Optimization of *Agrobacterium*-Mediated Genetic Transformation in *indica* Rice

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Abstract

This experiment was conducted during the period from August 2004 to April 2005 in the Biotechnology & Genetic Engineering Laboratory of the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh. In this experiment, an efficient and reproducible protocol for the production of transgenic rice plants was developed by inoculating mature embryo of three *indica* rice genotypes with *Agrobacterium tumefaciens* strain LBA4404 carrying a binary vector pBI121, which contains selectable marker gene *nptII* conferring resistance to kanamycin and the *GUS* reporter gene. The transformation experiment was performed by optimizing two important parameters: infection time and co-cultivation period. Infection was most effective when explants were inoculated for 15 minutes (72.59% *GUS* positive) and co-cultivated for four days (70.37% *GUS* positive) with *Agrobacterium*. Among the varieties, BRRI dhan-33 showed the highest response to *GUS* assay (65.33% *GUS* positive). Transformation percentage (putative) was also highest in BRRI dhan-33 (4%). 10 minutes infection time and co-cultivation period of three days was found effective for regeneration of shoots (2.45% and 4.45%, respectively). Among the varieties, BRRI dhan-33 produced the highest percentage (61.11%) of rooted shoots.

Keywords: transformation, *indica* rice*, GUS, Agrobacterium*

Introduction

Rice is the synonym for food in Bangladesh and has been traditional source of carbohydrates and proteins since the prehistoric days. Grown on some 25 million acres, rice forms nearly 80% of the total food intake of Bangladesh (Kaul et al., 1982). In Bangladesh, rice covers an area of 11,059 thousand hectares, yielded 3,448.2 kg ha^{-1} and the production is 38,134 thousand metric tons (SAIC, 2003). This sector generates about 35% of the total foreign exchange earnings and provides nearly 40% of total national employment (48% of rural employment), about 2/3 of total calorie supply, about 1/2 of the total protein intake of an average person and contributes 18% to the total GDP of the country (Alam, 1997; Bhuiyan and Karim, 1999; Husain et al., 2001).

Not only for Bangladesh, rice is the staple food for the largest number of people on Earth. In more recent years annual increase in crop yields and production have only gradually increased suggesting that the genetic potential for maximum crop yields is close to being attained. According to one estimate, to meet the demand global rice production must reach 800 million ton in 2005 from 585 million ton in 2003 (Virk et al., 2004). To meet this challenge, new strategies to elevate rice productivity and yields must be devised to accommodate the growing demand for food. Such strategies will include genetic modification to raise the ceiling for maximum yield and to increase yield potential under stressful environment (Bhuiyan and Karim, 1999). Although conventional breeding will continue to play a major role in increasing crop yield, laboratory based techniques, such as genetic transformation to introduce novel genes into crop plants, will be essential in complementing existing technologies (Ingram, 2001).

Recent advances in plant tissue culture and molecular biology (biotechnology) have made it possible to produce engineered crops that resistant to a variety of biotic and abiotic stresses, and that can help to substantially reduce or even eliminate the huge crop losses (Vasil, 1998). Biotechnology can significantly strengthen rice breeding program and help to produce new varieties with higher yield potential and greater yield stability. These should improve the efficiency of rice production and allow an expansion of the rice growing area. Biotechnology will enable rice breeders to achieve results more quickly and efficiently and will help them to attain breeding goals not feasible using conventional techniques. Biotechnology will complement, not replace breeding (Khush and Toenniessen, 1991).

In recent years, the successful transformation of rice by *Agrobacterium* had been reported, although monocotyledonous plants were considered to be outside the host range of *A. tumefaciens* (Gould et al., 1991; Raineri et al., 1990). Transformed rice callus was first reported by Chan et al. (1992) but no transgenic plants were obtained. But the next year Chan et al. (1993) reported the generation of first transgenic japonica rice plants by inoculating immature embryos with *Agrobacterium*, the transferred genes to the progeny by Southern blot analysis.

The inherent difficulties in the establishment and maintenance of calli and the regeneration of plants from explants, encourage the search for other more friendly methods for transformation of rice. The successful production of transgenic plants of these cultivars will open the way for introducing various agronomically useful traits into any commercially grown cultivars, providing the best opportunity for maximizing yields with other qualities.

This current research work was set up for a transformation system using *Agrobacterium*. The work had been planned and undertaken to study the transformation potentiality of three *indica* rice varieties in accordance with the optimization of preculture and co-cultivation time required for *Agrobacterium* mediated genetic transformation.

