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Agrobacterium-Mediated Transformation of PsCBL and PsCIPK Gene in Indica Rice (Oryza sativa L.)

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Abstract: *CBL and CIPK genes are play a major role during abiotic stresses through Ca^{2+} mediated signaling pathway. This study was aimed to investigate efficient Agrobacterium mediated transformation of the two indica rice cultivars viz., BRRI dhan 47 and BRRI dhan 53, independently with PsCBL and PsCIPK gene of pea (Pisum sativum) to enhance abiotic stress tolerance of the rice cultivars. With different hormonal combination tested, the maximum callus induction 85% and 71% was observed on MS medium supplemented with 2 mg/l 2,4-D, 1 mg/l BA and 0.5 mg/l NAA from the mature seeds of BRRI dhan 47 and BRRI dhan 53 respectively. Three weeks old calli were again preculture in fresh initiation medium. Afterwards precultured calli immersed in Agrobacterium suspension (strain LBA4404, OD600 = 1.0) and co-cultured at 26 ± 2°C in dark for 2 days. The maximum transformation efficiency (54%) was achieved with infection of callus explants for 60 min along with use of 150 µM acetosyringone. The best plant regeneration frequency of BRRI dhan 47 and BRRI dhan 53 was 64% and 74% respectively observed on MS medium supplemented with 1 mg/l BA, 1 mg/l Kinetin, 0.5 mg/l NAA and kanamycin. Root formations of the regenerated shoots were performed in MS basal medium without supplementing any growth regulators. PCR amplification of the GUS gene from isolated DNA of the regenerated plantlets confirmed transformation of PsCBL and PsCIPK gene independently along with GUS gene into the genome of the rice cultivars.*

Keywords: *2,4-D, BRRI dhan, callus, CBL, CIPK*

I. Introduction

Abiotic stress is a serious constraint in agricultural crop production worldwide. Due to climate change, rice plants experience different types of abiotic stresses, like drought, high salinity and low temperature. These stress conditions have a negative impact on rice production. It requires development of abiotic stress tolerant varieties for better productivity or to prevent crop yield reduction. Drought is a world-wide problem that seriously influences grain production. Increasing human population and global climate change make the situation more serious (Hongbo *et al.*, 2005) [1]. Rice, as a paddy field crop, is particularly susceptible to water stress (Tao et al., 2006) [2]. It is estimated that 50% of world rice production is affected by drought (Bouman, *et al.* 2005) [3]. Water deficit is becoming increasingly frequent in irrigated areas due to falling water tables. In total, world rice production uses about 1,578 km^3 of water, which is 30% of the fresh water used worldwide. The international scarcity of water threatens the sustainability of the irrigated rice ecosystem. Of all the cereals, rice (*Oryza sativa*) is most susceptible to damage from water deficit (Lafitte and Bennet, 2003) [4]. Drought and salinity are two of the most complex stress tolerances to breed for, as the type (combinations of heat and drought or sodicity and salinity), timing in relation to plant growth stage and intensity of the stress can all vary considerably (Witcombe *et al.* 2008) [5].

Plant genetic engineering provides an opportunity to incorporate novel resistance traits into rice. In recent years, the successful transformation of rice by Agrobacterium had been reported, although monocotyledonous plants were considered to be outside the host range of *A. tumefaciens*. Insertion of the reporter gene β-glucuronidase (GUS) 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) [6]. Signaling pathways induced by abiotic stresses is very complex as it involves coordinated action of many genes. Most abiotic stresses can elicit an increase of cytosolic free Ca^{2+} concentration in almost all eukaryotic cells and the change of Ca^{2+} concentration has been generally accepted as a second messenger to transduce the cellular responses to extracellular stimuli (Berridge *et al.*, 2003; Hofer and Brown, 2003; Kolukisaoglu *et al.*, 2004) [7, 8, and 9]. So, those proteins recognizing the changes of Ca^{2+} concentration play an important role in signaling. In plant cell, many calcium sensors have been identified which include calmodulin (CaM) and calmodulin-related proteins (Luan *et al.*, 2002) [10], Ca^{2+}-dependent protein kinases (CDPKs) (Pandey *et al.*, 2000) [11] and CBL (calcineurin B-like) protein (Cheong *et al.*, 2003) [12]. Nagamia *et al*. (2007) [13] transformed *katE* gene (a catalase gene) of *Escherichia coli* into indica rice variety Kasalath conferring improved salt tolerance of transgenic rice plants. Enhancing salt tolerance attribute by *katE* was also reported by other authors (Prodhan *et al*., 2008; Motohashi *et al*., 2010) [14 and 15]. Li *et al.*

