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Agrobacterium-mediated Genetic Transformation of Rice var. BRRI Dhan 58

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Abstract

Agrobacterium mediated genetic transformation of BRRI Dhan 58 was conducted by using immature embryos following indirect regeneration. High percentage of callus induction at 96.5% was obtained when seeds of BRRI dhan 58 were cultured on modified MS medium supplemented with 2.5 mg/l 2, 4-D under dark condition. The maximum regeneration response was rerecorded when MS was supplemented with 3 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l Kn. Genetic transformation was performed using *A. tumefaciens* strain LBA4404 harboring pCAMBIA1301 plasmid carrying the marker genes for β-glucuronidase (*GUS*) and hygromycin resistance (*hptII*). Integration of the *GUS* gene into the genome of the rice plants was confirmed by PCR. The leaf segments of the PCR positive transformed plants showed the expression of *GUS*. The results of this study would be an effective tool for crop improvement and functional studies of gene on rice plant.

Introduction

Genetic transformation by using *Agrobacterium tumefaciens* is a commonly used technique for the transfer of foreign genes into higher plants and mostly used for dicotyledons. As the monocot plants including rice, wheat, corn etc. are not regular hosts to *Agrobacterium,* some alternative methods have developed for the cereal improvement namely rice protoplast transformation (Shimamoto et al. 1989, Datta et al. 1992) and particle bombardment (Christou 1996). The transformation and regeneration of indica rice are highly genotype-specific, tedious and time consuming (Nishimura et al. 2007,

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Sahoo et al. 2011). Some indica rice varieties were improved earlier by *Agrobactrerium*-mediated genetic transformation (Rashid et al. 1996, Khanna and Raina 2002, Ignacimuthu and Arockiasamy 2006) which involved the regeneration of plants from transformed embryogenic calli or anther calli (Jiang et al. 2004, Arnold et al. 1995). There are several other factors such as the addition of acetosyringone, media with 2.4 -D, acidic pH and high osmotic pressure played a significant role for the induction of vir gene (Turk et al. 1991, Usami et al. 1988) during *Agrobactrerium*-mediated transformation of rice. Many reports are available that actively growing embryogenic calli or immature embryos can be infected by *A. tumefaciens* (Hiei et al. 1997). In addition, shoot apices have also been used as gene receiver (Park et al. 1996, Rogers and Bendich 1994) in Japonica cultivar. Considering the importance of genetic transformation in functional genomics it is essential to develop an easy, reproducible and highly efficient transformation and regeneration protocol for indica rice. The present study was undertaken with the objective to develop an efficient plant regeneration system from mature seeds of indica rice (BRRI dhan 58) and to evaluate the possibility to achieve high transformation efficiencies for regular uses.

Materials and Methods

The seeds of Indica rice (*O. sativa* L.) cultivar namely BRRI dhan 58 was collected from Bangladesh Rice Research Institute (BRRI), Gazipur, Bangladesh and used in this study for *in vitro* regeneration and genetic transformation. Mature seeds of BRRI dhan 58 were dehusked carefully and taken in a beaker containing distilled water. After that, Twin-80 (1 or 2 drops) was added and the mixture was shacked for 5 min in a shaker. Seeds were then washed several times with distilled water to remove the influence of Twin-80. The seeds were again surface sterilized twice with 0.1% (w/v) $HgCl_2$ solution for 5 min by vigorous shaking flowed by rinsed with sterile distilled water several times to remove traces of mercuric chloride completely. Treated seeds were then dried on to a filter paper.

