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BRIEF COMMUNICATION

Genetic engineering of *Oryza sativa* by particle bombardment

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Abstract

Genetic engineering of rice (*Oryza sativa* L. cv. Pusa basmati 1) using synthetic *Cry*1Ac gene has been achieved by "particle bombardment". Scutellar tissues excised after 5 - 6 d from mature seeds cultured on induction medium were bombarded using gold particles coated with a mixture of C*ry*1Ac and marker genes on medium with osmoticum. Bombarded tissues were subjected to 30 mg dm^{-3} hygromycin selection for two cycles. The selected calli after GUS assay were transferred to shoot regeneration medium. Regenerated shoots were rooted and plantlets (T_0) were grown to full maturity. Polymerase chain reaction (PCR) analysis of T_0 plants using C*ry*1Ac specific primers revealed the presence of *Cry*1Ac gene in 65 % plants. Phenotypic assay, β-glucuronidase assay and PCR during T_1 generation revealed the inheritance of the C*ry*1Ac and marker genes along with the native plant genes.

Additional key words: embryogenic calli, hygromycin, regeneration, transgenic rice.

Rice has been cultivated for more than 7000 years as a major crop and currently supports more than 50 % of world's population, thus has emerged as a model crop for the analysis of genome and proteome of cereals. Intensive research has been aimed at the development of practical gene transfer systems. High velocity DNA-coated gold particles to transform intact plant cells are being used as a broadly applicable gene transfer system (Maqbool and Christou 1999). Transgenosis has been successfully used to develop insect resistance in several crops (Schuler *et al.* 1998, De Maagd *et al.* 1999, Ahmad *et al.* 2002). Initial rice transformation work was limited to *japonica* rice varieties but the transformation success has also been achieved with some *indica* rice varieties (Tang *et al.* 1999, Husnain *et al.* 2000). The present communication deals with the introduction of *Cry*1Ac gene along with the marker and reporter genes into a commercial semi dwarf basmati rice cultivar, Pusa basmati 1, and to study the transmission of transgene(s) to R_1 progeny.

Dehusked seeds were surface sterilized with $HgCl_2$ (0.1%) for 10 min and cultured on Murashige and Skoog (1962, MS) salts supplemented with 2.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.5 mg dm^{-3} kinetin (kin) + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar (induction medium) and incubated at 27 °C in dark. After 5 - 6 d of incubation scutellar tissues were excised and arranged in centre of a target plate (60 × 50 mm) containing medium with osmoticum [MS + 2.5 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} kin + 0.4 M mannitol + 30 g dm^{-3} sucrose + 8 g dm^{-3}] (Fig. 1*B*). After 4 h, these were bombarded with standard procedures using particle gun (*Bio Rad*, Hercules, USA). All calli were bombarded twice with 4 h interval. A plasmid contained GUS A and hygromycin resistance genes under the control of CaMV 35S promoter, another plasmid pUBC contained a synthetic *Cry*1Ac gene under the control of the maize ubiquitin promoter and nos terminator. After 16 h calli were again placed on proliferation medium (MS salts supplemented with 2.5 mg dm^{-3} 2,4-D, 0.5 mg dm^{-3} kin and 250 mg dm^{-3} cefotaxime and incubated in the dark.

Different concentrations of hygromycin (*Sigma-Aldrich*, St. Louis, USA), *i.e.*, 20, 40 and 50 mg dm^{-3} were tested to check the optimum concentration required to inhibit the growth of non-transformed callus. Hundred calli pieces were plated on MS medium containing various concentrations of the antibiotic. Death of nontransformed

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Abbreviations: BAP - benzylaminopurine; 2,4 D - dichlorophenoxyacetic acid; kin - kinetin (6-furfurylaminopurine); GUS - β-glucuronidase; MS - Murashige and Skoog; PCR - polymerase chain reaction.
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calli within 22 d confirmed reliability of selection. Following bombardment, after 2 d on proliferation medium the embryogenic calli were placed on an initial selection medium (MS + 2.5 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} kin + 30 mg dm^{-3} hygromycin + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar). Selection was carried out for two cycles of two weeks each. After 14 d of initial selection healthy calli were transferred to fresh medium containing same concentration of hygromycin to provide escape free selection. Fourteen days after second selection, actively growing calli were placed on MS regeneration medium (MS + 0.5 mg dm^{-3} NAA + 2.0 mg dm^{-3} BAP + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar + 30 mg dm^{-3} hygromycin). A proportion of the plantlets regenerating under hygromycin selection was chosen for further analysis.