(2011) [16] found over-expression of *OsTPS1* gene enhances abiotic stress tolerance in rice. Transgenic expression of barley *HvCBF4* gene in rice resulted in an increase in tolerance to drought, high-salinity and low-temperature stresses without stunting growth (Oh *et al*., 2007) [17]. Over-expression of *OsLEA3-1* in rice improved grain yield under drought conditions (Xiao *et al*., 2007) [18]. All of the aforementioned reports were performed through cocultivation of *A. tumefaciens* with calli derived from mature seeds or embryos.

II. Materials and methods

The study was conducted in the Plant Genetic Engineering Laboratory, Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet, Bangladesh. The detail of materials used and analytical methods employed during this study is given below:

2.1 Explant sterilization: Mature seeds of two indica rice cultivars; BRRI dhan 47, BRRI dhan 53 were used as explant materials for callus induction. For these purpose, healthy seeds were dehusked and surface-sterilized with 70% ethanol (v/v) for 2 min, followed by 10 min in 0.1% (v/v) $HgCl_2$ with the addition of two drops of tween 20 and then dried on sterile filter paper.

2.2 Embryogenic callus induction: For callus induction, seeds of two indica rice cultivars are inoculated in test tube on callus induction medium and incubated at 28 ± 1°C in dark. Non-embryogenic calli (compact and non-friable calli that develop root like structures) were discarded and only embryogenic calli (friable calli) were selected. These embryogenic calli were cut into small pieces and sub-cultured onto fresh callus induction medium and kept at 28 ± 1°C in dark.

2.3 *Agrobacterium* strains and vector constructs: Two *Agrobacterium tumefaciens* strains LBA4404 harboring the binary vector pBI121/*PsCBL* and pBI121/*PsCIPK* respectively were used for transformation in this study. Both binary vectors contain *uidA* (*GUS*) gene (Jefferson *et al.,* 1986) [19] encoding - glucuronidase (GUS) and *nptII* gene (Herrera-Estrella *et al.,* 1983) [20] encoding neomycin phosphotransferase II (nptII) conferring kanamycin resistance within the right border (RB) and left border (LB) of T-DNA region of the constructs. But, only pBI121/*PsCBL* contains *PsCBL* gene (Mahajan *et al*., 2006) [21] and pBI12 1/*PsCIPK* vector contains *PsCIPK* gene (Mahajan *et al*., 2006) [21] within the LB and RB. (Figure 2.1 and 2.2)

