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Establishment of *Agrobacterium*-Mediated Genetic Transformation in Popular Rice Cultivar BRRI Dhan-29 through Reporter GUS Gene Expression System in Bangladesh

M. Ruhullah [1], M. S. Rahman [1], Md. Sarafat Ali [2]*, G.M. Mohsin [3] and M. Khalekuzzaman [4]*

[1] Lal Teer Seed Ltd., Bashon, Gazipur, Bangladesh
[2] Department of Biotechnology and Genetic Engineering, Bangabandhu Sheikh Mujibur Rahman Science & Technology University, Gopalgonj-8100, Bangladesh
[3] Department of Agriculture, Noakhali Science and Technology University, Bangladesh
[4] Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh

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Abstract: *Agrobacterium*-mediated genetic transformation was done in BRRI dhan-29 using *Agrobacterium* strain LBA4404 harboring the plasmid pBI121. The plasmid carries CaMV35S promoter driving the expression of *GUS* and Kanamycin resistant genes. The β-glucuronidase (*GUS*) gene served as a reporter gene in the histochemical assay and the neomycin phosphotransferase II (*npt*II) gene used for the identification of resistance to kanamycin. The incubation period of 30 minutes was found to be the most effective for infection and integration of foreign genes into callus. Transformed calli were selected along with a control in the regeneration medium containing 100 mg/l of kanamycin. Embryogenic calli were found to show the best transformation ability producing 30 % *GUS* positive result in histochemical assay after 3 days of co-cultivation. *GUS* expression was also observed in the leaf and root of putative plants prior to final confirmation of transformation through PCR amplification.

Key words: *Agrobacterium,* BRRI Dhan-29, callus, *GUS,* Transformation.

Introduction

Rice is a staple food of Bangladeshi people and almost every people depends on rice. Furthermore, this country is a spot land of forming an exciting climax of weather and rice production decreasing tremendously due to climatic change by the means of global warming and urbanization. Every year, 1.8 % rice consumers are greater in intensity [1] in Bangladesh. In Bangladesh, especially Indica-type rice provides the staple food for more than half of the world population. To satisfy the greater demand and ensure food security of the ever increasing population, more sustained production of indica-type rice is needed.

Generally, in conventional breeding extensive back cross method is applied to introduce one gene for the improvement of existing cultivar. It takes a long time for comes up conclusion where as transgenic method may allow scientist for introducing one or two characterized gene at a time within shortest possible time. Nevertheless, it also provides the re-introduction of genes that have been extracted and modified to give altered properties. Several gene transfer methods have been developed in order to introduce target DNA into the rice cell, of which, *Agrobacterium tumefaciens* mediated transformation harboring the plasmid pBI121 has been well established on ac-

*Corresponding authors (M. Khalekuzzaman & Md. Sarafat Ali)
E-mail: < kzaman63@ru.ac.bd; sarafatbiotech@gmail.com >

count of its simplicity, low cost, and low copy number of transgene integration. Another reason might be pBI121 plasmid has the potentiality to one-step replacement of the β-glucuronidase (*GUS*) gene with another gene and can quickly create an over expression vector for the gene.

It is more practical to use reporter genes which can be easily screened. Reporter genes are extremely useful especially in optimizing transformation protocol, where easy detection of transformants is desirable. Most commonly used reporter genes are β-glucuronidase (*GUS*), Chloramphenicol acetyl transferase (*CAT*), Nopaline synthetase (*NOS*), Octopine synthetase (*OCS*) and firefly luciferase (*Lux*). The GUS gene originally isolated from *E. coli* is usually the reporter gene of choice for plant genetic research for several reasons. Plant lacks appreciable *GUS* activity (with very few exceptions). Various β-glucuronic acid substrates are available for detection of *GUS* expression *in vitro*. It has the ability to tolerate N-terminal fusion. Stable expression of the *GUS gene* in the transformed leaves/ shoots assayed histochemically.

So, the aim of the present study is to establish an efficient tissue culture-regeneration technology and the protocol of DNA transfer of reporter GUS gene transformation system in the local varieties of rice (BRRI Dhan-29) in Bangladesh.

