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Vol. 14(31), pp. 2415-2423, 5 August, 2015
DOI: 10.5897/AJB2015.14556
Article Number: 2C5FA1854554
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Agrobacterium mediated genetic transformation and regeneration in elite rice (*Oryza sativa* L.) cultivar BRRI dhan56

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Received 11 March, 2015; Accepted 29 June, 2015

***Agrobacterium*-mediated genetic transformation of rice (*Oryza sativa* L.) cultivar BRRI dhan56 was carried out in this study. *Agrobacterium tumefaciens* strain LBA 4404, which harbors the plasmid pIG121 that carries the genes for ß-glucuronidase gene, served as a reporter gene in the histochemical assay and the neomycin phosphotransferase II (*NPT II*) gene for the identification of resistance to kanamycin was used for genetic transformation. Twenty days old embryogenic calli from mature embryos of highly regenerating rice cultivar BRRI dhan56 were used to co-cultivate with 0.8 to 0.9 OD_{600} *Agrobacterium* for 25 min and the cultured was continued on agar medium for this study. The transformed colonies were selected by using 50 mg/L kanamycin and 50 mg/L rifampicin and confirmed by colony PCR. The PCR positive colonies were isolated to transform by using calli of indica rice cultivar BRRI dhan56. Putative leaf and root segments from plantlets obtained from transformation experiment with the plasmid pIG121 were GUS positive. Integration of the introduced gene into the genome was demonstrated by PCR. The maximum transformation efficiency of 32% was obtained by using 500 mg/L cefotaxime as a bacteriostatic agent to inhibit growth of *Agrobacterium*. In this study, 100 µM acetosyringone in co-cultivation medium and co-cultivation for 3 days were the optimum conditions for maximum transformation. The expression of GUS gene revealed that the calli were successfully transformed. The results of this study would be an effective tool for crop improvement and gene-function studies on the model monocot plant rice.**

Key words: *Agrobacterium, Oryza sativa* L., acetosyringone, β-glucuronidase, cefotaxime, plasmid, phosphotransferase, rice, transformation.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most significant cereal crops of Poaceae family cultivated for more than 10,000 years (Sasaki, 2005). It was the first major cereal crop regenerated into whole plant from tissue culture (Vasil, 1983). It is an economically very important crop in the world, with more than half of the world's population depending on it as a primary staple food (Lu, 1999). Its cultivation is concentrated for the most part in Asian

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countries, which together make up approximately 90% of the rice cultivation area in the world. Numbers of rice consumers are increasing at the rate of 1.8% every year (Islam et al., 2015). It is estimated that rice production has to be increased 50% by 2025 (Khush and Virk, 2000). Approximately 80% of the world rice production is based on indica rice varieties which are cultivated under subtropical and tropical conditions as long grain rice and thus securing a unique position in agriculture (Khush, 1997; Abbasi et al., 2010).

In recent years, some important agronomic traits of rice have been improved through biotechnological approaches (Hao et al., 2009; Skamnioti and Gurr, 2009; Cheng et al., 1998). Genetic transformation has become a valuable tool in targeted improvement and gene function studies in rice (Xu et al., 2012). Most indica rice varieties were adapted to by *Agrobactrerium*-mediated genetic transformation (Chan et al., 1992; Rashid et al., 1996; Khanna and Raina, 2002; Supertana et al., 2005; Ignacimuthu and Arockiasamy, 2006) involve regeneration of plants from transformed embryogenic calli, anther calli (Jiang et al., 2004; Arnold et al., 1995), and protoplasts. *Agrobacterium*-mediated transformation has significant advances of such as introduction of fewer copies of genes into the plant genome, high co-expression of introduced genes, transformation of relatively large segments of DNA and high fertility of transgenic plants (Cheng et al., 1997; Hiei et al., 1994; Datta et al., 1992). *Agrobacterium tumefaciens* mediated transformation has been well established, owing to its simplicity, low cost, as well as low copy number of transgene integration. However, the transformation efficiency is very low in the indica rice varieties. Most indica rice varieties also show a low rate of callus growth and low regeneration frequency in conventional culture (Nishimura et al., 2007; Larkin and Scrowcroft, 1981). Now *Agrobacterium*-mediated rice transformation has been successful in many cultivation of japonica, indica and javanica (Aldemita and Hodges, 1996; Hiei et al., 1994; Dong et al., 1996).

There are several factors which are involved in *Agrobacterium*-mediated gene transfer into rice. During co-cultivation, the addition of acetosyringone, media with 2,4-D, acidic pH and high osmotic pressure have been reported to be significant for the induction of *vir* gene expression on Ti plasmid (Turk et al., 1991; Usami et al., 1988). Most reports suggested to use actively growing, embryogenic calli derived from rice mature seed or immature embryos as receptors to be infected by *A. tumefaciens* (Hiei et al., 1997). In addition, shoot apices of tropical japonica have also been used as gene receiver (Park et al., 1996; Rogers and Bendich, 1994). In the present study, we report establishment of an efficient plant regeneration system from mature seeds of indica rice applicable to BRRI dhan56 as explants using different growth regulators and demonstrate their amenability to *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Plant materials

Indica rice (*O. sativa* L.) cultivar namely BRRI dhan56 was used as explants for study of *in vitro* regeneration and genetic transformation. Seeds were collected from Bangladesh Rice Research Institute (BRRI) Regional office, Rajshahi, Bangladesh.

Preparation of explants

Mature seeds of indica rice cultivars namely BRRI dhan56 were dehusked carefully and separately. Dehusked seeds were taken in beakers having distilled water and 1 or 2 drops of Twin-80 were added in the distilled water. The mixture was shacked for 5 min with orbital shaker. Seeds were then washed with distilled water for several times to remove the effect of Twin-80. Then seeds were bought in front of laminar air flow and surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 5 min by vigorous shaking twice. Treated seeds were rinsed with distilled water several times to remove mercuric chloride completely and blot dried on to a filter paper.

