHX4 is a 6.43 kb construct containing the hygromycin phosphotransferase (*hph*) gene fused with exon and first intron of the barely *shrunken 1 gene* (a gift from J. Finer, Ohio Sate University, Columbus, OH, USA) (Figure 1). Both plasmids were isolated and purified according to alkaline lysis and $CsCl_2$ density gradient centrifugation (Maniatis et al., 1982).

Particle bombardment

Transformation experiments were performed with the helium driven Biolistic™ PDS- 1000 particle delivery system (BioRad, Richmond, CA, USA) . Ultra pure plasmid DNAs, pHX4 *and* pUCGUS taken in 1:1 molar ratio were adsorbed on 1.0 m gold particles (Bio-Rad) according to Sanford et al. (1993) method and various physical parameters were optimized.

Friable embryogenic calli after 44, 56 and 68 days of proliferation were trimmed into 2-3 mm and centered in a petri dish (25- 30 pieces/dish) containing 20 ml of LS medium with 11.3 M 2,4-D, 0.2 M mannitol and sorbitol (NB medium for japonica rice calli) solidified with 2.5 g/L phytagel. Plates were incubated for 4 h in the dark before bombardment. Each plate was bombarded twice by rotating the plate by 90° at a helium pressure of 1100 psi, keeping the target distance as 9 cm, gap distance as 9 mm and travel distance as 3 mm. Vacuum of 28 inches of mercury (0.006 atm) was maintained in all experiments.

Selection and *GUS* assay

After 16- 20 h of bombardment, calli were transferred to medium with 50 mg/L hygromycin and incubated at 25±2°C under photoperiod of 16 h light using cool white fluorescent light. Histochemical *gus* assay of calli was carried out 24 h after co-bombardment and 24 days after selection (Jefferson et al., 1987). Frequency of gene delivery was determined by scoring number of blue spots on a callus clump and number of callus clump showing blue spot.

RESULTS AND DISCUSSION

In this study, initially an attempt was made for efficient induction of embryogenic calli from mature seeds of indica rice Rasi. For this four different basal media – MS (Murashige and Skoog, 1962), N6 (Chu et al., 1978) LS and B5 (Gamborg et al., 1968) were tested. Callus induction frequency was determined by measuring the fresh weight after 28 days of culture (data not shown).It was observed that LS medium was the best for induction and further proliferation. The initiation of callus tissue was observed after 10-14 days from the scutellar region (Figure 2A) of the mature seeds of indica cv Rasi with a frequency of 85.5%. 2,4-D at 11.3 has also been reported to induce optimum callusing in other cultivars of indica and japonica lines (Abdullah et al., 1986; Oinam and Kothari, 1995; Jain et al., 1996; Cao et al ., 1992; Saharan et al ., 2004). Seeds of japonica rice Taipei 309

