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GUS Gene Transformation in Rice (*Oryza sativa* L.) Variety BRRI Dhan-30 Mediated by *Agrobacterium tumefaciens*

[1]M. Asaduzzaman, [1]M.A. Bari, [2]M. Rahman, [3]M. Minami, [3]K. Matsushima and [3]K. Nemoto
[1]Biotechnology Laboratory, Institute of Biological Sciences,
University of Rajshahi, Rajshahi 6205, Bangladesh
[2]Department of Genetic Engineering and Biotechnology,
University of Rajshahi, Rajshahi 6205, Bangladesh
[3]Graduate School of Agriculture Science, Shinshu University, Nagano-Ken, Japan

Abstract: *Agrobacterium* mediated transformation has already been reported in rice but under the present investigation efforts has been made to establish the transformation protocol in Bangladeshi rice variety BRRI dhan-30. High percentage of callus induction at 97.3% was obtained when seeds of rice (*Oryza sativa* L.) cv. BRRI dhan-30 were cultured on modified N_6 medium supplemented with 3% sucrose, 2.0 mg L^{-1} 2, 4-D and 0.8% agar under dark condition. Maximum 88.28% calli induced shoots and were obtained from BRRI dhan-30 in the suitable regeneration medium was MS medium supplemented with 3% (w/v) sucrose, 2.0 mg L^{-1} BAP+0.05 mg L^{-1} NAA+3% sorbitol and 0.8% agar. *Agrobacterium* mediated gene transfer protocol for rice variety BRRI dhan-30 were performed using *Agrobacterium tumefaciens* strain LBA4404, which harbored the plasmid pCL3 containing genes for β-glucuronidase (GUS) and kanamycin resistance. It was found that kanamycin concentration up 50 μg mL^{-1} were effective for selection of transformants. Putatives transformants as indicated by kanamycin test were subjected to GUS assay. GUS activities were found in rice calli after co-cultivation.

Key words: Rice, callus induction and regeneration, scutellum, *Agrobacterium* mediated transformation, assay

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal and the staple food for more than two billion peoples, predominantly in developing countries (FAO, 1995). According to FAOSTAT (2005) rice is the world's single most important food crop and a primary food for more than a third of the world's population, mainly in the tropics. But rice improvement through conventional breeding methods has met with considerable success; however, international attention has focused on developing new techniques for genetic manipulation of this crop. This genetic engineering requires the *in vitro* culture of rice tissues from which whole plant can be regenerated conveniently and it is important for the improvement of crops (Potrykus, 1990). However, rice yield and quality are affected by pests and diseases, as well as by environmental stress. Plant genetic engineering provides an opportunity to incorporate novel resistance traits into rice (Pipatpanukul *et al*., 2004).

To meet the growing demand for rice, biotechnological intervention for its improvement using genetic engineering is becoming increasingly important. Such interventions hinge on the development of efficient and reproducible transformation protocols for agronomically superior and popular rice varieties grown in rice-consuming countries like Bangladesh. Of the two major types of cultivated rice *indica* and *japonica*, wide difference exists between their tissue culturability; the former being less responsive than the latter. Most of the high-yielding and agronomically superior varieties of *indica* rice grown in India and Bangladesh do not have reproducible transformation protocols available for them (Himani *et al*., 2007). Thus, it is important to establish parameters for the transformation of popular *indica* rice varieties (Visarada *et al*., 2002). Two transformation protocols are generally available for rice, namely *Agrobacterium*-mediated and biolistics. *Agrobacterium*-mediated transformation has several advantages, such as higher transformation efficiency, the ability to transfer large pieces of DNA, minimal re-arrangement of transferred DNA, integration in low copy numbers and low cost, etc. Amongst *indica* rice, reproducible transformation protocols have been reported only in a few