Hygromycin resistant, GUS expressing plants, washed thoroughly under running tap water were hardened by placing them in test tubes containing tap water and cotton to give support. The cotton was removed after four days. The hardened plants were finally transferred to the soil in the plastic pots (Fig. 1*G*) and kept in glasshouse (temperature around 32 °C, relative humidity 80 % and 16-h photoperiod).

Small sectors of hygromycin resistant calli and different tissues from plantlets, leaf segments, whole plantlets, root portions and ovary were immersed in the X-gluc solution (Klein 1988) for 72 h at 37 °C and examined for the presence of GUS (blue sectors).

For PCR analysis genomic DNA was isolated as described by Edwards *et al.* 1991, from leaf tissues of the hygromycin resistant plants. PCR analysis was carried out using primers for *Cry*1Ac forward primer 5^1-GCTCTCCGCGAGGAAATGCG-3^1, reverse primer 5^1-CACGTGGCTCAACCTGTGGG-3^1. PCR analysis was carried out in reaction volume of 0.025 cm^3 containing the template genomic DNA (100 ng), forward

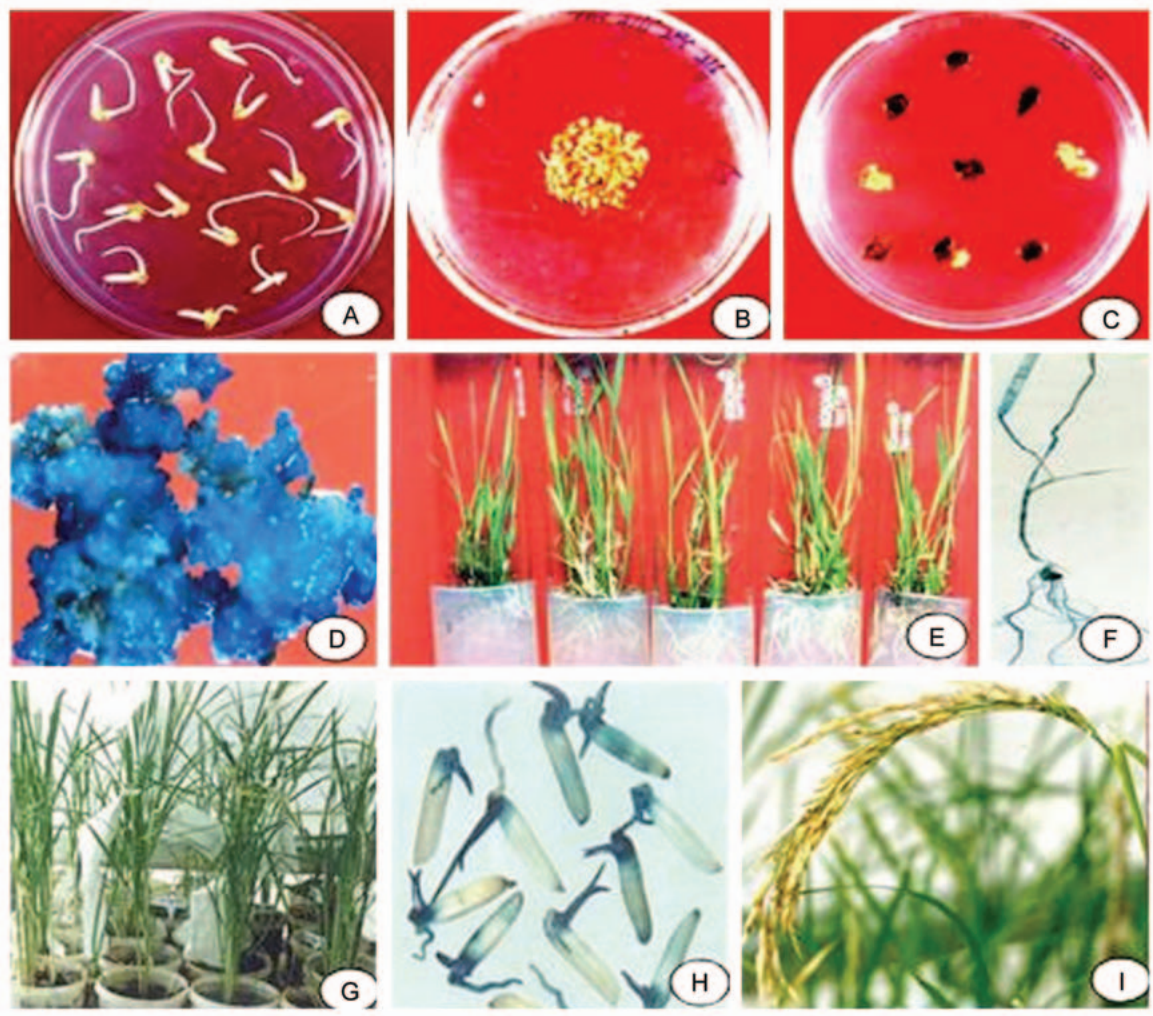

Fig. 1. *A* - Preparation of target tissues (scutellar calli) by culturing mature seeds on callus induction medium; *B* - 6-d-old scutellar calli arranged in the centre of the Petri dish for bombardment; *C* - selection of transformed tissues on medium containing 30 mg dm^{-3} hygromycin; *D* - GUS assay of putative transgenic calli showing blue coloration; *E* - regeneration of normal green shoots and root development; *F* - GUS positive transgenic plantlet; *G* - transgenic plants at flowering stage; *H* - GUS positive dehusked grain; *I* - normal seed set in the transgenic plant.

Table 1. Data on various morphological traits (T_0 generation) of transgenic plants. Non transformed Pusa basmati 1 plant served as control.

Plant	Plant height [cm]	Panicle length [cm]	Flag leaf length [cm]	Flag leaf width [cm]	Fertility
1	72.10 ± 1.24	22.65 ± 1.20	26.00 ± 2.00	0.93 ± 0.05	fertile
2	77.12 ± 2.00	23.00 ± 0.88	30.30 ± 1.25	1.33 ± 0.05	partial fertile
3	58.33 ± 8.94	18.51 ± 2.16	43.32 ± 1.05	0.80 ± 0.05	sterile
4	68.32 ± 2.15	23.00 ± 0.88	28.30 ± 1.00	0.86 ± 0.00	fertile
5	72.10 ± 1.25	22.65 ± 1.20	26.00 ± 2.00	0.93 ± 0.00	partial fertile
6	67.42 ± 1.65	20.00 ± 0.30	29.60 ± 1.17	1.11 ± 0.19	partial fertile