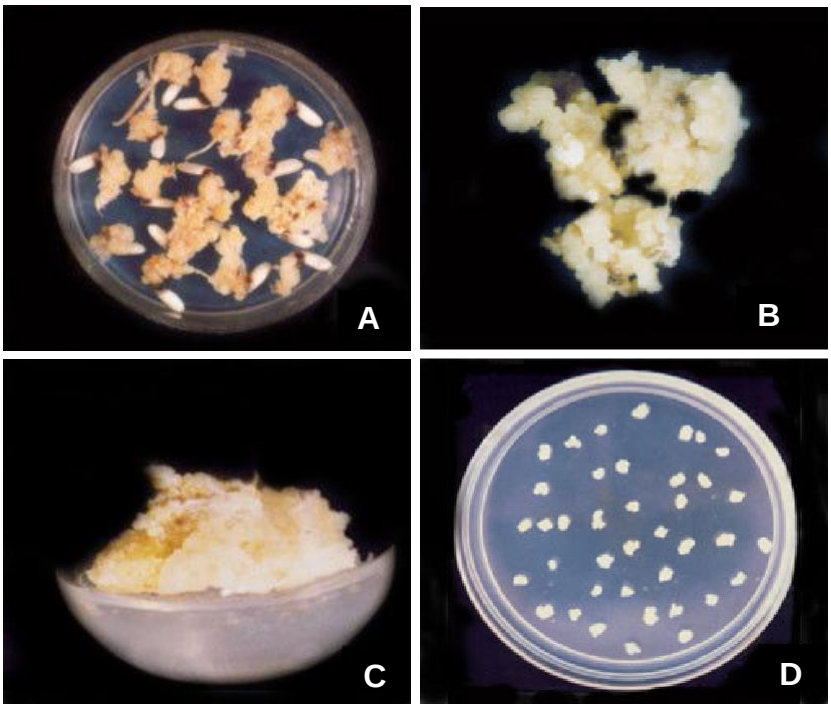

Figure 2. Tissue culture response of indica (A, B) and japonica rice (C, D). (**A**) Induction of embryogenic callus from mature seeds after four weeks of culture. (**B**) Enlarged view of 44 day's old sub cultured callus**.** (**C**) Highly regenerable 44 days old callus. (**D**) Finely trimmed calli (2-3 mm) prior to particle bombardment.

Table 1. Effect of age on transient expression of *gus gene* and production of hygromycin resistant calli in indica and japonica rice transformed via Biolistic^{TM} particle gun

Variety	Age of calli (days)	No. of calli bombarded	*GUS* expression				Callus transformation (%)
			24 h after co-bombardment		24 days after co-bombardment		
			GUS+*	%	*GUS*+**	%	
Rasi	44	390	88 (178)	49.4	13 (33)	39.3	15.5
	56	260	52 (120)	43.3	9 (34)	26.4	24.2
	68	250	31 (120)	25.8	8 (31)	25.8	23.8
Taipei 309	44	210	83 (105)	79.0	9 (37)	24.3	35.2
	56	180	58 (80)	72.5	10 (40)	25.0	33.3
	68	150	46 (78)	58.9	2 (27)	7.4	37.5

*Values in parenthesis indicate total number of calli selected for histochemical *gus* staining.
** Values in parenthesis indicate total number of hygromycin resistant calli.
The data has been collected from 9 independent experiments each with 16-18 plates with calli of different ages.

exhibited elongation of shoots followed by proliferation of white to yellow callus development on NB medium (Figure 2C) as observed earlier (Visarada and Sarma, 2002). Portions of friable embryogenic sectors subcultured on fresh medium increased their fresh weight (Figure 2B) In this study, friable, nodular, white to pale yellow embryogenic calli separated from non-embryogenic portions has been used as starting material (Figure 2D) to optimize parameters for gene delivery using PDS-1000/He driven particle delivery system. Out of various combinations of helium pressure, target distance and gap distance tried, a maximum frequency of gene delivery was observed when calli were bombarded at 1100 psi helium pressure keeping the target distance