Callus induction

For callus induction, three seeds were inoculated per test tube on callus induction medium (MCI) and incubated at 26 ± 2°C in dark. MCI was prepared using by basal MS salts containing all vitamins (Murashige and Skoog, 1962) supplemented with 2.5 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.50 mg/l naphthalene acetic acid (NAA), then the medium was solidified 0.8% agar before autoclaving 21 min at 121°C and 1.07 kg cm^{-2} and pH of the medium was adjusted to 5.8 before addition of agar. After 20 days, non-embryogenic calli (compact, non-friable calli that develop root like structures) were discarded and only embryogenic calli were selected. These embryogenic calli were cut into around 3 equal halves, then sub-cultured again onto fresh MCI and kept for 4 days (dark, 26 ± 2°C) before transformation with *A. tumefaciens*.

Agrobacterium strain

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

Culture of *Agrobacterium* strain

Primary culture of *Agrobacterium* strain was prepared by inoculating single colony from a freshly streaked plate which contains 5 ml of autoclaved liquid YEP medium (10 g/l bactopeptone, 10 g/l yeast extract, 5 g/l sodium chloride and pH 7.0) supplemented with 25 mg/l streptomycin, 50 mg/l rifampicin and 50 mg/l kanamycin. The culture was incubated for 20 hours on a rotatory incubator shaker at 120 rpm in dark at 28°C. Secondary culture of *Agrobacterium* strain was prepared in a 500 ml baffled flask containing 50 ml YEP medium (supplemented with same antibiotics as used for primary culture) by adding together 0.4% of the primary culture and grown under same environment. Once the OD_{600} reached ~1.0, *Agrobacterium* cells were pelleted by centrifugation at 8000 × *g* for 15 min at 4°C. The cells were re-suspended in MS re-suspension medium containing 150 µM acetosyringone (MS salts, 6 8 g/l

sucrose, 36 g/l glucose, 3 g/l KCl, 4 g/l $MgCl_2$ and pH 5.2) to adjust the OD_{600} of the bacterial suspension to 0.3.

Co-cultivation and selection of transformed calli

Sub-cultured (four days) embryogenic calli were collected and Agro-infected by immersing them in the *Agrobacterium* culture (LBA4404) for 20 to 25 min with blinking gentle shaking at 50 rpm. The Agro-infected calli were dried on sterile filter paper for 5 to 6 min. Calli were then transferred to the co-cultivation medium containing 10 g/l glucose, pH 5.2, 150 µM acetosyringone (Ali et al., 2007) and incubated at 26 ± 2°C in the dark for around 48 h. Once slight growth of *Agrobacterium* appeared around most of the calli, the calli were rinsed 8 to 10 times with 500 mg/l cefotaxime in sterile distilled water, dried on sterile filter paper and transferred onto first selection medium (MCI containing 500 mg/l cefotaxime and 100 mg/l kanamycin) and incubated for 13 days at 26 ± 2°C in dark. After the first selection, brown or black calli were removed and only creamish healthy calli were shifted to the fresh MS media for second selection and maintained at 26 ± 2°C in dark. After second selection for 11 days, micro calli could be observed which were finally transferred to fresh MS media for third selection and allowed to proliferate for five days at 26 ± 2°C in dark.

Regeneration of transformed calli

After third selection, black or brown microcalli were discarded and only granular 'macrocalli' were transferred onto MS media containing two or three growth regulators viz. 6 benzylaminopurine (BAP), kinetin, naphthalene acetic acid (NAA), pH 5.8; 250 mg/l cefotaxime and 50 mg/l kanamycin added after autoclaving. These microcalli were incubated at 26 ± 2°C in dark for seven days for the first phase of regeneration. During the second phase of regeneration, these were shifted to fresh same regeneration medium and incubated in light for four days. The regeneration frequency was calculated as per the formula: Regeneration frequency (%) = [(Number of microcalli regenerating shoots) / (Number of microcalli incubated)] x 100.

For development of vigorous roots, the regenerated shoots were shifted to rooting media and finally transfer to soil pot for hardening.

Histo-chemical GUS assay

Infected calli after co-cultivation as well as regenerated shoots of putative transformants were assayed for transient GUS activity according to the procedure described by a researcher (Jefferson, 1987). Tissue segments were immersed overnight in assay buffer containing 20% methanol, 500 µM potassium ferrocyanide, 500 µM potassium ferricyanide, 5 mM EDTA, 50 mM sodium phosphate buffer (pH 7), 0.5% triton X- 100 and 20 mM X-Glu. Then treated explants were transferred to 95% ethanol for preservation and the explants were observed for visualization of color products under zoom stereomicroscope.

DNA isolation and PCR analysis

Genomic DNA was collected from transformed and untransformed calli by using the CTAB method. PCR analyses were carried out by using two GUS primers namely; forward 5' CCTGTAGAAACCCCAACCCG 3' and reverse 5' TGGCTGTGACGCACAGTTCA 3' for amplification of GUS gene transformants. The reaction mixture (20 µl) of PCR consists of 1.0 µl DNA template, 2.0 µl 10× buffer, 1.0 µl (2.5 mM) dNTPs, 2.0 µl (25 mM) $MgC1_2$, 1.0 µl of each primer (F/R), 0.4 µl *Taq* DNA polymerase and ddH_2O 13 µl. Reaction procedures were carried out at 94°C for 4 min and followed by 25 cycles at 94°C for 1 min, 56°C for 45 s and 72°C for 1 min. After the final cycle, the reactions were maintained at 72°C for 5 min before completion. Finally, PCR products were analyzed on 1% agarose gel with 0.5× TBE buffer.