Corresponding Author: M. Asaduzzaman, T396-0021, Sakurahaitsu-206, Ina-Shi, Oaza Ina 3070-1, Nagano-Ken, Japan
Tel: 81-0265-73-2054

varieties (Lin and Jhang, 2005; Garg *et al*., 2002; Wang *et al*., 2002; Zhang *et al*., 1997; Mohanty *et al*., 1999; Aldemita and Odges, 1996; Sridevi *et al*., 2005; Datta *et al*., 2006), including the recently reported (Datta *et al*., 2006). *Agrobacterium*-mediated transformation protocol for *indica*-type rice varieties, BR29 and IR64. Among the currently available plant transformation techniques, *Agrobacterium* method, a simple natural gene transfer system, is the most widely used one for genetic improvement of several crop species including rice (Rao and Rao, 2007). The tissue culture system is suitable for *Agrobacterium*-mediated transformation and different factors affecting transformation efficiency are investigated. (Yookongkaew *et al*., 2007). To date, all the successful reports on *Agrobacterium*-mediated transformation of rice have been based on *Agrobacterium* preinduction and/or cocultivation in the presence of Acetosyringone (Khanna and Raina, 1999; Datta *et al*., 2000; Hashizume *et al*., 2006) or based on co-cultivation in presence of suspension culture of potato cells, a rich source of phenolic compounds (Chan *et al*., 1993). No transient expression of the GUS gene was observed in the absence of Acetosyringone even when using a super virulent *Agrobacterium* strain and 100 mM of acetosyringone was reported to be optimum for transient expression in rice (Azhakanandam *et al*., 2000).

The rice cultivar used for this transformation experiment was BRRI dhan-30, which is a glutinous rice widely grown in Bangladesh. To develop transformation protocols for one such variety (BRRI Dhan-30), an *Agrobacterium*-mediated co-cultivation has been described in this article. Insertion of the reporter gene glucuronidase (GUS) gene and the selectable marker, hygromycin phosphotransferase (hptII) gene, into several cultivars of *indica* rice using *Agrobacterium*-mediated transformation was also subsequently demonstrated (Rashid *et al*., 1996). Recently, Datta *et al*. (2000) introduced the chimeric chitinase gene in rice cultivars by using *Agrobacterium*. This research was set up to verify a transformation system using *Agrobacterium*-mediated transformation into *indica* rice. It was the first attempt in Bangladesh to transform rice plant with target genes. Transformed rice callus was first reported by Chan *et al*. (1992) but no transgenic plants were obtained (Chan *et al*., 1993). The successful production of transgenic plants of this cultivar would open the way for introducing agronomically useful traits into any commercially grown cultivars, providing the best opportunity for maximizing yields. Most of the rice transformation work reported was based on *japonica* varieties. However very little research had been done on improving Bangladeshi local rice varieties using modern biotechnological methods.

MATERIALS AND METHODS

BRRI dhan-30, popular *indica* type long grain rice (*Oryza sativa* L.) was used in the present investigation. Seeds of this variety were collected from Regional Rice Research Institute, Rajshahi, Bangladesh. Three week old scutellum derived calli of rice cv. BRRI dhan-30 was used as the explants for transformation in this experiment. This experiment was conducted from 1998-2004 in the Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Callus induction: For callus induction N_6 (Chu *et al*., 1975) medium supplemented with 2.0 mg L^{-1} 2, 4-D was used. The medium was solidified with 0.8% agar and pH of the medium was adjusted to 5.8 before addition of agar. Explant scutellum from mature embryos was cultured on N6 medium and incubated at 25±2°C in dark for callus induction. Calli were sub cultured on the same medium after fifteen days interval. The embryogenic callus inducing frequency was determined after three weeks of incubation of explant on callus inducing medium in the dark. The compact but fragile, nodular, creamy yellowish and light yellow in color calli were considered as embryogenic.