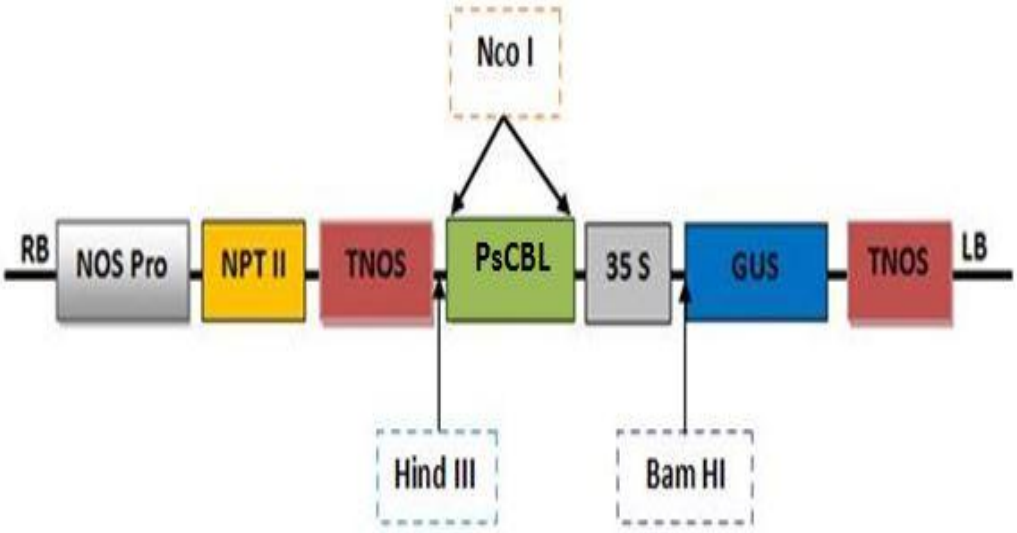


Figure 2.1: Schematic diagram of a part of the T- DNA region of plant expression vector pBI121/*PsCBL*. Abbreviation: RB, right border; *NOS pro*, nopaline synthetase promoter; *NPTII*, neomycin phosphotransferase II encoding gene; *TNOS*, terminator of nopaline synthetase; *PsCBL*, calcinurin B-like protein encoding gene of *Pisum sativum*; *35S*, Cauliflower mosaic virus 35-S promoter; *GUS*, β-glucuronidase encoding gene and LB, left border. *Nco* I, *Hind* III and *Bam* HI are restriction enzymes for the restriction sites respectively.

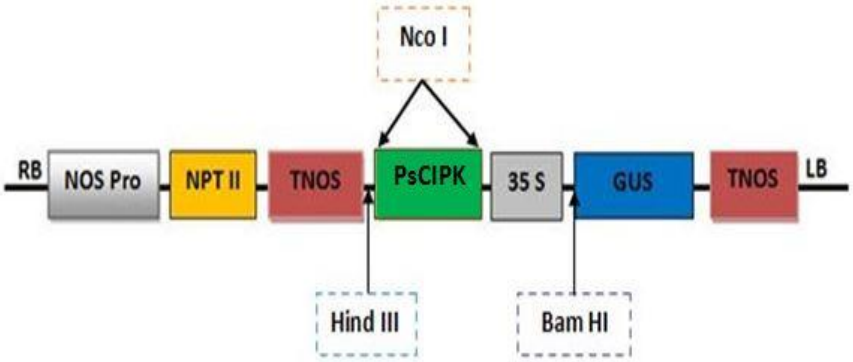


Figure 2.2: Schematic diagram of a part of the T- DNA region of plant expression vector pBI121/*PsCIPK*. Abbreviation: RB, right border; *NOS pro*, nopaline synthetase promoter; *NPTII*, neomycin phosphotransferase II encoding gene; *TNOS*, terminator of nopaline synthetase; *PsCIPK*, CBL interacting protein kinase encoding gene of *P. sativum*; *35S*, Cauliflower mosaic virus 35-S promoter; *GUS*, glucuronidase encoding gene and LB, left border. *Nco* I, *Hind* III and *Bam* HI are restriction enzymes for restriction sites respectively.

2.4 Preparation of *Agrobacterium* culture: The *Agrobacterium* culture was maintained in liquid LB medium supplemented with 50 mg/l kanamycin. The culture was incubated in a shaker at 150 rpm in dark at 30°C. *Agrobacterium* cells were pelleted by centrifugation and when the O.D. 600 reached to approximately 1.0. The MS resuspension media were fortified with various concentration of acetosyringone (100 µM, 150 µM and 200 µM) to increase the efficacy of the infection of *Agrobacterium* to plant cells.

