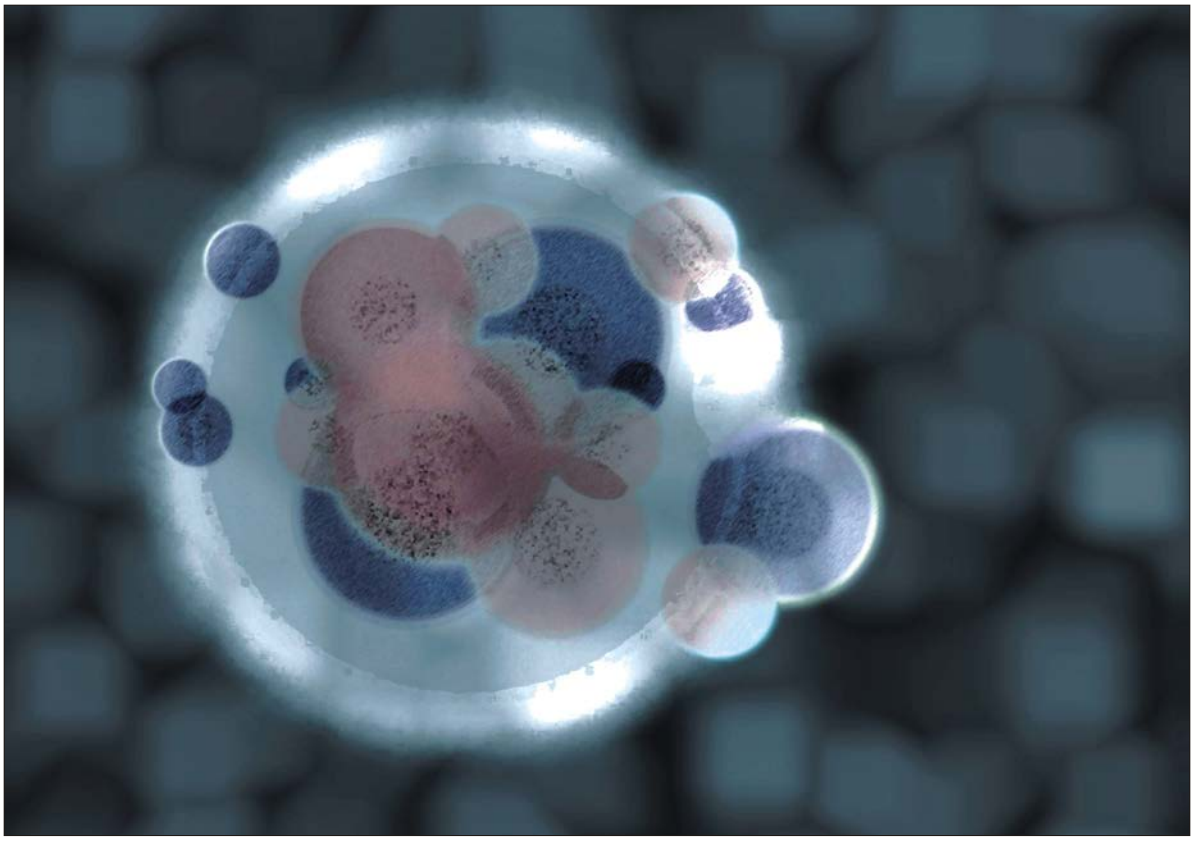

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 4 (2): 207-211, 2004
ISSN 1727-3048

Regeneration Study of Some Indica Rice Cultivers Followed by *Agrobacterium*-Mediated Transformation of Highly Regenerable Cultivar, BR-8

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Abstract: Some Bangladeshi indica rice (*Oryza sativa* L. var.) cultivars were subjected to tissue culture to study their regenerability in terms of regeneration percent and total number of regenerated plantlets obtained for a fixed sample size per variety. Regeneration potential was found to be the highest (88%) for BR-8 and the lowest (22%) for BR-31. Highest number of plants per seed were obtained for BR-8 and lowest for BR-31. Highly regenerating Indica rice cultivar BR-8 was subjected to genetic transformation mediated by *Agrobacterium tumefaciens* EHA105 harbouring the virulent plasmid pCAMBIA1301. Successful transformation events in the infected calli with this strain were followed by transient GUS assay using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) as a substrate. The frequency of transformation in terms of transient GUS assay was found to be 40%.