The sterilized mature seeds of indica rice cultivar BRRI dhan 58 were incubated in callus induction medium [MS salts and vitamins, proline 65 mg/l, casein hydrolysate 30 mg/l, 2,4-D 2.5 mg/l, BAP 0.15 mg/l, sucrose 30 g/l, phytagel 4 g/l, agarose 2 g/l, pH 5.8] at 26 ± 2°C for 21 days in dark condition. The mature seed derived callus was then sub-cultured in the same callus induction medium at 26 ± 2°C for 7 days and used for *Agrobacterium* mediated genetic transformation. *A. tumefaciens* strain LBA4404 and pCAMBIA1301 were used for genetic transformation. The T-DNA region of the plasmid pCAMBIA1301 carries GUS gene and hygromycin-resistance (*hptII)* gene; both are under the control of CaMV35S promoter. A single PCR positive *Agrobacterium* colony was taken in 5 ml liquid culture of YEM medium and incubated at 28°C over-night in a rotary shaker. One ml of primary culture was inoculated in 100 ml of YEM liquid medium containing Streptomycin 25 mg/l, Rifampicin 10 mg/l and Kanamycin 50 mg/l and again incubated in a rotary shaker at 28°C for over-night. When the optical density (OD_{600}) of

Agrobacterium culture was reached in between 0.6 - 1.0, the culture was centrifuged (10 min at 4,000 rpm at 20°C) and the cells were pellet down. The pellet was again resuspended in 10 - 15 ml (depending on the pellet size) of liquid MS media containing 150 mM acetosyringone (AS).

The mature seed derived calli were then immersed and swirled by hand for 20 min in *Agrobacterium* suspension, then blotted dry on a filter paper finally transferred to co-cultivation media [MS salts and vitamins, proline 65 mg/l, casein hydrolysate 30 mg/l, 2,4-D 2.5 mg/l, BAP 0.15 mg/l, sucrose 30 g/l, Phytagel gL^{-1}, agarose 2 g/l, acetosyringone 150 mM/l, pH 5.8] and incubated at 26 ± 2°C for 48h in dark. After 48h, the infected calli were washed with sterile distilled water containing 300 mg/l cefotaxime and blotted dry on sterile Whatman paper, then transferred to selection medium [MS salts and vitamins, proline 65 mg/l, casein hydrolysate 30 mg/l, 2,4-D 2.5 mg/l, BAP 0.15 mg/l, sucrose 30 g/l, phytagel 4 g/l, agarose 2 g/l, cefotaxime 300 mg/l, pH 5.8.] containing 50 mg/l hygromycin for 15 days. After 15 days of selection, embryogenic calli were separated and transferred to fresh selection medium and incubated at 26 ± 2°C in dark. After two rounds of selection (15 days each), the resistant proliferated calli were transferred to regeneration media [MS salts and vitamins, BAP 3 mg/l, NAA 0.5 mg/l, Kn 1 mg/l, sucrose 30 g/l, agarose 2 g/l, pH 5.8.] containing 40 mgL^{-1} hygromycin and kept in dark for 5 days and transferred to light (16h photo period). The regenerated shoots were transferred to rooting media containing MS salts and vitamins, sucrose 20 g/l, phytagel 4 g/l, glucose 10 g/l, pH 5.8. The rooted plants were then transferred to vermiculite pots for hardening. The hardened plants were transferred to soil pots and kept in green house.

The histochemical assay of *GUS* expression was performed in the leaves of the transformed plants according to the method of Jefferson (1987), using 5-bromo-4-chloro-3-indolyl glucuronidase (X-gluc) as a substrate. The incubation temperature for this assay was 37°C. Genomic DNA was extracted from young leaf tissues of transformed and untransformed rice plants by CTAB method. PCR analysis was carried out to confirm the presence of GUS gene in the transformed plant by using GUS specific forward (ATCACCGAATACGGCGTGGA) and reverse (AGGCTGTAGCCGACGATG) primers. The 50µl reaction mixture contained 1µg DNA, 2mM dNTP mixture, 1 µM each of each of forward and reverse primers and 1 unit of Taq DNA polymerase, and 10× Taq buffer. The condition of the PCR was 98°C for 5 minutes followed by 28 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute. This was followed by one cycle of 10 minutes at 72°C.

Results and Discussion

The swelling of the embryos were observed 2-3 days after culture on MS medium containing different concentration of 2, 4-D (Table 1). It was observed that different concentration of hormone played a vital role for callus induction. The MS medium supplemented with 2.5 mg/l 2, 4-D was found most effective for callus induction for rice

variety BRRI dhan 58. About 96.5 % callus induction was observed in present of 2.5 mg/l 2, 4-D with compact and yellow in color (Table 1).