2.5 Cocultivation and selection of transformed calli: Embryogenic calli were subjected to preculture of three days onto fresh callus induction medium. The precultured embryogenic calli were collected and Agro-infected for 30 min and 60 min respectively by immersing them in the *Agrobacterium* resuspended media followed by drying on sterile filter paper for 5 min. Agro-infected calli were then transferred to the cocultivation medium and incubated for 2 days for at 28 ± 1°C in the dark. Once slight growth of *Agrobacterium* appeared around most of the calli. The calli were rinsed 8-10 times with 300 mg/l cefotaxime in sterile distilled water followed by drying on sterile filter paper. The cocultivated calli were then transferred onto first selection medium and incubated for 14 days at 28 ± 1°C in dark and incubated for 10 days at 28 ± 1°C in dark. However, after the first selection, brown or black calli were removed and only creamish healthy calli were shifted to the fresh selection media for second selection and maintained at 28 ± 1°C in dark for 10 days.

2.6 Regeneration of putative transformants: Black or brown microcalli were discarded after second selection and only granular microcalli were transferred onto regeneration media. These microcalli were incubated at 28 ± 1°C in dark and subcultured on the same media at every 7 days of interval until shoots proliferation were started. The regenerated shoots were shifted to the rooting media for root development and maintained at 28 ± 1°C in 16 h photoperiod.

2.7 GUS histochemical assay: The putative transformants were histochemically detected for the presence of *GUS* gene following the procedure of Jefferson *et al*. (1987) [19]. The samples were incubated in GUS assay solution, containing the stocks of 2 mM X-gluc in DMSO, 100 mM tris HCl (pH 7.5), 50 mM NaCl, 2 mM potassium ferricyanide and 0.1 % triton X-100, overnight at 37°C and cleared in 75 % ethanol to clean the tissue prior observation and the GUS activity was recorded as blue spots.

2.8 Polymerase Chain Reaction (PCR): Plants regenerated from GUS positive explants were used for DNA isolation and polymerase chain reaction (PCR) analysis confirmed that GUS positive shoots contained T-DNA. PrimerpairsForward5´-TTTGCAAGTGGTGAATCCCGACCT-3´andReverse5´AGTTTACGCGTTGC TTCCG CC AGT-3´ were used. PCR amplification was done in an oil-free thermal cycler following the PCR profile of 95°C for 2 minutes (initial denaturation) followed by 35 cycles of 1-minute denaturation at 95°C, 1-minute annealing at 54°C and elongation or extension at 68°C for 1 minute. After the last cycle, a final step of 5 minutes at 72°C was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4°C. PCR reactions were performed on each DNA sample with 15 µl reaction mixture containing 10X Buffer 2.5µl, dNTP (2.5mM) 1µl, $MgCl_2$ (50mM) 2µl, Taq Poly (5U/µl) 0.6µl, Forward Primer (4µM) 0.75µl, Reverse Primer (4µM) 0.75µl, Template DNA (20ng/µl) 2.0µl, ddH_2O 5.4µl. After amplification 2 µl loading dye was added to the amplification product for separation using 1% agarose gel

(containing Ethidium Bromide) electrophoresis. Electrophoresis was performed at 100 V for 1 hour. DNA ladder 100 bp (Invitrogen) was run alongside the reactions. Expected amplified DNA fragment was observed on UV trans-illuminator in Gel Documentation system.

III. Results and discussions

In this investigation, two gene constructs (*PsCBL* and *PsCIPK*) along with GUS gene were used for development of abiotic stress tolerant rice through Agrobacterium-mediated genetic transformation in two rice varieties (BRRI dhan 47 dhan and BRRI 53). The results obtained from this investigation are described and discussed under the following heading:

3.1 Optimization of conditions for callus initiation:

The maximum callus induction 85% and 71% respectively was observed on MS medium supplemented with 2 mg/l 2,4-D, 1 mg/l BA and 0.5 mg/l NAA from the mature seeds of BRRI dhan 47 and BRRI dhan 53. In an earlier report, 20 days old calli from mature seeds were used as explants. Here it was improved by using 21 d old calli as explants for quick and efficient transformation.

3.2 Optimization of co-cultivation conditions:

Usually the *Agrobacterium* overgrowth is observed on explants after co-cultivation. The overgrowth in calli kept in co-cultivation media for 48 h was more transformation efficiency as compared with 72 h. Different concentration of cefotaxime (200 mg/l, 250 mg/l and 300 mg/l) was used on selection media to prevent *Agrobacterium* overgrowth. Cefotaxime at 300 mg/l concentration was found to be efficient concentration for preventing bacterial overgrowth.