Key words: Transformation, *Agrobacterium*, regeneration, *in vitro* culture, indica rice, genotype

INTRODUCTION

Genetic transformation is an essential aspect of genetic engineering which offers the potential for the introduction of specific genes from any source into existing elite plant lines, thus accelerating the development of new plant varieties with agronomic traits. Prior to genetic transformation, regeneration study is necessary because high regenerability is an important prerequisite for successful transformation via *Agrobacterium tumefaciens*-mediated or other transformation techniques. Strategies to improve plant regeneration frequencies in cereals, including rice, have been steadily evolving during the last decade[1-7]. Plants can be regenerated *in vitro* by several methods. Plantlets may be derived from the outgrowth of a pre-existing structure such as an apical meristem or an auxillary bud, or the plant may be formed *de novo* from a cultured cell. However, regeneration capacity varies with genotype and even among cells within the same plant[8].

Although genetic transformation of dicot plants mediated by *Agrobacterium* is well established and stable transgenic plants expressing foreign genes have been produced[9-11]; genetic transformation of monocot plants could not be developed because these plants do not serve as a natural host for *Agrobacterium tumefaciens*. Consequently, alternative procedures for transformation of monocots like PEG-mediated DNA transformation of protoplasts[12,13] or electroporation of protoplasts[14-15] and the particle bombardment method[16-17]. However, these methods are still inefficient, time consuming, costly and require complex procedures. Furthermore, multiple copies of genes may be integrated into the genome and fragmentation and rearrangements of genes as well as high frequency of sterile plants were found to occur, especially in the Indica varieties[8]. On the other hand, *Agrobacterium*-mediated transformation appears to be more efficient in producing simple patterns of gene integration. The later technique also has the advantage that no sophisticated laboratory or expertise and protoplasts are required. Efforts to manipulate *Agrobacterium* to transform monocots have therefore, continued[18,19].

An efficient *Agrobacterium tumefaciens*-mediated transformation of Japonica varieties of rice using strain LBA4404 (pToK233) containing the *vir* B, *vir* C and *vir* G genes of supervirulent pTiBO542[20,25,21] added to the binary vector, has been reported and confirmed and extended to the Indica rice varieties IR72 and TCS10 in the last decade[22-24].

The objective of this study was to standardize a genetic transformation technique for a highly regenerable Bangladeshi HYV-type cultivar BR-8.

MATERIALS AND METHODS

Plant material: Rice seeds BR-2, BR-5, BR-8, BR-17, BR-31, Patjak, FR-13A were supplied by Bangladesh Rice

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Research Institute (BRRI), Gazipur. The first five varieties released by BRRI are high yielding varieties, whereas Patjak is a medium yielding variety grown in the Hawor area of Tangail and released by Bangabondhu Sheikh Mujibur Rahman Agricultural Univesity and FR-13A is a flood tolerant variety from Eastern India.

Surface sterilization of mature seeds: Dehusked mature seeds were surface sterilized first with 95% ethanol for 2-3 min and then 70% clorox (5.25% sodium hypochlorite purchased from The Clorox Co. U.S.A.) supplemented with 1-2 drops of Tween-20 for 30 min. Seeds were then rinsed three times with sterile deionized water.

Initiation and growth of callus: Calli were initiated from mature seeds of all seven varieties on MS[27] media supplemented with 2 mg l^{-1} 2,4-D, 0.3 g l^{-1} casein hydrolysate, 10 mg l^{-1} thiamine, 3% sucrose, 0.01% myo-inositol, pH 5.8, 0.3% phytagel which was used as gelling compound. Callus initiation was carried out in the dark for 5-7 days at 27±1°C. Callus induction was calculated as follows:

$$\% \text{ callus induction} = \frac{\text{Number of callus}}{\text{Number of mature seeds inoculated on MS media}} \times 100$$

7-10 days old calli were transferred into the same fresh callus induction media (MS media).

Plant regeneration: After 15-25 days on callus induction media, the intact calli were transferred to regeneration media (MS media supplemented with 2 mg l^{-1} BAP and 0.2 mg l^{-1} NAA, pH 5.8) for the germination of somatic embryos. Cultures were kept under 10-14 light/dark at 27°C. After plant development, percent callus regeneration and average number of plants per callus were calculated as follows:

$$\% \text{ callus regeneration} = \frac{\text{Number of callus producing plantlets}}{\text{Total number of calli transferred to regeneration media}} \times 100$$

$$\text{Average number of plants per seeds} = \frac{\text{Total number of plants}}{\text{Total number of regenerable callus}}$$

After plant development, plantlets were transferred to rooting media (MS media supplemented with 0.2 mg l^{-1} IBA, pH 5.8) and kept under 10-14 light/dark regime until sufficient roots were formed. From seed sterilization to root formation all the steps were performed in a laminar airflow cabinet to maintain sterility.