7	66.33 ± 1.72	24.00 ± 0.58	24.30 ± 1.93	0.86 ± 0.03	fertile
8	85.12 ± 1.65	22.65 ± 1.20	30.30 ± 1.94	0.92 ± 0.04	fertile
9	77.12 ± 1.75	24.70 ± 1.36	34.00 ± 1.88	1.33 ± 0.08	partial fertile
10	67.34 ± 0.00	22.50 ± 0.67	40.17 ± 1.04	0.70 ± 0.02	partial fertile
11	61.11 ± 1.25	18.00 ± 1.59	25.00 ± 1.25	0.92 ± 0.04	fertile
Control	66.35 ± 1.65	24.00 ± 0.85	25.00 ± 1.25	0.92 ± 0.04	fertile

and reverse primers (70 ng each), dNTPs (20 μM), PCR buffer 1X (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 9) and Taq polymerase 1 unit (*Pharmacia*, Uppsala, Sweden). Conditions were as follows: 95 °C for 7 min, 54 °C for 1 min, and 72 °C for 1 min (1 cycles) followed by 95 °C for 1 min., 54 °C for 1 min and 72 °C for 1 min (30 cycles). Final cycle was carried out at 72 °C for 7 min. A 0.01 cm^3 aliquot from each reaction was used for electrophoresis in 0.8 % agarose gel.

The cultured seeds of Pusa basmati 1 resumed proliferation of scutellar calli after 3 - 4 d. In general, the scutellar tissues of cereals including rice have been found to be very responsive to tissue culture (Arencibia *et al.* 1998, Hagio 1998). Pusa basmati 1 exhibited excellent response to callus induction as more than 90 % of the cultured seeds exhibited proliferation of scutellar tissues (Fig. 1*A*). The scutellar calli appeared slight yellowish in colour. Addition of proline (560 mg dm^{-3}) in the tissue culture medium improved embryogenesis in the developing calli. This may be because of the role of proline in the plant metabolism and it can also be attributed to its buffering role for stabilizing the pH of the culture medium. Following bombardment, 1 - 2 calli per bombardment were capable of normal growth on selection medium (Fig. 1*C*). Out of total 5056 scutellar calli bombarded, 693 calli were selected (hygromycin 30 mg dm^{-3}). Hygromycin has been found to be very safe and effective agent for selecting transgenic tissues in rice (Christou 1992). However, different rice cultivars possess different sensitivity to hygromycin. In Pusa basmati 1 hygromycin at concentration of 30 mg dm^{-3} has been found to be optimum for effective selection which allowed no escape of untransformed controls, whereas, higher concentrations , *i.e.*, 40 or 50 mg dm^{-3} were toxic.