Callus regeneration: The embryogenic calli were induced from scutellum explants for three weeks in N6 medium was used for regeneration studies. The calli induced from scutellum explant was sub cultured on the same medium for three days under 1000 lux light at 25±2°C for better proliferation. The proliferated calli were finally transferred to regeneration media MS (Murashige and Skoog, 1962) and N_6 supplemented with different concentrations of phytohormones. The calli were kept in light at 14 h day/10 h night cycle at 25±2°C. Regenerated plants were transferred to MS medium for production of elaborated roots.

***Agrobacterium* strains and culture:** The *Agrobacterium* stain LBA4404 (pCL3) harboring with GUS was constructed and provided by Prof. P. Nick, Frieburg University, Germany. *Agrobacterium* strains LBA4404 (pCL3) was grown in 50 μg mL^{-1} kanamycin containing LB medium (Tryptone 10 g L^{-1}, yeast extract 5 g L^{-1} and NaCl 10 g L^{-1}) solidified with 12 g of bactoagar at pH = 7 for 3 days at 28°C. Two days prior to inoculation, sterile

wooden sticks were used to select single colonies of pCL3 allowed to grow overnight in 50 mL of LB media with 50 μg mL^{-1} kanamycin on a 200 rpm shaker at 28°C.

Inoculation and co-cultivation: The generative embryogenic calli of BRRI dhan-30 rice were inoculated by placing them in 5 mL of the *Agrobacterium* suspensions of GUS in LB medium. The *Agrobacterium* suspensions were rocked for 5 min at 5 rpm at room temperature with a desktop rocker. The inoculated calli were vacuums infiltrated for 5 min at 20 Hg. After inoculation in these suspensions, the calli were blotted dry on sterile filter paper, to remove excess moisture and then plated on culture plates containing 4.3 g L^{-1} Murashige and Skoog (MS) salts, 0.1 g L^{-1} myo-inositol, 0.002 g L^{-1} glycine, 25 g L^{-1} sucrose, 0.50 mg L^{-1} kinetin, 0.050 g L^{-1} arginine, 1 mL B5 vitamins, 1000 ppm acetosyringone and 1.75 g L^{-1} phytogel. Plated calli were then co-cultivated for 2-3 days in the dark at 2°C. After 3 days the explants were removed from the co-cultivation media and rinsed 2-3 times in sterile water, before being assayed for GUS activity. The effect of acetosyringone to the co-cultivation media was evaluated in this research, to have an effect on calli-mediated DNA transformation.

Assay of GUS activity: The co-cultivated rice calli were taken in a falcon tube with 1 mL GUS substrate buffer (5 mM potassium ferrocyanide, 0.1 M sodium phosphate buffer pH 7.0, 20 μL of 0.3% v/v Triton X-100) and incubated at 37°C for 25 min. Then the treated calli were subjected to vacuum infiltration for 5 min at 20 Hg. The chemical X-gluc was then added to the substrate buffer and again the materials were put under vacuum infiltration for 7 min at 20 Hg. The explants were removed from the vacuum and placed in 37°C for 24-38 h. Within 24 h explants were examined under microscope for (GUS) gene expression, observed as blue spots (Fig. 3).

Effect of acetosyringone during co-cultivation: Various studies (Khanna and Raina, 1999; Gould *et al*., 1997) examined that without acetosyringone, there would be a complete absence of GUS expression, the quickest and most visual proof of transformation. Five different concentrations viz. 100, 200, 400, 600 and 1000 ppm were tested using calli in each concentration. The effectiveness of the concentration of acetosyringone was observed by the presence of blue color from GUS assays performed on the calli. The results show that as the concentration of acetosyringone increased, the number of blue GUS spots/calli also increased (Table 4). However, between 400 and 1000 ppm the results (i.e., the number of blue spots) were not significantly different. Therefore, using acetosyringone at 1000 ppm was decided upon for the use of co-cultivation medium.