Materials and methods

Agrobacteriun tumefaciens strain and selectable marker

The bacterial strain used for infection in the transformation was *Agrobacterium tumifaciens* strain LBA4404 with the binary plasmid pBI121. The binary vector pBI121 has the background of pBIN19. It contains a scorable reporter gene GUS (β-glucurunidase) driven by a CaMV35S promoter and NOS terminator and a selectable marker gene *npt*II fused between promoter and terminator encoding for the enzyme neomycin phosphotransferase conferring kanamycin resistance [2].

Culture media for *Agrobacterium* strain

Luria-Bertani (LB) medium: 10 g/l Bacto-tryptone, 5 g/l Yeast extract, 10 g/l Sodium chloride, 15 g/l Agar (for solid medium) supplemented with the proper selective agent (kanamycin 50 mg/l).

Plant material

Mature zygotic embryo of *Indica* rice genotypes (*Oryza sativa* L.) namely BRRI dhan-29 was used as an explant for the study of genetic transformation. Seeds were collected from Bangladesh Rice Research Institute (BRRI) Regional office, Rajshahi, Bangladesh.

Chemical and reagents

A combination of Twin-80; 0.1 % (w/v) $HgCl_2$ and sterilized distilled water was used as a sterilizing solution. MS [3] media were used for callus induction and their subsequent subculture. Growth regulators were added separately to the media, according to requirements. 4.33 g Na_2HPO_4, 3.03 g NaH_2PO_4, 5.0 g Triton X 100, 1.86 g Na_2EDTA, 106 mg K^+ Ferrocyanide, 3.3 ml hydrogen peroxide and 5 ml X-Gluc solution, were used as histochemical reagents.

Establishment of embryogenic calli from mature embryos

Preparation of explants

Mature seeds of three *indica* rice cultivars BR-29 were dehusked carefully taken in beakers having distilled water. Adding 1 or 2 drops of Twin-80 the mixture was shacked for 5 minutes with orbital shaker. Seeds were then again washed with distilled water for several times to remove the effect of Twin-80.

Callus induction

For callus induction, dehusked mature surface sterilized (0.1 % w/v $HgCl_2$) seeds were placed in MS basal media supplemented with different concentrations of 2,4-D and NAA combinedly on induction of callus from mature seeds of mentioned rice cultivar. The pH of the medium was adjusted to 5.8 before autoclaving for 21 min at 121°C and 1.07 kg cm^{-2}. Culture tubes were sealed with cotton plug and the explants were incubated in the dark at 26±2°C.

Histological study

Histological analysis of the embryogenic calli was performed according to Boissot *et al.*[4]. Embryogenic calli were collected 5, 10, 15 and 20 days after cultured on callus induction medium. Then, the samples were dehydrated in a graded

series of ethanol (70 %, 95 % and 100 %) for 1 h each one and embedded in paraffin wax. Samples were cut into 9-12 µm sections by microtome and were stained with safranin and calcofluor white stain. Microscopic features of different days of calli were dissected and the micrographs of the histological study were taken under 10X and 40X using a B-350 OPTIKA (Italy) contact face and inverted microscope.

Genetic transformation

Culture of Agrobacterium strain

A loop full of bacteria was inoculated in to the liquid LB medium supplemented with proper selection (kanamycin 50 mg/l) in a conical flask and placed on a rotary shaker incubator at 120 rpm for about 20 hours to obtain OD_{610} of 0.8-0.9 for bacterial concentration. The incubation temperature was maintained at 28°C. *A. tumefaciens* containing pB1121 vector was added to acetosyringone at a final concentration of 100 µM, incubated at 22°C for 4 h [5]. The suspension was then centrifuged at 3000 rpm for 10 minute. The supernatant was discarded and the bacterial pellet was suspended in liquid MS medium.

Inoculation of explants

For agro-infection embryogenic calli were immersed in the bacterial suspension for about 30 minutes. Then the explants were blotted dry on sterile filter paper and transferred for co-cultivation on normal MS medium. The plates were sealed with parafilm and kept in a dark chamber for three days. After co cultivation, the explants were transferred to the regeneration medium (BAP 2.0 mg/l + 1.0 mg/l NAA + 1.5 mg/l KIN) containing carbenicillin (100 mg/l) until shoots initiation and the same time uninfected explants were inoculated on regeneration medium as control for the first cycle and second cycle selection.