Table 1. Effect of 2, 4-D on percent callus induction, texture and callus color of rice cv. BRRI dhan 58 cultured on MS under dark condition.

Treatment (MS+ 2, 4-D) (mg/l)	% callus formation (Mean ± SE)	Texture of the callus	Color of the callus
1.5	42.6 ± 3.8	Compact	Pale
2.0	73.3 ± 2.4	Compact	Yellow
2.5	96.5 ± 0.7	Compact	Yellow
3.0	82.2 ± 1.6	Compact	Yellow

To know the effect of optimal hormonal combination on regeneration response the MS medium were supplemented with different hormonal combinations and the results are showed in Table 2. The maximum regeneration response (83.3%) was rerecorded when the medium is supplemented with 3.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kn. The number of shoots per callus was also highest (6.13) in this hormonal combination. Therefore, further *Agrobacterium*-mediated transformants were regenerated in The MS medium containing 3.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kn.

Table 2. Effect of BAP, NAA and kinetin on regeneration response of rice cv. BRRI dhan 58 cultured on MS.

Media formulation (MS + BAP + NAA + Kn) (mg/l)	%±SE of shoot formation	Number of shoot/callus
2.0 + 0.4 + 0.5	67.4 ± 2.30	3.89
3.0 + 0.5 + 1.0	83.3 ± 3.15	6.13
4.0 + 0.6 + 1.5	52.7 ± 3.55	4.20

After infection with *Agrobacterium* the embryogenic calli were co-cultivated for 2 days on normal MS media and sub cultured on selection medium for 15 days containing hygromycin (50 mg/l). After selection, the resistant proliferated calli were transferred to regeneration medium (BAP 3.0 mg/l + 0.5 mg/l NAA + 1.0 mg/l Kn) containing hygromycin (40 mg/l). The regenerated shoots were transferred to rooting media and transferred to vermiculite pots for hardening in confined laboratory condition. The hardened plants were transferred to soil pots and kept in green house (Fig 1a-i).