Histochemical GUS assay of the selected calli was performed to check the introduction and expression of reporter GUS gene. Randomly selected 13 calli portions were tested, out of which, 6 were GUS positive. Most of the calli carried a single blue stained sector, sometimes with slight diffusion of the colour into adjacent cells. The distribution of blue sectors on callus bombard twice, tended to be more uniform (Fig. 1*D*). It was observed that, blue cells occurred usually on embryogenic callus regions. It was interesting to note that some of the transgenic calli and plants were very strong GUS positive as the tissue appeared blue even after 1 h of keeping in the X-gluc solution at room temperature (Fig. 1*F*). After two cycles of selection, 693 selected calli were placed on regeneration medium and complete plants were obtained after 4 weeks of incubation. Total 55 independent putative transgenic plants (Fig. 1*E*) were regenerated and these grew rapidly and continued normal growth on medium containing 30 mg dm^{-3} hygromycin (Fig. 1*E*). Hygromycin resistant, GUS expressing 17 plants were hardened for 10 d.

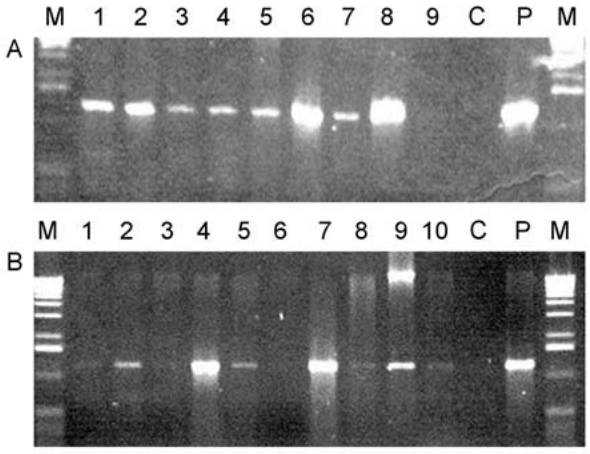

Fig. 2. PCR analysis of T_0 (*A*) and T_1 (*B*) plants.

All the transferred plants established in the soil and exhibited normal growth, flowering and seed set (Fig. 1M). PCR analysis was carried out by taking leaf segments from the glasshouse grown plants. All the 17 plants were screened for the presence of *Cry*1Ac, out of which 11 were PCR positive and some plants were

very strong PCR positive (Fig. 2*A*). This observation clearly shows that more than 65 % putative transgenic plants carried *Cry*1Ac gene. During PCR analysis, non transformed plants (C) were incubated as negative control and a positive control (P) (*Cry*1Ac DNA) was incubated to check the proper amplification. All the T_0 plants kept in the greenhouse were raised to maturity to record data on various morphological traits along with the non-transformed control (Table 1). It was observed that some of the plants were fully fertile and some were partially fertile whereas some completely sterile plants were also observed in the same growth conditions. This differential fertility may be attributed to delayed effects of culturing and transgene effects. In rice, sterility of transgenics (particularly those inducd by particle bombardment) has been noticed (Sudhakar *et al.* 1998). Mature T_1 seeds collected from to plants were cultured on MS medium containing 30 mg dm^{-3} hygromycin to eliminate hygromycin sensitive non-transgenic segregants. The hygromycin caused yellowing, stunted growth and necrosis of the susceptible seedlings within two weeks. Hygromycin resistant plants exhibited normal growth and tillering.

The germinating T_1 seeds were put in the X-gluc solution to study the inheritance of GUS gene from T_0 to T_1 generation (Fig. 1*H*). Hardened T_1 seedlings were further assessed for GUS expression by taking their leaf and root segments. Most of the hardened seedlings exhibited fairly good GUS expression. Inheritance of GUS and other reporter genes and marker genes has been reported in some earlier studies on genetic transformation (Wang *et al.* 1988, Ye *et al.* 1990, Gosal and Gosal 2000). Total 10 T_1 plants were grown to maturity in the glasshouse and these were screened for *Cry*1Ac using PCR analysis; of the 10 T_1 plants thus screened for *Cry*1Ac, 8 were PCR positive (Fig. 2*B*). It was interesting to note that in the T_1 generation the plants exhibited improved growth and better fertility. Our results have shown that *Cry*1Ac and GUS gene are integrated and highly expressed in basmati rice genome, but there is need to improve the regeneration efficiency of the transformed tissue.

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