Selection of transformed cells and tissues

After proliferation shoots were cultured on selection medium containing kanamycin (50-150 mg/l) to select transformed tissues on the basis of controls. Initially, kanamycin was used 50 mg/l then increased with each subculture at 21 days intervals up to 150 mg/l. During each subculture the dead and deep brown tissues were discarded and green shoots and shoot buds were sub cultured to fresh medium containing the next higher concentration of kanamycin.

Positive and negative control

For positive control, explants neither infected nor placed in the selection medium, rather they were placed in the regeneration medium. Cultures were sub cultured after every 15 days. For negative controls, explants were not infected by bacteria but were placed in the selection medium. They began to die after 7-10 days in kanamycin (100 mg/l).

Substrate for β-glucuronidase

The most widely used substrate for histochemical assay of *GUS* is 5-Bromo-4-Chloro-3-indolyl β-D-glucuronide (X-gluc). This substrate gives a blue precipitate, very insoluble and highly colored indigo dye 5,5′-dibromo, 4,4′-dichloro-indigo (CIBr-Indigo) at the site of *GUS* enzyme activity. This dye is formed by oxidative dimerization of the colorless indolyl derivatives that results from *GUS* hydrolysis of X-gluc.

Preparation of histochemical reagents for GUS assay

The solution was prepared by dissolving chemicals one by one in 300 ml of distilled water while stirring in a beaker. X-Gluc solution was prepared by dissolving 250 mg in Dimethyl sulfoxide (DMSO). The final volume was made to 500 ml by adding distilled water and filter sterilized.

Method of histochemical GUS assay

Infected calli after co-cultivation as well as regenerated shoots of putative transformants were assayed for transient *GUS* activity. Explants were dipped into histochemical reagent solution and incubated for 24-48 hours at 37°C. Then treated explants were transferred to 95 % ethanol for preservation and the explants were observed under zoom stereoscopic microscope (Japan, model-SDZ-TR-P).

Result and discussion

Callus induction

Callus induction observed from mature seeds of popular rice variety in Bangladesh (BRRI

Dhan-29) cultured on MS basal media supplemented with different concentration and combination of 2,4-D (1.0 and 2.0 mg/l) and NAA (0.5, 1.0 and 1.5 mg/l). The result obtained from this experiment was shown in Table 1. Among the six hormonal concentrations and combination callus formation was highest (85 %) in BRRI dhan-29 when the medium was supplemented with 2.0 mg/l 2,4-D + NAA 1.0 mg/l NAA and callus formation was lowest (35 %) when the medium was supplemented with 1 mg/l 2,4-D + 0.5 mg/l NAA. A similar result was recorded by Islam and Khalekuzzaman [6] in BRRI Dhan-29 as 92.0 % in MS having 2.0 mg/l 2,4-D + 1.0 mg/l NAA among four combinations of 2, 4-D and NAA. Callus induction in rice greatly affect on the choice of explants, concentration and combination of hormonal and nutritional element [7,8]. Hiei *et al*.[9] reported that scutellum derived callus was the most amenable explant for *Agrobacterium* mediated transformation. In the present study, mature rice seeds were used to obtain scutellum derived calli and the maximum callus induction frequency was obtained (85 %). The texture of callus was mostly observed friable when the hormonal concentrations were low and were found compact callus at higher concentrations. Degree of callusing was excellent (++++) in case of the hormonal treatment 2.0 mg/l 2,4-D + 1.0 mg/l NAA and 2.0 mg/l 2,4-D + 1.5 mg/l NAA; good (+++). Color of callus was mostly creamy in single hormonal treatment, while the inverse situation was obtained in that of combined.