Materials and Methods

Plant Materials

Three varieties of *indica* rice namely, BRRI dhan-29, BRRI dhan-33 and Binnatoa were used in that experiment.

Agrobacterium Strain and Plasmid

Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used for infection in the transformation experiment. This strain contains plasmid pBI121 of 14 KDa (binary vector).This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct:

The *udiA* gene (Jefferson, 1986) *encoding* GUS (β-glucuronidase), driven by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation.

The *nptII* gene (Herrera-Estrella et al., 1983) encoding neomycin phosphotransferase II (*nptII*) conferring kanamycin resistance, driven by NOS promoter and NOS terminator.

The bacterium also contains plasmid pAL4404 which is a disarmed Ti plasmid (132 KDa) containing the virulence genes.

Media Used

YMB (Yeast extract Mannitol Broth) and LB (Luria Broth) medium were used for bacteria culture and maintenance, MS medium supplemented with 1 mg L^{-1} 2,4-D and 50 mg L^{-1} L-Tryptophan for callus induction and co-cultivation. Along with these 100 μM acetosyringone was added to enhance the bacterial activities.

MS liquid medium supplemented with 500 mg L^{-1} cefotaxime were used for washing of explants after co-cultivation, for Post-cultivation and shoot differentiation MS medium supplemented with 6 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA and 200 mg L^{-1} cefotaxime were used. MS medium supplemented with 6 mg L^{-1} kinetin and 0.5 mg L^{-1} NAA along with increasing rate (20 to 30 mg L^{-1}) of kanamycin and decreasing rate (100 to 75 mg L^{-1}) of cefotaxime were used for selection and regeneration and MS medium + 0.5 mg L^{-1} IBA + 50 mg L^{-1} kanamycin + 50 mg L^{-1} cefotaxime were used for rooting

Sterilization and Placement of Seed

Mature seeds of three *Oryza sativa* var. *indica* were dehusked manually without interrupting the embryo. The dehusked seeds were then washed thoroughly in distilled water followed by dipping in 70% ethyl alcohol for three minutes with vigorous shaking and washing. Surface disinfection's was done by the use of 30% clorox for fifteen minutes.

Sterilized mature seeds were cultured directly as explant in MS medium supplemented with different concentrations of hormones and sucrose required as per treatment. Ten explants were horizontally placed in a petridish containing about 20 mL solid medium.

Infection and Incubation

The *Agrobacteria* grown in liquid LB media were used for infection and incubation maintaining the optical density of OD_{600}=0.6 to get suitable and sufficient infection of the explants, freshly excised explants were wounded and immersed in a culture of the preinduced bacteria for 5-15 minutes with gentle shaking and then transferred them to co-cultivation medium.

GUS (β-glucuronidase) Histochemical Assay

From each batch of explants following each transformation experiment, randomly selected co-cultured tissues were examined for *GUS* histo-chemical assay. For this experiment co-cultured explant tissues were immersed in X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution and were incubated at 37°C overnight. A characteristics blue color would be the expression of *GUS* (*β-glucuronidase*) gene in the plant tissue. Proper control for *GUS* histochemical assay was done with the explants having no *Agrobacterium* infection.

After X-gluc treatment explants were transferred to 70% alcohol for degreening. Following degreening explants were observed under stereomicroscope.

Results and Discussion

Histochemical *GUS* (*β*-glucuroronidase) Assay

Depending on the variety, incubation time and co-cultivation time with *Agrobacterium,* variation in histochemical assay of *GUS* repoter gene in explants tissue was noticed (Table 1).

Effects of Varieties

All the three varieties responded positively in *GUS* histochemical assay, where BRRI dhan-33 showed the highest (65.93%) and Binnatoa showed the lowest (56.39%) expression to *GUS* assay (Figure 1). Control explants did not show any response to the assay (Figure 2).

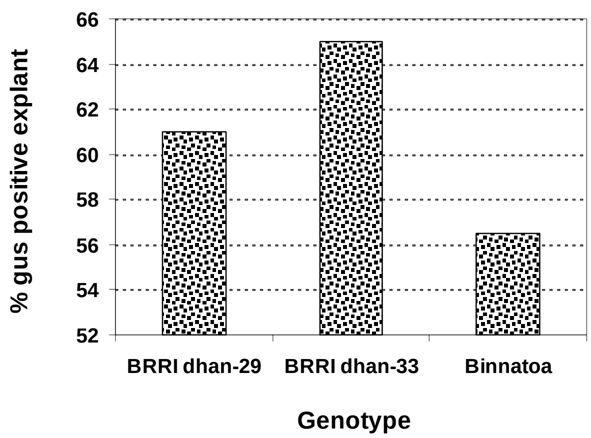

Figure 1 Response of different genotypes towards GUS assay.