RESULTS AND DISCUSSION

Callus induction

Transformation of rice using *Agrobacterium* mediated methods and subsequent regeneration are dependent on several factors such as the choice of explants, hormonal concentrations of the medium used and nutritional supplements highly affects the callusing and regeneration(Lin et al., 1995; Katiyar et al., 1999). A reporter (Hiei et al., 1994) reported that scutellum derived callus was the most amenable explants for *Agrobacterium* mediated transformation. In the present investigation, seeds of elite indica rice cultivar BRRI dhan56 was used as explants for *Agrobacterium* mediated transformation. Calli were produced by using different types of hormonal concentrations of 2, 4-D (1.5 to 3.0 mg/l), NAA (0.5 to 2.0 mg/l), 2, 4-D + NAA (1.5+1.0 to 3.0+1.0 mg/l), 2, 4-D + BAP (1.50+0.10 to 2.50+.50 mg/l) and NAA + BAP (1.0+0.20 to 2.0+1.50 mg/l) were used and 2, 4-D + NAA (2.0+1.0 mg/l) was showed the highest result where 95.22% explants produced calli in BRRI dhan56 (Figure 1A and B). All the recorded data are given in Table 1. With the increase of concentration of 2, 4-D above 2.0 mg/L the callus induction efficiency was reduce in rice cultivar. This indicate that the use of low concentration of 2, 4-D was enough for production of high amount of callus in rice. It was revealed from the results of tissue culture experiments that BRRI dhan56, showing the best callus induction with combinations of 2, 4-D and NAA.

Similar result for the callus induction in rice were also reported by others (Rashid et al., 2003; Islam et al., 2013; Roly et al., 2013, 2014; Islam et al., 2014a; Islam et al., 2014b). After 12 days, calli of BRRI dhan29 was sub cultured in MS medium containing different concentrations of 2, 4-D for development of Embryogenic calli (Table 2). The highest Embryogenic calli (95.22%) showed MS media with 2, 4-D + NAA (2.0+1.0 mg/l). Different days (10, 15, 20, 25 and 30 days) of calli were tested as suitable for transgenic rice BRRI dhan56. For *Agrobacterium*-mediated genetic transformation, twenty days of old calli are suitable for transgenic rice BRRI dhan29 (Figure 1C and D). Regeneration responses of embryogenic calli derived from mature seeds were influenced with the concen-trations and combinations of 2,4-D and NAA present in the regeneration media. Similar results were reported by some researcher (Pandey et al., 1994; Islam et al., 2013) who observed a high regeneration frequency for the medium containing high levels of 2, 4-D and NAA and after a certain levels 2, 4-D and NAA, callus induction frequency decreased.

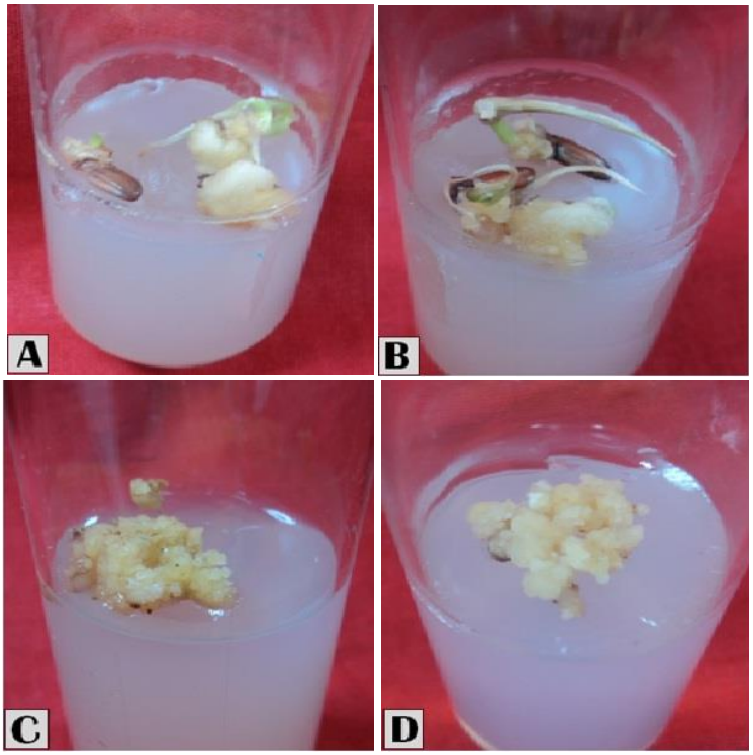

Figure 1. Different types of calli from mature embryo in elite rice cultivar BRRI dhan29: **(A)** and **(B)** Ten days old callus, **(C)** and **(D)** Embryogenic calli derived from mature embryo on callus induction medium.

Table 1. Effects of different hormonal combinations (2, 4-D, NAA, 2, 4-D + NAA, 2, 4-D + BAP and NAA + BAP) for callus formation from zygotic embryo of BRRI dhan56.