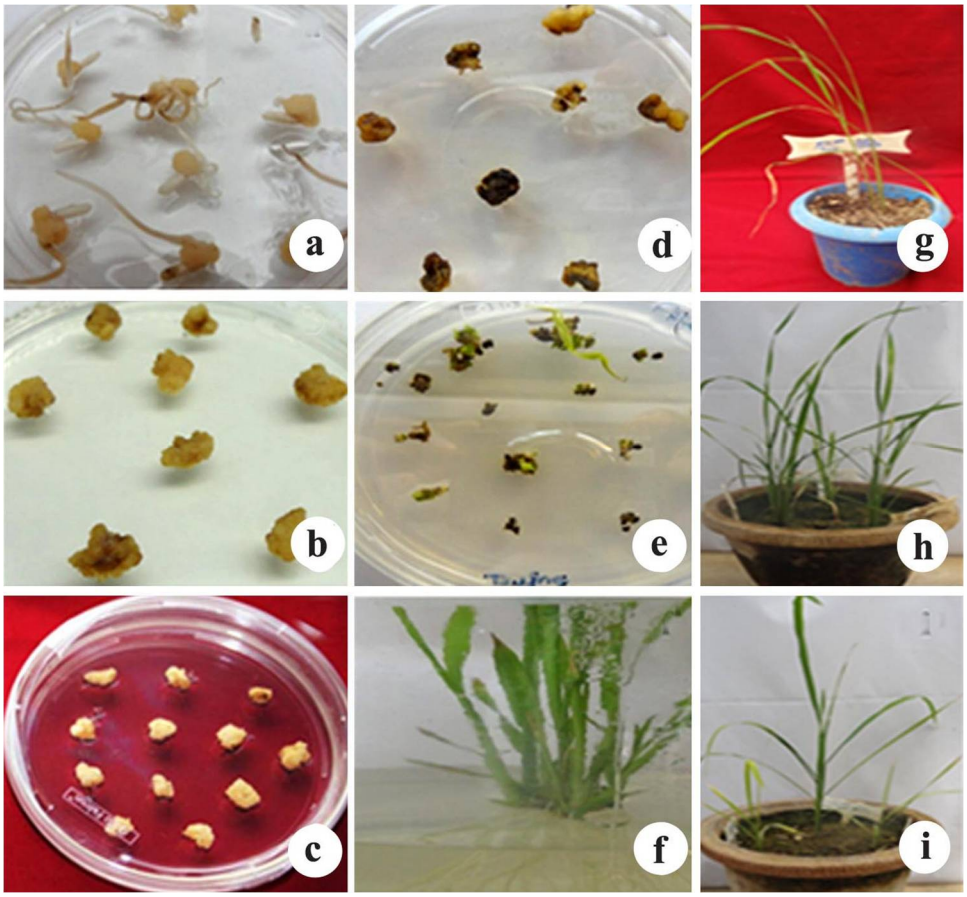

Fig 1. Different stages of plant regeneration of BRRI dhan 58 mature seeds transformed with pCAMBIA1301. a. Mature seeds in callus induction medium for 21 days. b. Calli sub-cultured on fresh callus induction medium for 7 days. c. *Agrobacterium* in infection of mature seeds derived calli. d. Selection of transformed calli on selection medium having hygromycin 50 mg/l. e. The regeration of shoots from the resistant calli in MS regeneration medium. f. Shoots in rooting medium. g. Hardening of plant in vermiculite. H-i. Plants in soil.

After hardening, the transformed plants were confirmed by examining the expression of *GUS* gene. The genomic DNA was isolated from the leaf tissues of the transformed and untransformed plant and PCR analysis was carried out. The GUS expression was observed in the transformed plants while no expression was found in the untransformed plant (Fig 2a). The leaves of some PCR positive and non-transformed plant were also analyzed by the histochemical staining methods [Fig 2b (i-ix)]. The appearance of blue colour confirmed the presence of the GUS gene in the transgenic plants [Fig 2b (ii-ix)]. All primary transformed plants grew normally and produced normal seeds after self-pollination.

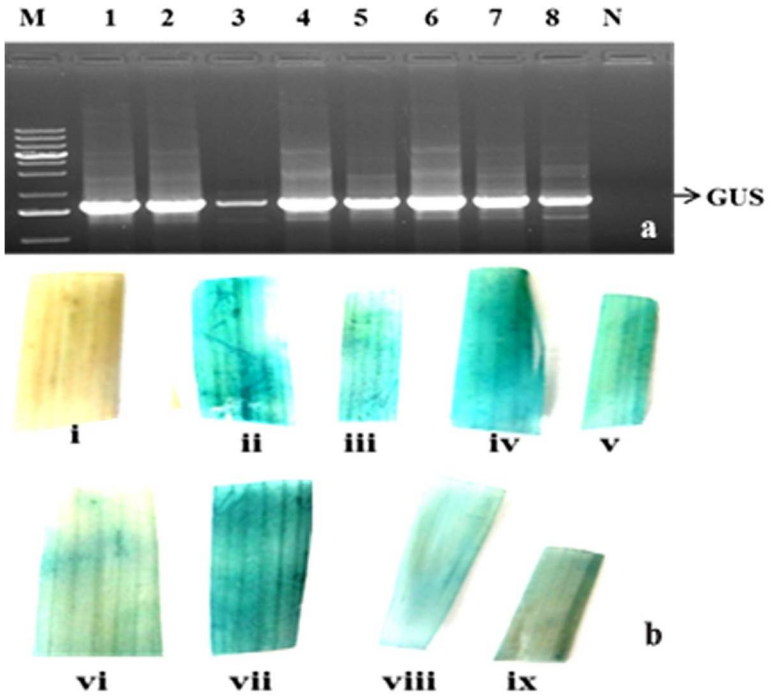

Fig. 2. Confirmation of transgenic status of the rice plant. a. PCR amplification of transgene from genomic DNA. M: Molecular marker, 1-8 numbers is the transformed lines and N: Negative control. b. Level of GUS expression in leaves (i to ix), i: leaves of untransformed plant.