Transplantation: Plantlets with sufficient roots were washed with running tap water and then transplanted to small pots containing fertile soil. Plants along with the pots were covered with transparent moist polythene bags to prevent desiccation. Plants were sprayed with water to maintain humidity. Polythene bags were removed after 7-10 days. Healthy plantlets were transferred to the field after two weeks exposure to normal natural conditions.

Plant material for *Agrobacterium*-mediated transformation: Rice seeds of BR-8 provided by Bangladesh Rice Research Institute (BRRI), Gazipur, is a high yielding variety and has a high regeneration ability (~90%) from mature embryo-derived callus.

***Agrobacterium* growth and infection:** 7-10 days old scutellar calli were used for *Agrobacterium* infection. *Agrobacterium vir* helper strain EHA105 harbouring pCAMBIA1301 vector, which contains CaMV35S hygromycin gene in the transfer region was used for transformation. *Agrobacterium* strain was first grown on LB medium containing kanamycin (50 mg l^{-1}) for two days at 28-29°C or until a bacterial lawn was obtained. Then suspension of *Agrobacterium* was made by scraping and re-suspending a few loops of bacteria into liquid LB medium without antibiotic but containing 250 μM acetosyringone, followed by growing with vigorous shaking at 250 rpm for 8-10 h prior to use for infection and transformation. The scutellar calli were submerged in the prepared bacterial suspension for 30 min with 10 min of air vacuum and with occasional shaking. The calli were then blotted dry on sterile filter papers and cocultivated for 72 h in the dark by placing on solidified MS media supplemented with 10 g l^{-1} glucose, 250 μM acetosyringone, pH 5.2. The co-cultivation was done in the dark at 27°C by placing the plates upside down.

Washing and selection: Calli co-cultivated with *Agrobacterium* for 3 days were washed with shaking in sterile water containing 250 mg l^{-1} cefotaxime over 3-4 h with several changes to remove the bacteria. The calli were blotted dry on sterile filter paper. Some of the calli were used for transient GUS assay and the remaining calli were transferred to MS medium containing 250 mg l^{-1} carbenicillin and 250 mg l^{-1} cefotaxime to kill *Agrobacterium* attached to the calli and 50 mg l^{-1} hygromycin to select transgenic calli. Calli were incubated in the dark at 27°C for 6-8 weeks.

The calli were subcultured on same fresh medium once every two weeks. Small transgenic hygromycin resistant embryogenic calli start to appear after 4 weeks of selection on hygromycin.

Transient GUS assay: Transient expression of GUS activity in transiently transformed calli was assayed histochemically as described by Jefferson[28]. Infected calli were assayed for transient GUS expression using 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (X-gluc) as a substrate. The substrate was dissolved in 100 µl dimethyl formamide and made upto a final concentration of 1 mg ml^{-1} with 50 mM phosphate buffer at pH 7.0. Calli or tissues were fixed at pH 5.6 by placing in 10 mM MES, 0.3% formaldehyde and 0.3 M mannitol and vacuum infiltrated for 2 min. The tissues were then washed several times in 50 mM phosphate buffer and incubated in X-gluc for 16 h at 37°C. Buffers and substrates were filter-sterilized and sterile eppendorf tubes used for assay to avoid false positives by contaminating *E. coli*.

RESULTS AND DISCUSSION

Plantlets of seven rice varieties were regenerated from induced callus. The callus induction, callus regeneration, average plants per seed of all seven varieties are shown in Table 1. These seven varieties were grouped together as highly regenerating, intermediately regenerating and poorly regenerating on the basis of regeneration percent and total plantlets obtained for a fixed number of embryos cultured.

High regeneration percent was found for BR-8 (88%) and BR-5 (75%). Patjak and BR-17 showed intermediate regeneration, which were 62.5 and 49%, respectively. Finally FR-13A, BR-2 and BR-31 showed poor regeneration ability. The regeneration steps were repeated three times.