PGRs	PGR concentrations (mg/l)	Percentage explants induced calli	Color of calli	Morphological features of calli
2,4-D	1.5	90.66±0.21	Pale yellow	Compact
	2.0	93.44±0.32	Pale yellow	Compact
	2.5	90.66±0.50	Pale yellow	Compact
	3.0	87.59±0.41	Pale yellow	Compact
NAA	0.50	41.75±0.31	Pale yellow	Compact
	1.00	62.86±0.09	Pale yellow	Compact
	1.50	71.84±0.34	Light pale yellow	Compact
	2.00	67.53±0.20	Pale yellow	Compact
2,4-D + NAA	1.5+1.0	91.11±0.12	Pale yellow	Compact
	2.0+1.0	95.22±0.19	Light pale yellow	Compact
	2.5+1.0	93.44±0.31	Pale yellow	Compact
	3.0+1.0	86.89±0.23	Pale yellow	Compact
2,4-D + BAP	1.50 + 0.10	43.12±0.18	Brownish	Friable
	2.00 + 0.10	64.75±0.34	Brownish	Friable
	2.00 + 0.20	74.32±0.52	Brown	Friable
	2.50 + 0.50	66.56±0.37	Brown	Friable
NAA +BAP	1.00 + 0.20	42.98±0.17	Bright yellow	Compact
	1.50 + 0.50	73.78±0.29	Bright yellow	Compact
	1.50 + 1.00	86.65±0.44	Bright yellow	Compact
	2.00 + 1.50	64.54±0.27	Bright yellow	Compact

Table 2. Effects of different hormonal combinations (2, 4-D, NAA, 2, 4-D + NAA, 2, 4-D +BAP and NAA + BAP) on development of embryogenic calli after 12 days of sub culture of BRRI dhan56.

PGRs	PGR concentrations (mg/l)	Percentage of embryogenic calli	Color of calli	Morphological features of calli
2,4-D	1.5	80.67±0.12	Pale yellow	Compact
	2.0	85.45±0.15	Pale yellow	Compact
	2.5	82.66±0.32	Pale yellow	Compact
	3.0	77.59±0.09	Pale yellow	Compact
NAA	0.50	41.86±0.07	Pale yellow	Compact
	1.00	59.43±0.34	Pale yellow	Compact
	1.50	66.53±0.51	Light pale yellow	Compact
	2.00	57.78±0.32	Pale yellow	Compact
2,4-D + NAA	1.5+1.0	82.11±0.31	Pale yellow	Compact
	2.0+1.0	88.22±0.50	Light pale yellow	Compact
	2.5+1.0	83.44±0.21	Pale yellow	Compact
	3.0+1.0	77.89±0.09	Pale yellow	Compact
2,4-D + BAP	1.50 + 0.10	43.90±0.14	Brownish	Friable
	2.00 + 0.10	62.52±0.21	Brownish	Friable
	2.00 + 0.20	71.32±0.44	Brown	Friable
	2.50 + 0.50	66.67±0.32	Brown	Friable
NAA +BAP	1.00 + 0.20	39.96±0.08	Bright yellow	Compact
	1.50 + 0.50	71.07±0.19	Bright yellow	Compact
	1.50 + 1.00	84.32±0.33	Bright yellow	Compact
	2.00 + 1.50	71.08±0.21	Bright yellow	Compact

Genetic transformation of rice callus

In our present investigation, *Agrobacterium* strain LBA4404 harboring the plasmid pBI121 contains kanamycin resistance gene (npt*II*) as the selectable marker gene. Kanamycin was tested as a selective agent for transgenic rice BRRI dhan56. Infected calli from mature seeds of BRRI dhan56 were cultured on shoot induction medium containing various concentrations (50, 75, 100, 125 and 150 mg/l) of kanamycin. Shoot regeneration was greatly inhibited by the increased concentration of kanamycin (Figure 2). The highest response was 32.0% of the explants regenerated shoots in the presence of 100 mg/l kanamycin and there was no shoot regeneration with 150 mg/l or greater concentrations of kanamycin. The effects of different incubation periods (15, 20, 25, 30 and 35 min) 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 32% *GUS* positive result was found with 25 min incubation period. On the other hand, the lowest 10% *GUS* positive result was found with 15 min incubation period (Figure 3). Optical density (OD) of *Agrobacterium* strain is an important factor for genetic transformation in rice. In this study, five

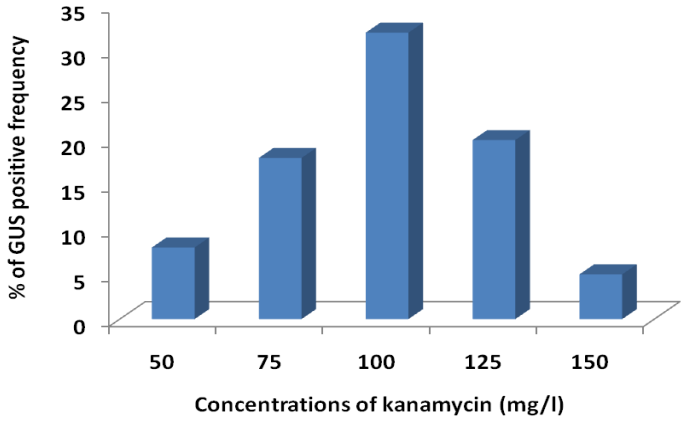

Figure 2. Effect of kanamycin concentrations (50, 75, 100, 125 and 150 mg/l) on shoot regeneration from calli of mature seed of elite rice cultivar BRRI dhan56.

different optical density levels were tested viz., 0.6, 0.8, 0.9, 1.0 and 1.1 (Figure 4). The highest number of GUS expressing calli was observed at OD 0.8 to 0.9. It was clearly confirmed that the bacterial strain LBA4404 (pBI121) showed highest peak of performance in lower

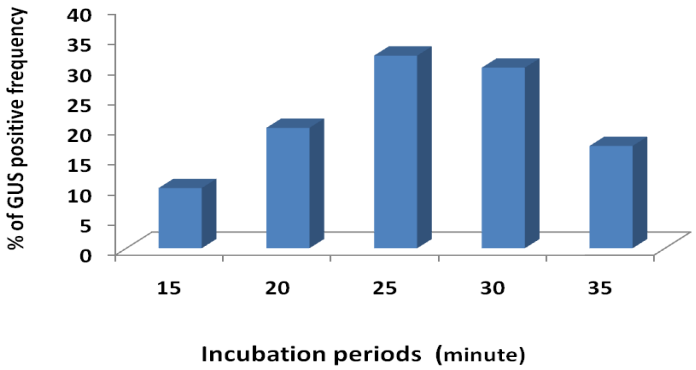

Figure 3. Transformation efficiency (%) of the 3 week old calli of BRRI dhan56 incubation time with *Agrobacterium* strain LBA 4404 which harboring the plasmid pIG121 at 15, 20, 25, 30 and 35 min.