Transformation and regeneration depends on various factors such as explants, hormone concentrations and nutritive supplements etc. (Lin et al. 1995, Katiyar et al. 1999). Plant growth regulators played a significant role in callus cultures. The effect of growth regulators on callus culture and on plant regeneration in rice has been studied (Zhang et al. 1996, Marassi et al. 1996, Pipatpanukul et al. 2004). 2, 4-D is essential in the medium for induction and proliferation of rice callus (Pipatpanukul et al. 2004, Prodhan and Komamine 2007, Islam et al. 2015). In the present study, application of 2.5 mg/l 2, 4-D in the callus induction medium showed best response. In this concentration 96.5% callus induction was recorded. Similar results for the callus induction were previously observed by other scientist in rice plant (Rashid et al. 2003, Islam et al. 2013, Roly et al. 2013, 2014, Islam et al. 2014). In this study, transformed BRRI dhan58 were produced using *Agrobacterium* strain LBA4404 during the transformation experiments. In 2008, Hiei and Komari showed that transformation efficiency also affected by *Agrobacterium* strains. The results of the present study confirmed that seeds and calli of BRRI dhan 58 can be used as the target tissue for *Agrobacterium*-mediated genetic transformation and no need to use the supervirulent strain for rice transformation. Datta et al. (2000) reported rice transformation without using the super virulent strains of *A. tumefaciens*. However, some

studies showed that super virulent *A. tumefaciens* were useful in some rice callus transformation (Hauptmann et al. 1988, Hiei et al. 1994). In this study successful rice transformation was achieved without using vir inducer. Successful transformation of rice without using a vir gene was reported earlier by Raineri et al. (1990). However, the pre-treatment of *A. tumefaciens* with vir inducing compound, particularly acetosyringone and potato suspension, before co-cultivation was necessary for promoting the efficiency of transformation (Park et al. 1996, Godwin et al. 1991, James et al. 1993). Selective agents are needed to avoid off target transformants during transformation. In this experiment, hpt*II* (encoding resistance to hygromycin) was used for the production of transgenic rice. This aminoglycoside antibiotic cause death to plant tissues by inhibiting transcription and translation. Use of 50 mg/l hygromycin throughout the callusing as well as regeneration period was helpful to avoid off target transformants. Chan et al. (1992) reported transformation of *nptII* gene by adding 20 mgL^{-1} kanamycin in the selection media. Cheng et al. (1998) reported the presence of 50 mgL^{-1} hygromycin throughout the callusing as well as regeneration period to avoid development of the escapes. The present results showed, the CaMV35S promoter was useful for rice transformation. High level of gene expression with 35S promoter was also earlier reported (Hiei et al. 1994). There is also report that not CaMV35S, but rice *actin1* might be more effective promoter for driving high expression of gene (Park et al. 1996). Hauptmann et al. (1988) confirmed that the CaMV35S promoter expression was less effective in cereal cells than in dicot cells. In the study, lower efficiency in plant regeneration was observed and this might be due to prolonged period of tissue culture. The calli were three weeks old before co-cultivation and maintained more than four weeks on the selection medium to ensure no escape of non-transformants. Wu et al. (1998) reported the loss of morphogenetic capacity in the established transformed callus and recommended that reduction of the culture period improved plant regeneration efficiency.

This study described the use of *A. tumefaciens* strain LBA4404 and pCAMBIA1301 to transfer screen able and selectable marker genes into BRRI dhan 58. This study confirmed that rice seeds are the suitable target for *Agrobacterium*-mediated transformation. Further, molecular analysis showed that primary transgenic plants have stable integration of transgenes. This method can be used to transform novel genes into other rice cultivars particularly genes conferring abiotic and biotic (disease or pest) resistance.

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