Histological analysis of callus

The histological observations of the rice embryogenic calli at different developmental stages showed an internal organization of the calli. Observations in 21 days old embryogenic calli of BRRI dhan-29 revealed two types of cells. Thus, the external layers consisted of meristematic cells, which gave rise to clusters of embryogenic cells and somatic embryos.Whereas, interior of the callus consisted of parenchymatic cells with a less visible nucleus and many vacuoles. Some of these parenchymatic cells are broken and gave rise to the internal space of the callus [Fig. 1(e & f)].

Regeneration response of embryogenic callus

It is more difficult to regenerate shoot from scutellum derived calli of rice seeds. To maximize regeneration of shoots the concentration of cytokinin (BAP and KIN) and auxin (NAA) plays a vital role nevertheless, regeneration or organogenesis is highly dependent on its genotype. There-

Table 1. Effect of different concentrations combination of 2, 4-D and NAA on induction of callus from mature seed scutella of BRRI dhan-29

Treatments (mg/l)	Days to callus initiation	% of callus formation	Texture of callus	Color of callus	Degree of callusing
2,4-D 1.0 + NAA 0.5	11-14	35	C	PY	++
2,4-D 1.0 + NAA 1.0	11-13	60	C	PY	+++
2,4-D 1.0 + NAA 1.5	10-12	65	F	PY	+++
2,4-D 2.0 +NAA 0.5	10-12	75	C	PY	++++
2,4-D 2.0 + NAA 1.0	9-11	85	C	PY	++++
2,4-D 2.0 + NAA 1.5	9-11	80	C	PY	++++

PY = Pale yellow
F = Friable
++++ = Excellent
Cr = Creamy
C = Compact
+++ = Good
++ = Average

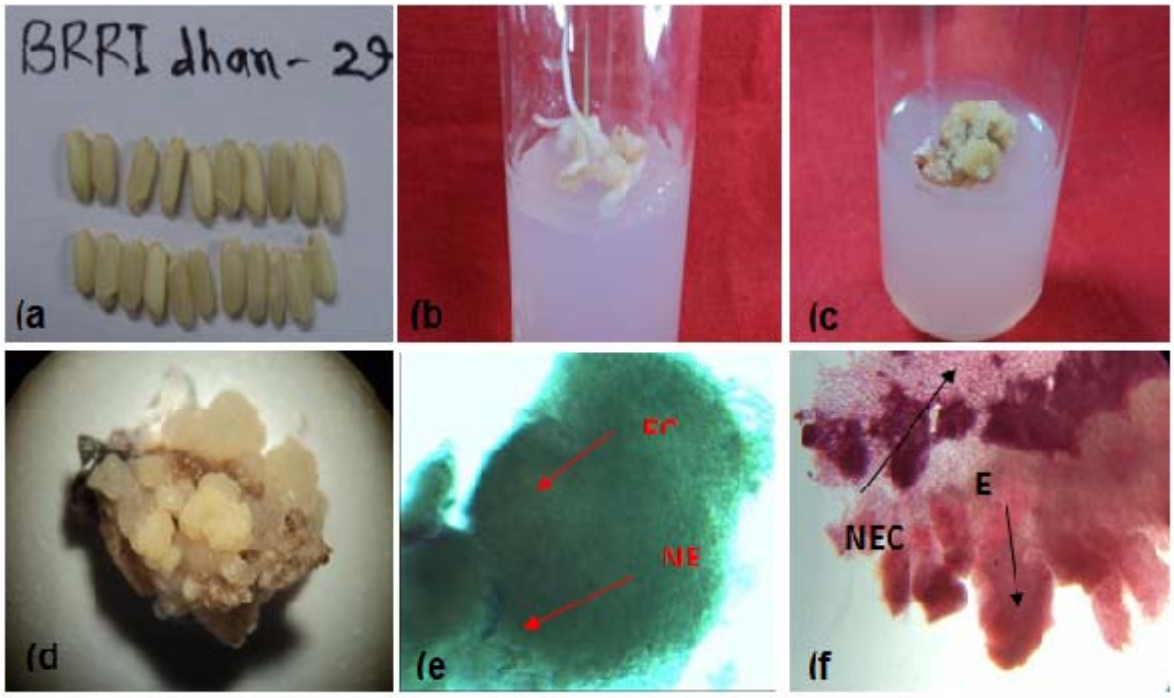


Fig. 1. (a): Explants (BRRI-29) used for callus induction; **(b):** Callus initiation after 10 days of inoculation; **(c):** Development of callus in MS medium with 2.0 mg/l 2,4-D + 1.0 mg/l NAA after four weeks of culture; **(d):** Somatic embryogenesis in globular shape embryo (GSE); **(e & f):** histology of embryogenic cali; embryogenic cells (EC), and non-embryogenic cells (NEC) of BRRI Dhan-29