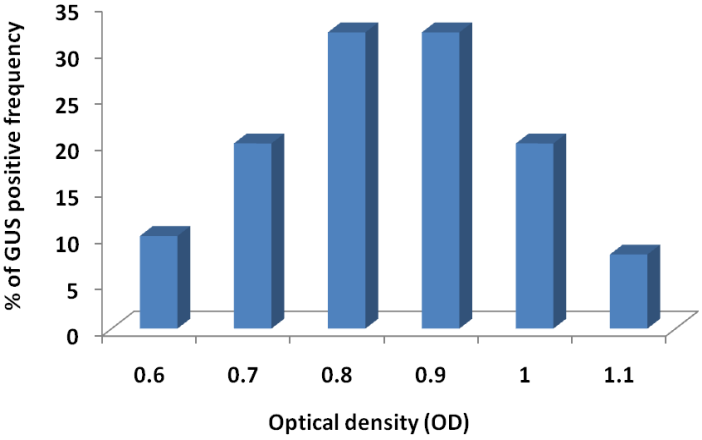

Figure 4. Transformation efficiency (%) of the 3 weeks old calli of BRRI dhan29 co-cultivated with *Agrobacterium* strain LBA 4404 which harboring the plasmid pIG121 at 0.6, 0.8, 0.9, 1.0 and 1.1 OD600.

range of OD and it gradually decreases with the increase of OD taken.

Duration of co-cultivation, the temperature of incubation, with *Agrobacterium*, concentration and the composition of bacteriostatic agent and duration of selection and concentration of antibiotic selection marker are the other important factors reported which affect *Agrobacterium* mediated transformation in rice (Okkels and Pederson, 1998; Katiyar et al., 1999). To find the optimum conditions for co-cultivation, different concentration of acetosyringone and the duration of co-cultivation were tested very carefully. The frequency of kanamycin resistant calli obtained following each variation in co-cultivation conditions was taken as the transformation frequency. For optimum transformation, the presence of 100 µM acetosyringine in co-cultivation medium, 500 mg/L cefotaxime to inhibit *Agrobacterium* growth and co-cultivation for three days were found to be the most suitable.

Stable integration of foreign gene and confirmation

After inoculation with *Agrobacterium,* the explants were co-cultivated for 3 days on normal MS media. The explants were then sub-cultured on regeneration medium (BAP 2.0 mg/l + 1.0 mg/l NAA + 1.5 mg/l KIN) containing carbenicillin. With 2 to 3 weeks of culture, the calli developed shoot buds (Figure 5A and B) and were sub-cultured on the same medium for shoot development (Figure 5C). After proliferation, shoot were transferred on selection medium with kanamycin and the same hormonal combination (Figure 5D) first cycle and second cycle selection. At the same time control plants were subsequently cultured on selection medium first cycle and second cycle selection and 90% shoots died after 21 days. A bacteriostatic agent namely cefotaxime was used to inhibit *Agrobacterium* growth after co-cultivation. It has been recorded that the use of high concentration of bacteriostatic agents may reduce the regenerability of the calli as they structurally resemble auxins. Further, in combination with other callus inducing hormones such as 2, 4 D may cause loss in regeneration potential (Lin et al., 1995; Okkels and Pederson, 1998). Regenerated shoots harvested from selection medium and transfer in the normal MS medium for vigorous root development (Figure 5E) before micro plant transfer in pot for establishment. After six days, a well-developed root system was observed in the plantlets. Then the plantlets transferred to soil survived under the normal environmental conditions and grew to maturity. With the purpose of confirm the presence of the transgene, calli were subjected to histochemical staining. GUS assay was performed according to Jefferson (1987) in two stages, first, After 3 days of co-cultivation in calli and second the leaves and roots from putative plants were taken and incubated in X-*gluc* buffer. Indigo blue coloration was observed in calli (Figure 6A), section of calli (Figure 6B), leaves and root (Figure 6C and 6D). Appearance of blue color following overnight incubation at 37°C with GUS assay revealed the presence of the transgene. GUS gene was amplified in transformed plant DNA and plasmid DNA where forward and reverse primer amplified 880 bp *GUS* gene segment (Figure 7). Compared to the non-transformed calli with regeneration frequency of 96% and transformed calli showed very low regeneration frequency (32%). A researcher (Kumar et al., 2005) recorded a transformation efficiency of 4.6 to 5.5% and 6.4 to 7.3% for two recalcitrant elite *Indica* rice cultivars, which were lower transformation frequencies than these results.