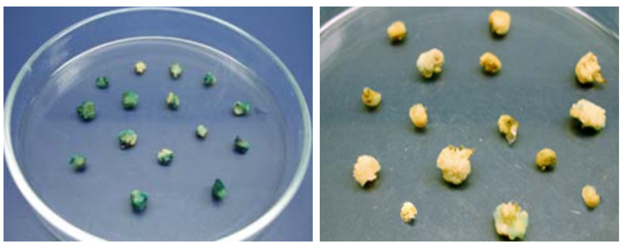

Figure 2 Histochemical localization of *GUS* activity (blue zone) at the entire surface of infected calli (left) with control explant (right).

Table 1 Effects of infection time and co-cultivation periods on *GUS* expression of infected calli of three rice genotype by *Agrobacteriu tumifaciens* strain LBA4404 containing binary plasmid pB1121.

Variety	Infection time (min)	Co-cultivation Periods (day)	No. of explants incubated	No. of explants assayed for *GUS*	No. of explants +ve for *GUS*	% of *GUS* + ve explants
BRRI dhan-29	5	2	50	15	6	40.00
		3	50	15	7	46.67
		4	50	15	8	53.33
	10	2	50	15	9	60.00
		3	50	15	11	73.33
		4	50	15	12	80.00
	15	2	50	15	11	73.00
		3	50	15	12	80.00
		4	50	15	13	86.67
BRRI dhan-33	5	2	50	15	6	40.00
		3	50	15	8	53.33
		4	50	15	9	60.00
	10	2	50	15	9	60.00
		3	50	15	11	73.33
		4	50	15	12	80.00
	15	2	50	15	10	66.67
		3	50	15	11	73.33
		4	50	15	13	86.67
Binnatoa	5	2	50	15	5	33.33
		3	50	15	6	40.00
		4	50	15	8	53.33
	10	2	50	15	7	46.67
		3	50	15	9	60.00
		4	50	15	10	66.67
	15	2	50	15	9	60.00
		3	50	15	10	66.67
		4	50	15	12	80.00

Effects of Co-cultivation Periods

Duration of co-cultivation periods of the explants with *Agrobacterium* is an important factor, which was found to influence genetic transformation. When calli were co-cultivated for four days gave the highest (70.37%) response to the *GUS* assay, indicating that the percentage of *GUS* positive explants increased with the increase of co-cultivation period (Figure 3).

Effects of Infection Time

Infection time is also an important factor in transformation experiments mediated by *Agrobacterium*. In the present study, calli were infected for 5, 10 and 15 minutes and observed that GUS^{+ve} response increased with the time of infection. Calli showed the highest response to the assay in case of 15 minutes infection time (72.59%) (Figure 4).

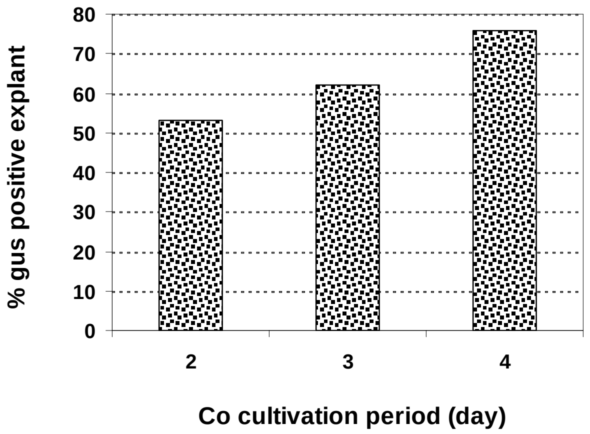

Figure 3 Response of different co-culture periods towards GUS assay.

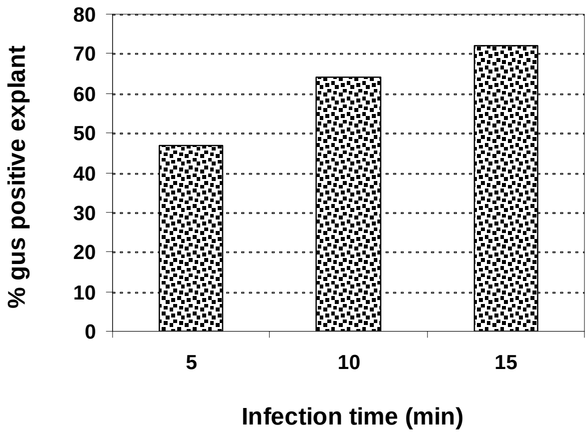

Figure 4 Response of different infection time towards GUS assay.