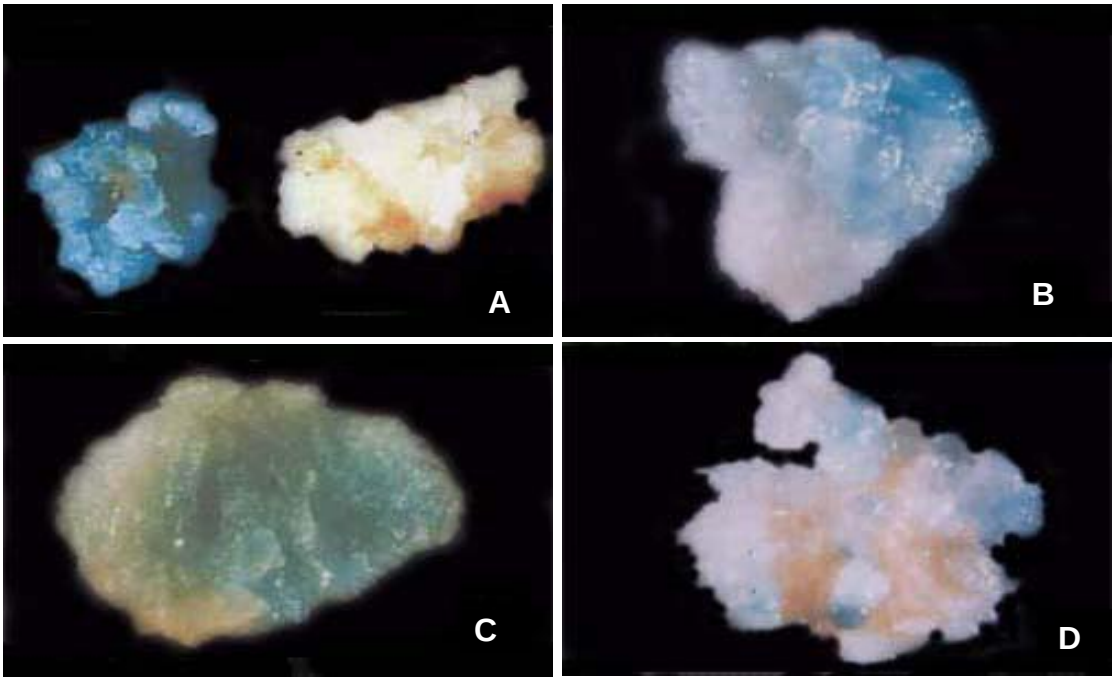

Figure 3. *gus* expression in 44 days old callus tissues of indica and japonica rice after 24 h of bombardment (A,B) and 24 days of selection (C,D). (**A**) Mature seed derived indica rice callus stained for *gus* expression along with control. (**B**) Embryogenic callus expressing *gus* expression from japonica rice after 24 h of bombardment. Intensive (**C**) and moderate (**D**) staining in hygromycin resistant callus of indica and japonica rice respectively after 24 days of selection.

as 9 cm and gap distance as 9 mm. No *gus* gene expression was observed when bombarded at helium pressure of 650 psi. Helium pressure of 1100 psi and target distance of 9 cm promoting high frequency of *gus* expression was shown previously (Zhang et al., 1996; Minhas et al., 1996; Chen et al., 1998a, Jiang et al., 2000 and Jain et al., 1996; Anoop and Gupta., 2004).

Effect of age of callus tissue on transient expression of *gus* gene after 24 h and 24 days of selection are presented in Table 1. Approximately 50% of the calli were tested for *gus* expression after 24 h of bombardment. Activity was always higher in 44 days old callus tissues followed by 56 and 68. Maximum activity (79%) was found with mature seed derived calli from Taipei 309 (Figure 3B) followed by the same source calli (49.4%) of Rasi (Figure 3A). Highest level of *gus* gene expression were found if calli were selected for transformation after 44 days of maintenance with a frequency varied between 49.4 and 79% (Table 1) . In most of the samples *gus* gene expression exhibiting blue stains were unevenly distributed on the surfaces. Similar observations have been previously reported for embryogenic calli 48 h after bombardment (Li et al., 1993 and Sivamani et al., 1996).

Resistance to antibiotic hygromycin B has been used as selection criterion for selecting transformed cells in this study as reported previously in developing transgenic rice (Lin et al., 1995; Sivamani et al., 1996; Duan et al., 1996; Zhang et al., 1996; Wu et al., 1997; Chen et al., 1998b; Abedinia et al., 2004). Sensitivity of calli to hygromycin was assessed before transformation experiments. Hygromycin at 50 mg/L was found to completely arrest the growth (data not shown). Remaining calluses bombarded with pHX4 and pUCGUS were selected for 24 days subculturing once in 12 days on callus induction medium containing hygromycin. In both varieties, frequency of *gus* expression, after selection ranged from 7.4 - 39.3%. The highest value of 39.3 was observed for seed derived calli of indica rice and the lowest value of 7 were observed for seed derived calli of japonica rice. Expression results scored for calli bombarded after 56 and 68 days of maintenance are gradually decreasing. In some of the clumps intensive blue staining could be detected (Figure 3C) and this could be due to the diffusion of *gus* reaction product to neighboring region. Similar expression patterns were reported earlier following particle bombardment in rice (Li et al., 1993), turf type Bermuda grass (Li and Qu, 2004), cowpea (Kononowicz et al., 1995), and Sweet potato (Prakash and Vardarajan, 1992).

Callus transformation frequency was found to be cultivar-dependent ranging from 15.5 to 27.6 and 33.3 to 37.5% for indica and japonica rice, respectively. Although indica rice calli exhibited lower transformation frequency than japonica, the frequency of *gus* positive calli was higher for indica rice calli after selection (Table 1). Unbombarded calli turned brown with in 6-8 days

whereas transformed calli though first turned brown, white patches of fresh resistant callus could be seen after 24 days with increase in size from 3-5 mm in diameter. Maximum callus transformation frequency of 37.5 was observed for 68 days maintained japonica rice followed by 35.2 for 44 days maintained calli.

In conclusion, our results have demonstrated that embryogenic calli initiated from seed embryos of both indica and japonica cultivars successfully exhibited co-transformation event after particle bombardment and hygromycin selection.

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