3.3 Optimization of concentration of acetosyringone:

Different concentrations of acetosyringone were used (100, 150 and 200 mM) on both bacterial suspension and co-cultivation medium with different duration (30 and 60 min). Bacterial infection for 60 min and co-cultivation for 48 h in the presence of 150 mM acetosyringone was found to be suitable for transformation.

3.4 Gus assay:

The assay for GUS gene was carried out for selected calli. The calli were submerged in fixation buffer kept at room temperature for 10 min. The fixation buffer was removed and the material was washed twice with 50 mM sodium phosphate buffer to remove fixative buffer. The tissue samples were stained with 1.5 mM of X-gluc, 50 mM sodium phosphate and 0.1% Triton X-100 and then kept at 37°C overnight in the dark. The calli which showed blue patches were recorded as positive transformants.

3.5 Callus proliferation on selection media:

The media which were supplemented with 300 mg/l cefotaxime along showed total killing of the untransformed explants after two round of selection. In the first round of selection the non-transformed parts of the calli died and became black. In the second round of selection, the transformed calli became more white and proliferated well. The well-proliferated calli were transferred to regeneration medium.

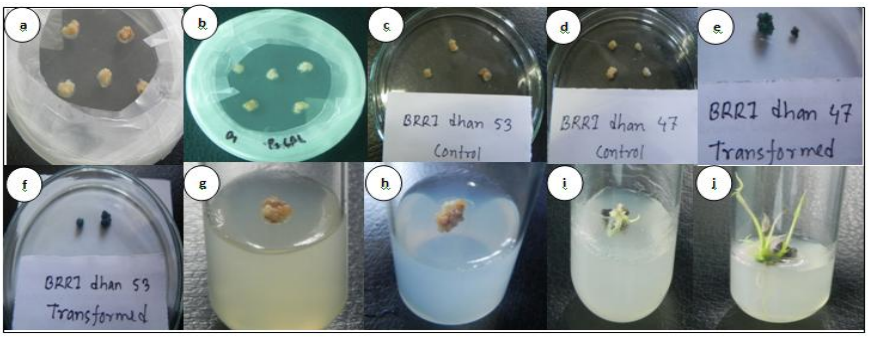
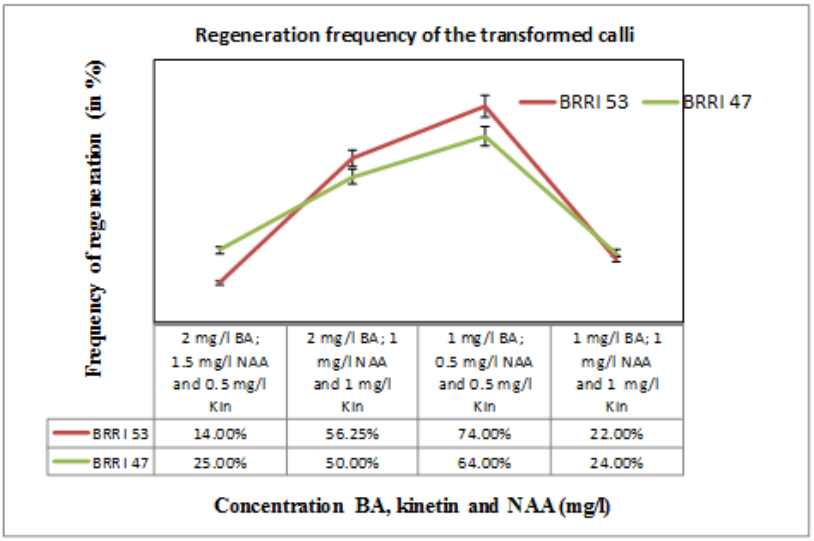


Figure 1: Co-cultivation for Agrobacterium strain (a) LBA4404 (pBI121CIPK) and (b) (pBI121CBL) with two selected cultivars; GUS histochemical assay of the cocultivated calli of the rice cultivars, (c) and (f) control and transformed BRRI dhan 53; (d) and (e), control and transformed BRRI dhan 47; BRRI dhan 53 (g) and BRRI dhan 47 (h) on 50 mg/l kanamycin selection pressure. Plantlets regeneration on selection media from transformed callus infected with LBA4404 (pBI121CIPK) (i) and (pBI121CBL) (j); BRRI dhan 53(i) and BRRI dhan 47(j).