fore, before using the above calli in genetic transformation, it was necessary to confirm its regeneration ability [10]. Out of nine treatments MS medium supplemented with BAP 2.0 mg/l + NAA 1.0 mg/l + KIN 1.5 mg/l formed highest percentage of shoots (Table 2). In some cases, roots originated and when they were transferred to soil, they did not survive. To solve the problem, separate experiments were set up to induce roots on shootlets for the regeneration of complete plant (Table 3). Among various concentrations of IBA the maximum percentage of cultures that regenerated roots was 86.23 % when the shoots were culture in media having 0.4 mg/l IBA. The highest number of roots per explant was 4.7 after 20 days of culture in media having 0.4 mg/l IBA.

Genetic Transformation

Infected embryogenic calli were co-cultivated for 4 days on normal MS media and sub cultured on regeneration medium (BAP 2.0 mg/l + 1.0 mg/l NAA + 1.5 mg/l KIN) containing carbenicillin. After 2-3 weeks, the calli developed shoot buds. Proliferated shoot buds were then transferred on selection medium with kanamycin and the same hormonal combination for the first cycle and second cycle selection [Fig. 2 (A)]. At the same time control plants were subsequently cultured on selection medium [Fig. 2 A (a)] for the first cycle and second cycle selection and 90 % shoots died after 21 days. Regenerated shoots harvested from selection medium and transfer in the normal MS medium for root induction. GUS assay was performed according to Jefferson [11] in two stages. Firstly, after 4 days of co cultivation in calli and secondly the leaves and roots from putative plants were taken and incubated in X- gluc buffer. Indigo blue coloration was observed in calli [Fig. 2 (B)], section of calli leaves and roots [Fig. 3 (a and b)]. Blue patches indicated GUS activity and confirming GUS expression in co-cultivated callus tissue. Conspicuous GUS positive (Blue color) regions were detected in the explants surface.

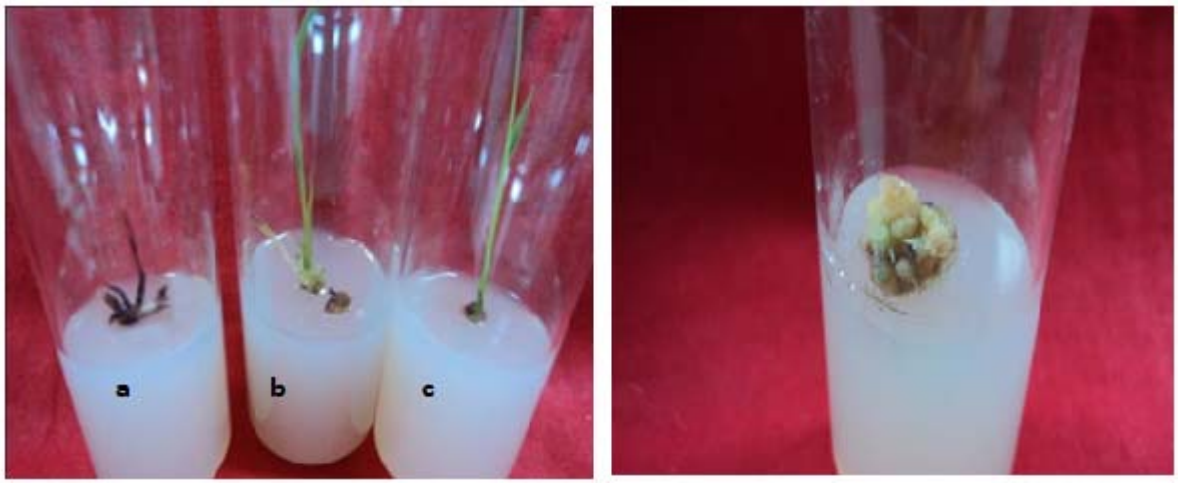
GUS gene was amplified in transformed plant DNA and plasmid DNA where forward and reverse primer amplified 880 bp *GUS* gene segment (Fig. 4).