Conclusion

In conclusion, we have established rapid multiple shoot induction and efficient plant regeneration method from seeds elite indica rice cultivar BRRI dhan56 in a genotype independent manner. The combination of 2, 4-

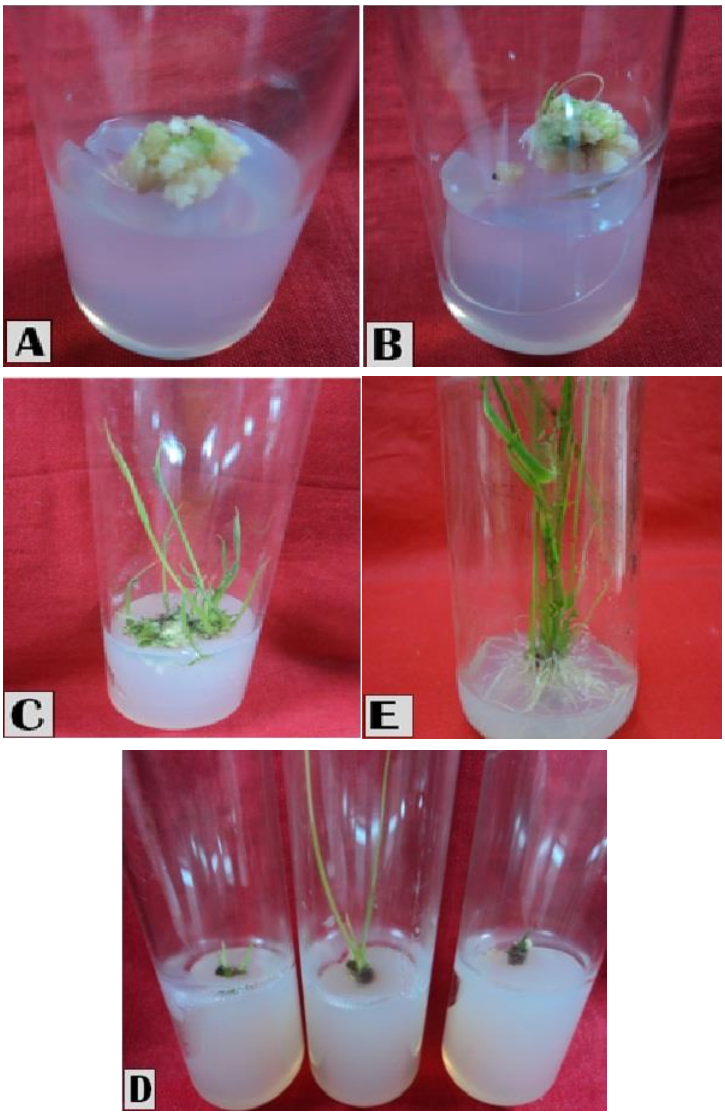

Figure 5. (A) Shoot bud development, **(B)** shoot initiation, **(C)** Shoot elongation, from transform calli in regeneration medium (BAP 2.0 + NAA 1.0 + KIN 1.5) containing 100 mg/l carbencillin. First cycle selection: **(D)** Shoots of transformed calli and **(E)** control in selection medium with 75 mg/l kanamycin.

D + NAA (2.0+1.0 mg/l) was shown to enhance the highest callus induction, where 95.22% explants produced calli in BRRI dhan56. Compared to the non-transformed calli with regeneration frequency of 96% and transformed calli showed very low regeneration frequency (32%). For optimum transformation, the presence of 100 µM acetosyringine in co-cultivation medium, 500 mg/L cefotaxime to inhibit *Agrobacterium* growth, OD_{600} 0.8-0.9 of *Agrobacterium* strain and co-cultivation for 3 days were found to be the most suitable. The method described was simple, inexpensive 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.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to acknowledge rice seeds provided by the Bangladesh Rice Research Institute (BRRI) Regional office, Rajshahi, Bangladesh. The authors are also

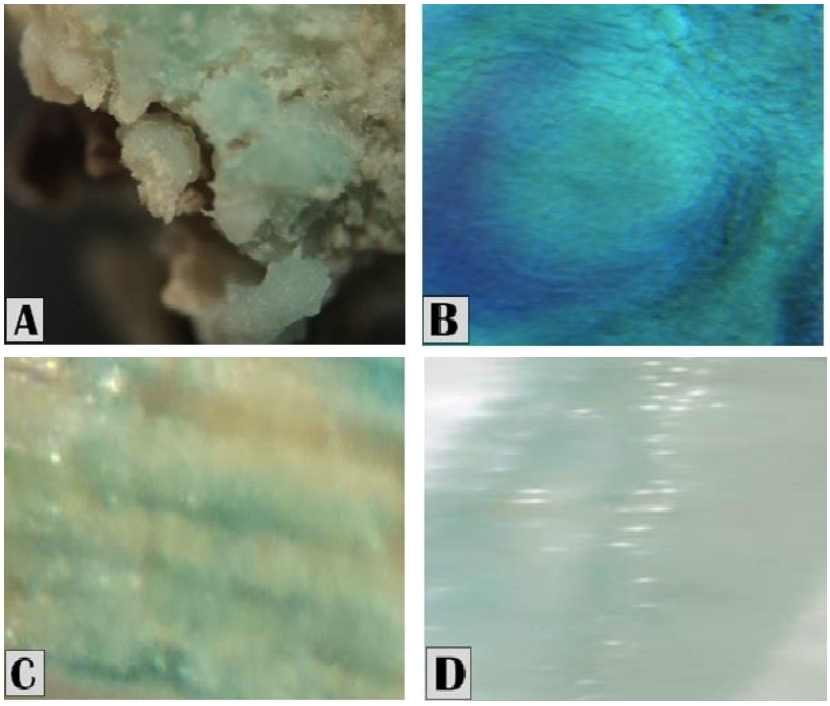

Figure 6. (A) Expression in transformed callus and **(B)** section of transform callus after 3 days of co-cultivation, **(C)** *GUS* expression in leaf segment and **(D)** root segment of putative plant.