RESULTS AND DISCUSSION

Callus induction: Results of callus induction performance of scutellum taken from BRRI dhan-30 variety was tested in N_6 medium supplemented with different concentrations of 2, 4-D and presented in Table 1. The table indicated that variations of hormonal concentrations played a major role in callus induction. N_6(Chu *et al*., 1975) supplemented with 2.0 mg L^{-1} 2, 4-D was found most effective in callus induction in rice variety and it gave 97.3% induction in BRRI dhan-30. This result is in agreement with the results obtained by others (Pipatpanukul *et al*., 2004; Zaidi *et al*., 2006), who reported that 2 mg L^{-1} 2, 4-D played an important role for embryogenic callus induction in rice. Lowest 40.7% induction was observed in lower concentrations (0.5 mg L^{-1}) of the hormone. In case of irrespective of hormonal concentrations, the N_6 medium supplemented with 2, 4-D was found more effective in callus induction. The use of 2, 4-D for callus induction in rice was also reported by Himani *et al*. (2007). When scutellum was used as explants callus formation started within 12-15 days of inoculation (Fig. 1).

However, regeneration from globular somatic embryo of rice was also reported by Rachmawati *et al*. (2004) from embryogenic calli of scutellum explants.

Callus regeneration: Plant regeneration from scutellum derived calli depends on a number of factors e.g., age of scutellum, type of derived calli, varieties of rice etc. The effect of age of scutellum derived calli for rice variety was tested and the results are shown in Table 2. The 21 days old calli of BRRI dhan-30 gave the highest percentage of regeneration (88.2%). Five days old calli

Table 1: Induction of callus from scutellum explant of BRRI dhan-30 rice variety on callus inducing medium

Treatments (mg L^{-1})	% of the callus formation (mean±SE) BRRI dhan-30	Texture of the callus	Color of the callus
N6 + 2,4-D 0.5	40.7±2.8	Compact	Pale yellow
N6 + 2,4-D 1.0	76.4±3.4	Compact	Yellowish
N6 + 2,4-D 2.0	97.3±0.5	Compact	Yellowish
N6 + 2,4-D 3.0	91.1±1.7	Compact	Yellowish
Mean±SE	76.4±12.7		

Table 2: Age effect of calli derived from scutellum on regeneration performance

Age of callus	Regeneration % in BRRI dhan-30 rice variety
2 days old	5.2±1.0
8 day old	21.1±2.8
21 days old	88.2±2.8

Table 3: Regeneration response of BRRI dhan-30 rice variety from scutellum derived calli on BAP with NAA in MS and N6 basal media

		BRRI dhan-30	
Regeneration media (RM)	Media formulation (mg L^{-1})	% ±SE of shoot formation	No. of shoot/callus
RM	MS+2 mg L^{-1} BAP+0.05 mg L^{-1} NAA+3% sorbitol	88.2±2.10	5.25
RM 1	MS+0.5 mg L^{-1} BAP+0.05 mg L^{-1} NAA	72.3±2.70	4.25
RM 2	MS+0.5 mg L^{-1} BAP	42.2±1.30	2.50
RM 3	N6+0.5 mg L^{-1} BAP+0.05 mg L^{-1} NAA	33.0±1.80	3.75
	Mean±SE	58.9±12.8	3.90

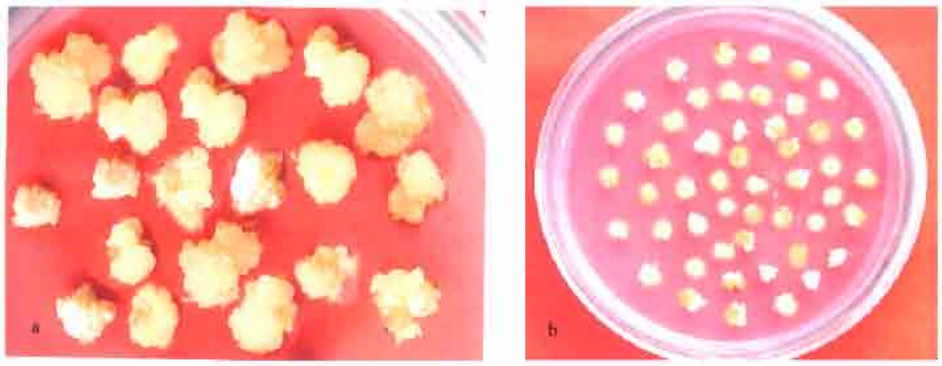

Fig. 1: Different types of scutellum derived calli, (a) embryogenic calli and (b) subculture of the embryogenic calli