Table 2. Regeneration response of embryogenic calli of BRRI Dhan-29 cultured on MS medium supplemented with different concentrations and combinations of BAP, NAA and KIN

Treatments (mg/l)	Days of shoot initiation	% of calli formed shoot	No. of shootper callus
BAP 1.0 + NAA 0.5	31-43	15	0.80 ± 0.02
BAP 1.0 + NAA 1.0	30-42	25	3 ± 0.175
BAP 2.0 + NAA 1.0	30-40	45	2 ± 0.00
BAP 2.0 + NAA 1.5	31-42	40	3 ± 0.25
BAP 1.0 + NAA 1.0 + KIN 1.0	28-40	45	3 ± 0.025
BAP 2.0 + NAA 1.0 + KIN 0.5	27-35	50	4 ± 0.012
BAP 2.0 + NAA 1.0 + KIN 1.0	26-32	50	5 ± 0.01
BAP 2.0 + NAA 1.0 + KIN 1.5	24-30	80	8 ± 0.125
BAP 2.0 + NAA 0.5 + KIN 1.5	29-36	65	6 ± 0.175

Table 3. Effect of different concentration of auxins on root induction from regenerated plantlets

Media composition ½ MS + IBA(mg/1)	% of root induction	Morphogenic response	
		Mean number of root per culture after 4 weeks	Mean length of the longest roots per culture after 4 weeks (in cm)
0.2	28.57	2.7	2.1
0.4	86.23*	4.7	2.9
0.6	64.28	4.6	2.8
0.8	42.85	3.9	2.6
1.0	21.42	2.1	1.9



(A) **(B)**

Fig. 2 (A): Second cycle selection of subculture of shoots after first cycle selection in medium with 100 mg/l kanamycin; transformed shoots (Sub Fig. b & c) and control (Sub Fig a); and **(B):** Gus expression in transformed callus

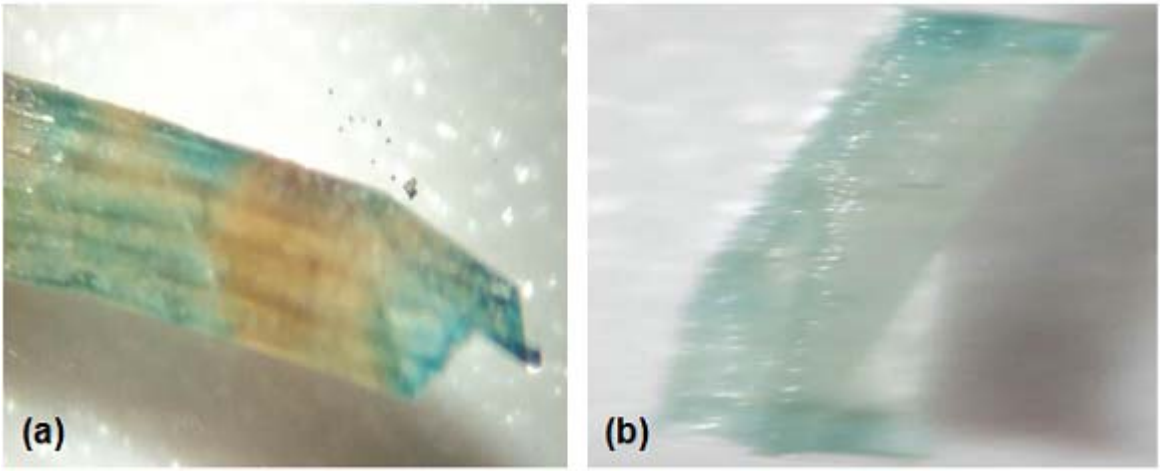


Fig. 3 (a): *GUS* expression in leaf segment; **(b):** *GUS* expression of root segment of putative plant