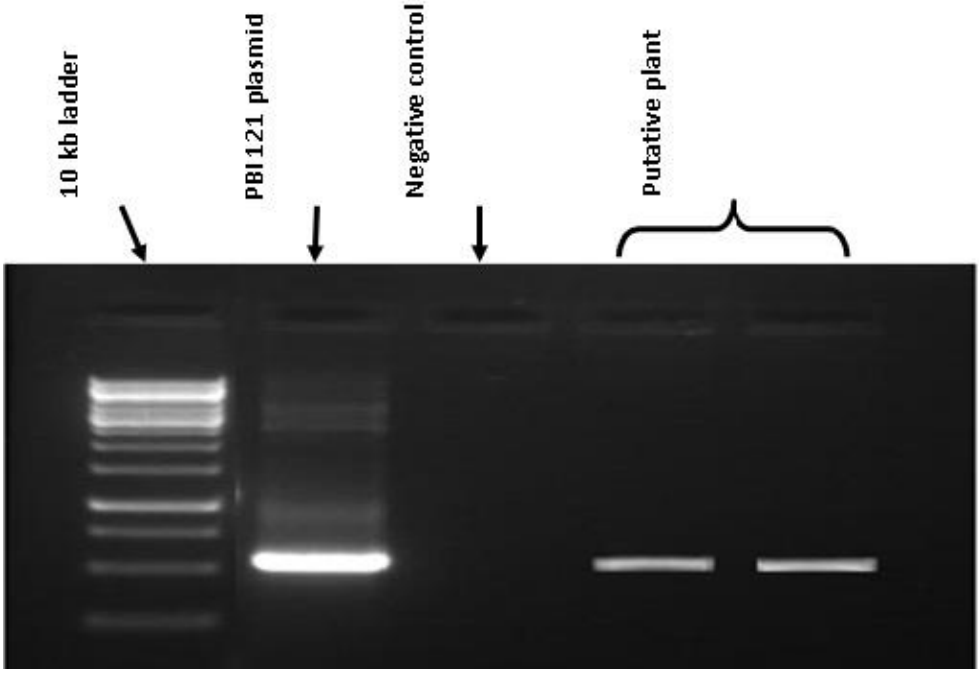

Figure 7. Confirmation of *GUS* gene through PCR analysis.

grateful to DNA and Chromosome Research Laboratory, University of Rajshahi, Rajshahi-6205, Bangladesh.

Abbreviations: GUS, β-Glucuronidase; **PCR,** polymerase chain reaction; **MS,** Murashige and Skoog; **2,**

4-D, 2,4-dichlorophenoxyacetic acid; **MCI,** callus induction medium; **OD,** optical density; **NAA,** 1-naphthaleneacetic acid; **BAP,** 6-benzylaminopurine.

REFERENCES

Abbasi FM, Ahmad H, Perveen F, Inamullah, Sajid M, Brar DS (2010). Assesment of genomic relationship between *Oryza sativa* and *Oryza australiensis*. Afr. J. Biotechnol. 9(12): 1312-1316.

Aldemita RR, Hodges T K (1996). Agrobacterium tumefaciens-mediated transformation of japonica and indica rice varieties. Planta 199(4): 612-617.

Ali S, Xianyin Z, Xue Q, Hassan MJ, Qian H (2007). Investigations for improved genetic transformation mediated by *Agrobacterium tumefaciens* in two rice cultivars. Biotechnology 6:138-147.

Arnold SV, Sabala I, Bozhkov P, Dyachok J, Filonova L (1995). Developmental pathways of somatic embryogenesis. Report, Dept. of Forest Genetics, SLU, Uppsala, Sweden.

Chan MT, Lee TM, Chang HH (1992). Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumifaciens*. Plant Cell Physiol**.** 33: 577-583.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol. 115(3): 971-980.

Cheng XY, Sardana R, Kaplan H, Altosaar I (1998). *Agrobacterium*-trasformed rice plants expressing synthetic cryIA(c) gene are highly toxic to stripped stem borer and yellow stem borer. Proc Nat Acad Sci USA 95: 2767-2772.

Datta K, Potrycus I, Dutta SK (1992). Efficient fertile plant regeneration from protoplasts of the *indica* rice breeding line IR-72 (*Oryza sativa* L.) variety. Aust J Agric Res. 51: 305-312.

Dong J, Teng W, Buchholz W G, Hall TC (1996). Agrobacterium-mediated transformation of Javanica rice. Mol. Breed. 2(3): 267-276.

Hao ZN, Wang J, Wang LP, Tao RX (2009). Influences of the disease resistance conferred by the individual transgenes, Pi-d2, Pi-d3 and Xa21, on the transgenic rice plants in yield and grain quality. Afr. J. Biotechnol. 8:4845–4848.

Hiei Y, Komari T, Kubo T (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol. Biol. 35(1-2): 205-218.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6(2): 271-282.

Ignacimuthu S, Arockiasamy S (2006). *Agrobacterium*-mediated transformation of an elite indica rice for insect resistance. Curr. Sci. 90:829–835

Islam MM, Haque ME, Alam SMM, Islam MA, Khalekuzzaman M, Sikdar B (2013). Morphological and Histological Observation of Embryogenic Calli Derived from Immature Embryo of BRRI Dhan28 (*Oryza sativa* L.) Variety. Res. Plant Biol*.* 3(5): 21-27.

Islam MM, Haque ME, Islam MA, Sikdar B, Khalekuzzaman M (2014a). Establishment of an Efficient Protocol for in Vitro Callus Induction and Regeneration System Using Mature Embryo in Elite Rice (*Oryza sativa* L.) Cultivars. Res. Plant Biol*.* 4(4): 09-20.

Islam MM, Khalekuzzaman M (2015). Development of Transgenic Rice (*Oryza sativa* L.) Plant Using Cadmium Tolerance Gene (*YCFI*) through *Agrobacterium* Mediated Transformation for Phytoremediation*.* Asian J. Agric. Res. 9(4):139-154.