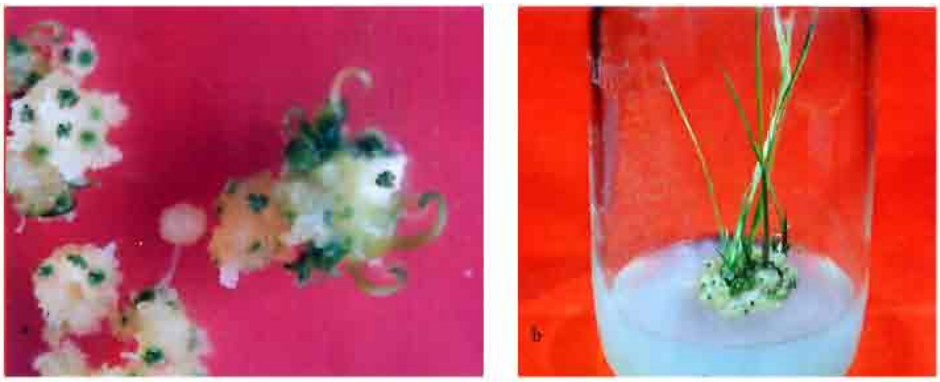

Fig. 2: Scutellum derived plants via regenerable callus, (a) plantlets developed from the embryoids and (b) scutellum derived plants

regeneration efficiency was very low, only 5.2%. Eight days old calli showed 21.1% of regeneration in BRRI dhan-30 variety of rice (Fig. 2).

The Table 3 shows that the variety BRRI dhan-30 gave highest regeneration response on medium RM (88.2%) while regeneration response on medium RM3 was the lowest (33.0%). The regeneration response on medium RM 1 was 72.3 and 42.2% regeneration was recorded on RM 2 for BRRI dhan-30.

***Agrobacterium*-mediated transformation:** After nearly two decades of research on rice transformation, few *indica* rice varieties have been successfully transformed despite significant progress in this field. Transformation of *indica* rice remains difficult for a number of reasons (Lin and Zhang, 2005). One prerequisite for high efficiency is an exceedingly effective and robust tissue culture system. Response of *indica* rice to callus induction and regeneration is genotype specific (Zaidi *et al*., 2006). In this study we have optimized media formulation for callus induction and regeneration for genetic transformation in an *indica* rice variety BRRI dhan-30.

Culturing of the *Agrobacterium* strain: Timing and temperature greatly affected virulence of the strain LBA4404 (pCL3). These strain had to be promptly removed from culture within 48 h. When these strains were left for a more extended period of time, the ability of the bacteria to cause transformation within the shoot calli decreased. Temperatures higher than 28°C also proved to