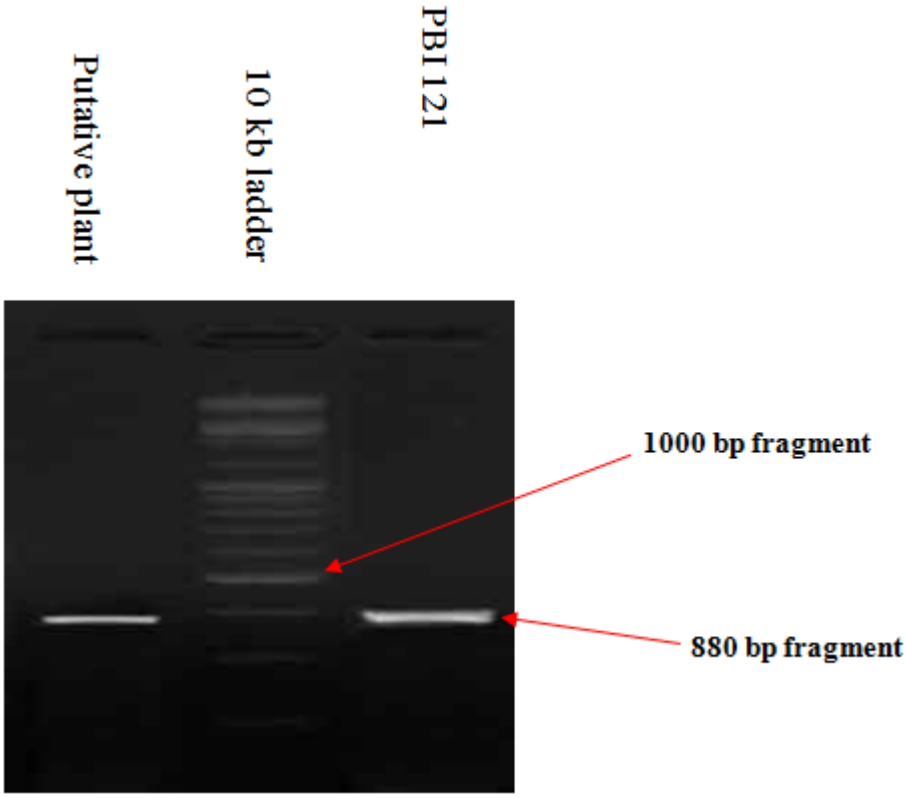


Fig. 4. Confirmation of *GUS* gene through PCR analysis

Choosing the correct concentration of antibiotic

Agrobacterium strain LBA4404 harboring the plasmid pBI121 contains kanamycin resistance gene (nptII) as the selectable marker gene. 100 mg/l concentration of kanamycin found to be suitable for successful selection. At concentration higher than 100 mg/l resulted in rapid and severe browning of the cells probably cause too much stress any surviving or transformed cells.

Effects of incubation period on transformation

The effects of different incubation periods (15,

20, 25, 30, 35 and 40 minutes) with the *Agrobacterium* strain on the calli were studied at a constant optical density (OD_{600} of ~0.9). In every case, 10 calli were infected and the highest 30 % GUS positive result was found with the 30 min incubation period. On the other hand, the lowest 10 % GUS positive result was found with 15 min incubation period (Fig. 5). These findings are consistent with Islam *et al*.,[1] and Islam and Khalekuzzaman [6].

Effect of Optical Density (OD)

The optical density of *Agrobacterium* is an important factor for genetic transformation. There are several studies describing the *Agrobacterium* mediated transformation using a range of concentrations of *Agrobacterium* from 0.5 to 1.5 OD_{600} [6,12,13]. The highest number of GUS expressing calli was observed at OD 0.8 - 0.9 (Fig. 6). Bacterial strain LBA4404 (pBI121) showed highest peak of performance in the lower range of OD and it gradually decreases with the increase of OD.