Shoot Initiation

After culturing on low selection media, callli were transferred to selection and regeneration media for shoot initiation. Calli started to initiate shoot buds after 30-45 days of incubation. The percentage of plant regeneration showed variation depending on varieties and co-cultivation periods. Results of this investigation are presented in (Table 2).

Presence of Kanamycin at 30 mg L^{-1} concentration in the selection media greatly influenced the emergence of putative transgenic shoot from the co-cultivated calli. Most of the calli failed to produce shoot and died in course of time. A few of the calli showed green spots and continued differentiating into shoots (Figure 5).

Among the varieties, BRRI dhan 33 showed the highest shoot initiation (4%) followed by BRRI dhan 29 (2.89%), while Binnatoa failed to produce shoot. Calli showed the highest (2.45%) shoots regeneration when they were infected for 10 minutes. Regeneration of putative transformed shoot was highest (4.45%) when calli were co-cultivated for 3 days. These results are concordance with Kumaria et al. (2001), stated that prolonged infection time adversely affects the callus growth and subsequent regeneration.

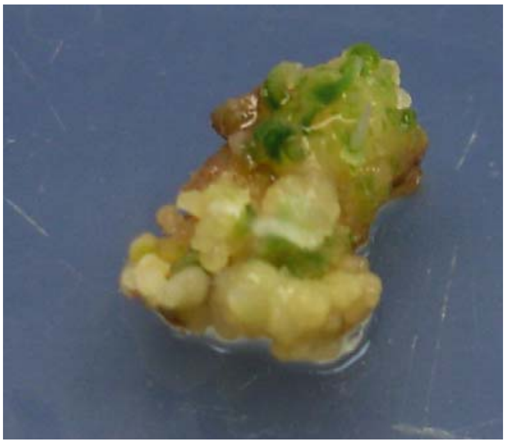

Figure 5 Gradual enlargement and development of green spots from *Agrobacterium* inoculated embryo derived callus on selection medium

Root Induction

Putative transgenic shoot regenerated from *Agrobacterium* infected calli were transferred to root induction medium (Figure 6). Percentages of rooted shoots are presented in Table 3.

Figure 6 Putative transgenic plantlets.

Table 2 Response of three genotypes towards towards plant transformation over different infection time and co-cultivation periods.

Variety	Infection time	Co-cultivation Period (day)	No. of explant incubated	No. of shoots produced	Average % of shoot produced	% plant transformation	Days required for shoot initiation
BRRI dhan-29	5	2	50	0	2.89	0	
		3	50	2		4	30-40
		4	50	1		2	30-45
	10	2	50	3		6	30-40
		3	50	6		12	30-45
		4	50	1		2	36-45
	15	2	50	0		0	
		3	50	0		0	
		4	50	0		0	
BRRI dhan-33	5	2	50	0	4.00	0	
		3	50	3		6	30-45
		4	50	0		0	
	10	2	50	3		6	30-40
		3	50	8		16	30-45
		4	50	1		2	30-45
	15	2	50	2		4	30-40
		3	50	1		2	30-45
		4	50	0		0	
Binnatoa	5	2	50	0	0.00	0	
		3	50	0		0	
		4	50	0		0	
	10	2	50	0		0	
		3	50	0		0	
		4	50	0		0	
	15	2	50	0		0	
		3	50	0		0	
		4	50	0		0	

Table 3 Number of transgenic shoots produced roots.

Genotype	No. of shoot incubated	No. of shoot showing root	% Root initiation
BRRI dhan-29	15	9	60
BRRI dhan-33	15	10	66.67

Conclusions

The transformation experiment was performed by optimizing two important parameters: infection time and co-cultivation period. Infection was most effective when explants were inoculated for 15 minutes (72.59% *GUS* positive) and co-cultivated for four days (70.37% *GUS* positive) with *Agrobacterium*. Among the varieties, BRRI dhan-33 showed the highest response to *GUS* assay (65.33% *GUS* positive). Transformation percentage (putative) was also highest in BRRI dhan-33 (4%). 10 minutes infection time and co-cultivation period of three days was found effective for regeneration of shoots (2.45% and 4.45%, respectively). Among the varieties, BRRI dhan-33 produced the highest percentage (61.11%) of rooted shoots. We have got a simple protocol for rice transformation but to make it more precise and reproducible further research considering many other factors with more laboratory facilities should be carried out.

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