Rice calli from somatic embryos would produce shoots and roots simultaneously when subjected to 2,4-D[16]. However, in our experiment, shoots and roots appeared together only in BR-2 and BR-17. In other varieties, some calli produced only shoots and some produced shoots and roots simultaneously. Regenerating calli (calli which give rise to plantlets) were usually fresh and creamy in colour and texture than non-regenerating calli.

This study revealed that all the varieties tested have regeneration capacity. BR-8 cultivar showed the highest regeneration potential (88%).

Table 1: Regenerability of seven Indica rice cultivars

Group plants	Variety	No. of seeds inoculated	Callus induction	Callus regener-ation %	Average plants per embryo	Plant range
High	BR-8	100	95	88	25	5-61
	BR-5	100	66	75	14	2-36
Medium	Patjak	100	82	63	13	2-28
	BR-17	100	63	49	12	2-16
Poor	FR-13A	100	95	36	7	2-14
	BR-2	100	100	31	7	2-7
	BR-31	100	85	22	6	2-11

Successful transformation events in the calli infected with *Agrobacterium tumefaciens* EHA105 (pCAMBIA1301) were followed by transient GUS assay using 5-bromo-4-chloro-3-indolyl, β-D-glucuronide (X-gluc) as a substrate. A good positive response (40%) for transient GUS assay was obtained. Blue spots (Fig. 1c) were visualised on the co-cultivated calli, which indicated positive transient GUS expression in the cells of calli. Negative control (Fig. 1b) control calli were subjected to exactly same treatment of transient assay] showed no blue colour. Positive control (transformed tobacco) showed the characteristic blue colour as in (Fig. 1a). An average of 5.9 and 7.6% efficiency for GUS positive calli in indica varieties, namely TCS 10 and IR72, respectively were reported by Aldemita and Hodges[23].

Transfer of foreign genes into monocotyledonous higher plants by *Agrobacterium tumefaciens* is a biological technique in plant transformation. Although there are many publications for successful transformation of Japonica rice and in some cases Indica rice with supervirulent strains LBA4404 (pTOK233) as mentioned earlier[22,1-7] limited success in Indica rice transformation due to recalcitrance of monocotyledonous plants to *Agrobacterium*-mediated transformation was observed. This is due to a block in one or more of the following steps involved in transformation:

I) chemotaxis of *Agrobacterium* towards wounded plant cells
ii) binding of *Agrobacterium* to plant cells
iii) induction of *vir* genes by plant signal molecules
iv) generation of T-DNA transfer intermediates
v) transfer of T-DNA transfer intermediates to plant cells and integration of T-DNA into plant genome.

It is therefore, clear that finding an appropriate match of regenerable cell types and stages with those cells, which can receive and express T-DNAs is critical to the successful transformation event.

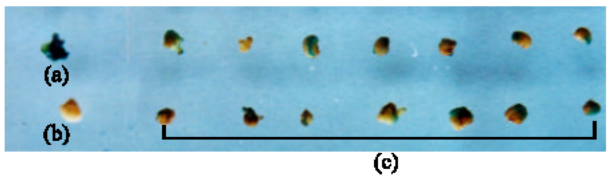

Fig. 1: Transient assay for histochemical GUS activity
a) Transformed tobacco leaf (positive control) showing the blue colour indicating the presence of GUS
b) Normal calli (negative control, no infection with *Agrobacterium*) showing no characteristic colour for GUS activity
c) X-gluc (substrate for GUS) positive blue spot of transiently transformed rice calli

Results of the present study showed successful infection and transformation of calli as evident from the positive histochemical transient GUS assay. However no stably transformed calli were obtained on hygromycin selection probably due to a defect in the critical steps of transformation as mentioned above. Calli viability may also be lost during washing with high concentration of cefotexime to make calli free from bacteria[26].

Reproducible transformation of Indica rice varieties with desired genes is very important for the development of cultivars with valuable agronomic traits in our country. Further work and optimisation of the transformation techniques will be needed to achieve the goal. Although we have yet been successful to get transgenic plants from the transformed calli, the good response in transient GUS assay indicates that this might be possible in near future.

ACKNOWLEDGMENTS

This research was supported by The Rockefeller Foundation grant No. RF96001#400 under the Rice Biotechnology Program.

The gift of *Agrobacterium tumefaciens* EHA105 (pCAMBIA1301) from Dr. Jefferson, R.A of CAMBIA, Australia is gratefully acknowledged.

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