Conclusion

In conclusion, we have established rapid multiple shoot induction and efficient plant regeneration method from seeds of popular rice cultivar BRRI Dhan-29 in a genotype independent manner. The method described was simple, inexpen-

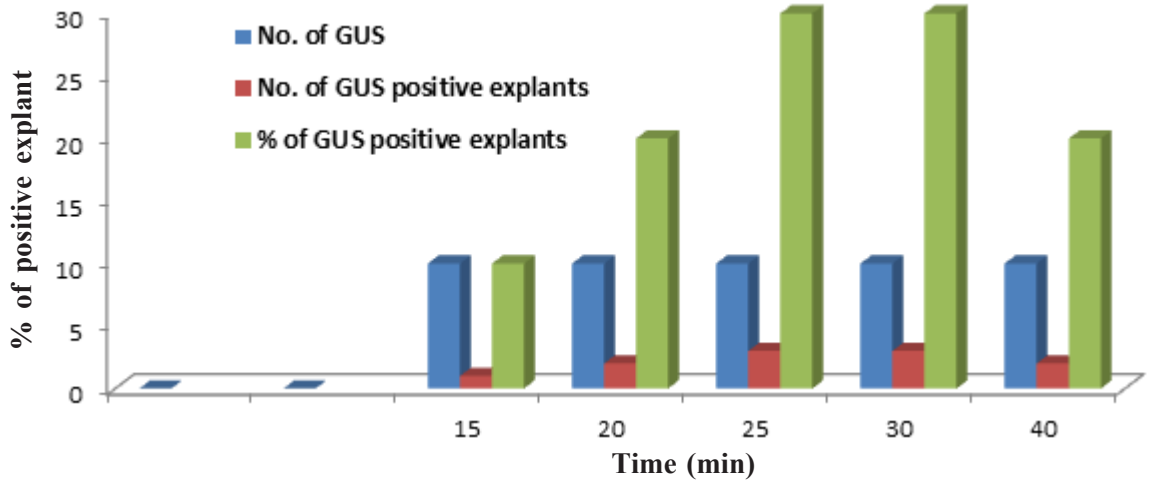


Fig. 5. Effects of different incubation periods on genetic transformation of calli explants of rice analyzed by transient *GUS* histochemical assay

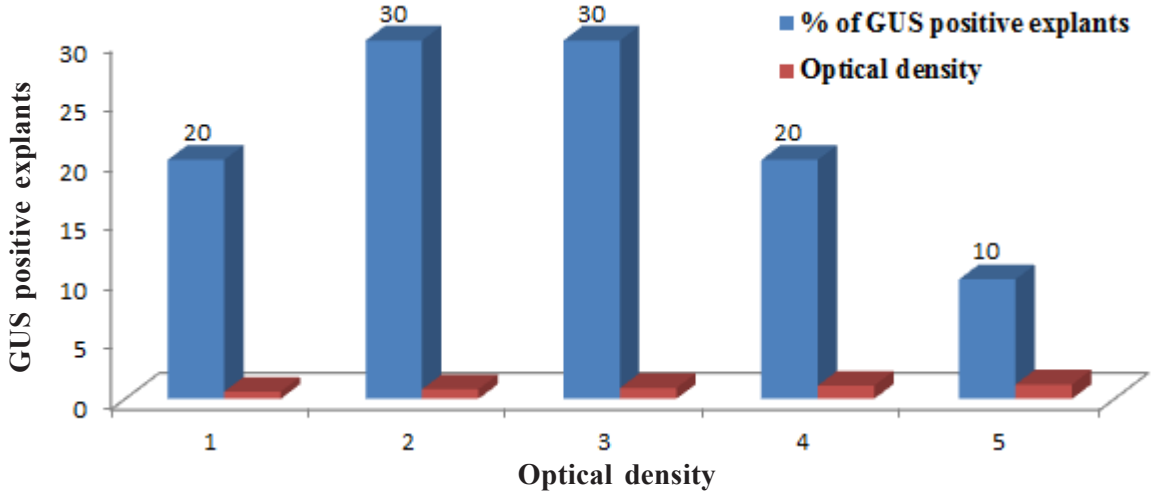


Fig. 6. Frequency of GUS positive explants with optical density at 600 nm

sive and does not require any advanced equipment. This study would be an effective tool for crop improvement and gene- function studies on the model monocot plant rice.

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