Islam MM, Roly ZY, Lee Y, Khalekuzzaman M (2014b). *In vitro* Propagation and Genetic Transformation System Using Immature Embryo in Elite Rice (Oryza sativa L.) Cultivars. Korean Soc. Breed. Sci. 2 (1): 88-96.

Jefferson RA (1987). Assaying chimeric genes in plants: The *GUS* gene fusion system. Plant Mol. Biol. Rep. 5: 385-405.

Jiang S, Chen CY, Cheng ZK, Cai R, Zhai WX, Zhu LH (2004) Analysis of the transgenic rice plants derived from transformed anther calli. Acta Genet. Sin. 31:1381–1387.

Katiyar SK, Chandel G, Singh P, Pratibha R (1999). Genetic variation and effect of 2,4-D in *in vitro* plant regeneration in *indica* rice cultivars. Oryza 36:254-256.

Khanna HK, Raina SK (2002). Elite indica transgenic rice plants expressing modified Cry1Ac endotoxin of Bacillus thuringiensis show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). Transgenic Res. 11:411–423.

Khush GS (1997). Origin, dispersal, cultivation and variation of rice. Plant Mol. Biol. 35:25–34.

Khush GS, Virk PS (2000). Rice breeding: Achievements and future strategies. Crop Improv. 27: 115-144.

Kumar KK, Maruthasalam S, Loganathan M, Sudhakar D, Balasubramaniam P (2005). An improved *Agrobacterium* mediated transformation protocol for recalcitrant elite *indica* rice cultivars. Plant Mol. Bio. Rep. 23: 67–73.

Larkin PJ, Scrowcroft WR (1981). Somaclonal variation a novel source of variability from cell culture for plant improvement. Theor Appl Genet. 60: 197-214.

Lin JJ, Garcia NA, Kuo J (1995). Plant hormone effect of antibiotics on the transformation efficiency of plant tissues by *Agrobacterium tumefaciens* cells*.* Plant Sci.109: 171-175.

Lu BR (1999). Taxonomy of the genus *Oryza* (*Poaceae*): historical perspective and current status. Int. Rice Res. Notes 24: 4-8.

Nishimura A, Aichi I, Matsuoka M (2007). A protocol for *Agrobacterium*mediated transformation in rice. Nat. Prot. 1: 2796-2802.

Okkels ET, Pederson MG (1998). The toxicity of plant tissue and to *Agrobacterium* of some antibiotics*.* Acta Hortic. 225: 199-207.

Pandey SK, Ramesh B, Gupta PK (1994). Study on effect on genotype and culture medium on callus formation and plant regeneration in rice (*Oryza sativa* L.). Indian J. Genet. Plant Breed. 54: 293-299.

Park SH, Pinson SR, Smith RH (1996). T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol. Biol. 32(6): 1135-1148.

Rashid H, Yokoi S, Triyama K, Hinata K (1996). Transgenic plant production mediated by *Agrohacterium* in *indica* rice. Plant Cell Rep. 15:727-730.

Rashid M, Cheema AA, Ashraf A, Qamar Z , Mahmood Z (2003). Development of basmati rice varieties through the use of induced mutations and related techniques. Pak. J. Bot. 35: 811-818.

Rogers SO, Bendich AJ (1994). Extraction of total cellular DNA from plants algae and fungi, in: S.B. Gelvin, R.A. Schilperoort, D.P.S. Verma (Eds.), Plant Molecular Biology Manual, 2nd ed., Kluwer Academic, Dordrecht, pp. D1/1–D1/8.

Roly ZY, Islam MM, Shaekh MPE, Arman MSI, Shahik SM, Das D, Haamem MME, Khalekuzzaman M (2014). *In vitro* callus induction and regeneration potentiality of aromatic rice (*oryza sativa* l.) cultivars in differential growth regulators. Int. J. Appl. Sci. Biotechnol. 2(2):156-163.

Roly ZY, Mahmudul M, Haque ME, Islam MA, Sikdar B (2013). *In vitro* multiple shoot regeneration from cotyledonary nodes, shoot tips and nodal segments of ridge gourd. Discov. Biotechnol. 4(9): 4-8.

Sasaki T (2005). The maped base sequence of the rice genome. Nature 436:793-800.

Skamnioti P, Gurr SJ (2009). Against the grain: safeguarding rice from rice blast disease. Trends Biotechnol. 27(3):141-150

Supertana P, Shimizu T, Shioiri H, Nogawa M, Nozue M, Kojima M (2005). Development of simple and efficient in planta transformation method for rice (Oryza sativa L.) using Agrobacterium tumefaciens. J Biosci. Bioeng. 100:391-397.

Turk SCJ, Melchers LS, den Dulk-Ras H, Regensburg-Tuink AJG, Hooykaas PJJ (1991). Environmental conditions differentially affect vir gene induction in different Agrobacterium strains. Role of the VirA sensor protein. Plant Mol. Biol. 16(6):1051-1059.

Usami S, Okamoto S, Takebe I, Machida Y (1988). Factor inducing Agrobacterium tumefaciens vir gene expression is present in monocotyledonous plants. Proc. Natl. Acad. Sci. 85(11): 3748-3752.

Vasil IK (1983). Isolation and culture of protoplasts of grasses. Int. Rev. Cytol. Suppl. 16:79-88.

Xu X, Liu X, Ge S, Jensen JD, Hu F, Li X, Dong Y, Gutenkunst RN, Fang L, Huang L, Li J, He W, Zhang G, Zheng X, Zhang F, Li Y, Yu C, Kristiansen K, Zhang X, Wang J, Wright M, McCouch S, Nielsen R, Wang J, Wang W (2012). Resequencing 50 accessions of cultivated and wild rice yields markers for identifying agronomically important genes. Nat. Biotechnol. 30:105-111.