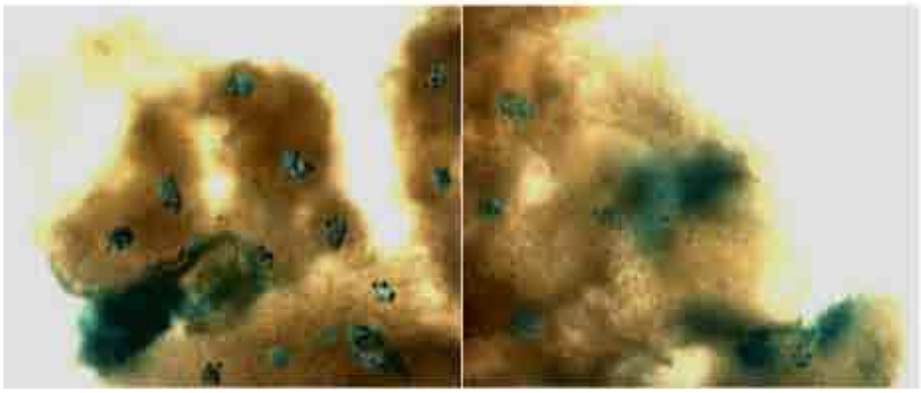

Fig. 3: Calli was transformed with GUS. Calli showed the specific competent tissue transformed with GUS, seen under microscope

be detrimental. Making fresh *Agrobacterium* suspensions for each experiment was routinely done since it aided in maintaining competency of the *Agrobacterium* suspension and increased chances of successful transformation.

The highly regenerative embryogenic rice calli were co-cultivated with *Agrobacterium* harboring with GUS gene. During co-cultivation the *Agrobacterium* was supposed to transfer its Ti plasmid along with GUS gene to the rice callus. *Agrobacterium*-mediated genetic transformation in different varieties of rice was also reported by Ignacimuthu and Arockiasamy (2006), Rachmawati and Anzai (2006), Zaidi *et al.* (2006), Hoque *et al.* (2005) and Pipatpanukul *et al.* (2004). After co-cultivation the callus tissue was subjected to GUS assay in order to be confirmed the GUS gene integration into the rice genome. Histochemical assay was performed on the co-cultivated rice calli according to the method described by Jefferson (1987) with some modifications. For microscopic observation treated rice calli were pressed on a slide covering with cover slide and observed under microscope (Fig. 3).

Effect of acetosyringone during co-cultivation: Various studies examined the importance of acetosyringone to *Agrobacterium* co-cultivation procedures (Himani *et al.*, 2007; Hoque *et al.*, 2005; Khan *et al.*, 2003; Rachmawati *et al.*, 2004; Rao and Rao, 2007; Yookongkaew *et al.*, 2007). These studies stated that without it, there would be a complete absence of GUS expression, the quickest and most visual proof of transformation. However, the problem was with the level of acetosyringone to use since concentrations to use ranged from 30-1000 ppm from different studies. Therefore, five different concentrations were tested; 100, 200, 400, 600 and 1000 ppm using calli in each concentration. The effectiveness of the concentration of acetosyringone was observed by the presence of blue color from GUS assays performed on the calli. The results are shown in Table 4.

Table 4: Acetosyringone effect on GUS activity as measured by the number of blue spots in the calli

Concentration of acetosyringone (ppm)	No. of blue spots/calli mean±SE
100	8.5±0.20
200	18.0±0.55
400	26.0±0.71
600	28.1±0.53
1000	29.0±0.47

The results show that as the concentration of acetosyringone increased, the number of blue GUS spots/calli also increased. However, between 400 and 1000 ppm the results (i.e., the number of blue spots) were not significantly different. Therefore, using acetosyringone at 1000 ppm was decided upon for the use of co-cultivation medium.

CONCLUSION

Application of plant biotechnology is gaining momentum in developed as well as in developing countries to improve the agricultural crops. In our country there have tremendous scopes for developing its agricultural crops by the application of plant DNA transgenic technology; in particular, to develop the high yielding, disease resistant and aromatic rice production would possible.

Most of the rice transformation work reported was based on *japonica* varieties, but *indica* rice, despite of its importance, was still under studied. However very little work had been done on improving Bangladeshi local rice varieties using modern biotechnological methods. There is an urgent need to under take such studies to produce

local transgenic rice varieties in order to exploit the prospective quality of aroma and ability to combating biotic and abiotic stresses. Bangladesh, as poor nation, holds the promise to develop metabolically engineered rice for important micronutrients like golden rice for supporting vitamin A.

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