3.6 Regeneration frequency:

To get better plant regeneration from mature seed-derived calli, various combinations of BA, NAA and kinetin were used in the regeneration media. The maximum plant regeneration of BRRI dhan 47 and BRRI dhan 53 was 64% and 74% respectively on MS medium supplemented with 1 mg/l BA, 0.5 mg/l NAA and 0.5 mg/l kinetin along with kanamycin. In case of rooting media, MS medium without any growth regulators produced maximum number of roots. It was observed that with the above conditions the root formation started within 4 days and maximum roots were obtained within 7 days.



	2 mg/l BA; 1.5 mg/l NAA and 0.5 mg/l Kin	2 mg/l BA; 1 mg/l NAA and 1 mg/l Kin	1 mg/l BA; 0.5 mg/l NAA and 0.5 mg/l Kin	1 mg/l BA; 1 mg/l NAA and 1 mg/l Kin
BRRI 53	14.00%	56.25%	74.00%	22.00%
BRRI 47	25.00%	50.00%	64.00%	24.00%

Figure 2: Regeneration frequency of the transformed calli on regeneration media under selective pressure where kanamycin 50 mg/l was used as selective agent.

3.7 Detection of Transformed *GUS* Gene by PCR Analysis:

Genomic DNA was isolated from putative transformed plants regenerated from callus explants of varieties BRRI dhan 47 and BRRI dhan 53 after transformation with both LBA4404 (pBI121CBL) and LBA4404 (pBI121CIPK) to assure the transformation at genomic level. To check the DNA quality, isolated genomic DNA was run on a 1% agarose gel for each sample.The *GUS* gene transformation at genomic level was detected by amplification using of primer. Primer amplifies 600bp DNA segment from *GUS* gene (Gambley *et al.*, 1993) [22]. The DNA isolated from transformed plantlets were produced bands of expected size 600 bp of the *GUS* fragment at the same position respectively. No band was produced in DNA isolated from the nontransformed control. The PCR results are shown in fig. 3.1. Expression of reporter gene at genomic level indicates the transformation of abiotic stress tolerance genes in rice varieties BRRI dhan 47 and BRRI dhan 53 used in the study. In both varieties, selected amplified DNA bands were found against GUS primer. This result confirms the stable transformation of the transgenes and successful integration in the rice genome.

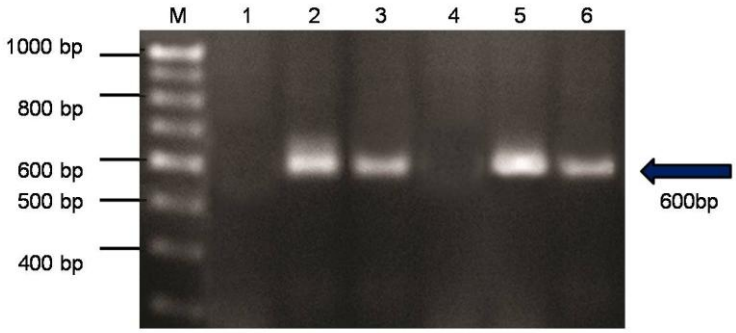


Figure 3: Detection of GUS gene by PCR of isolated genomic DNA from transformed rice varieties. M= Marker 100 bp, Lane 2 and 5, PCR product of transformed variety BRRI dhan 47 and BRRI dhan 53 respectively infected by Agrobacterium strain LBA4404 (pBI121CBL), Lane 3 and 6, PCR product of transformed variety BRRI dhan 47 and BRRI dhan 53, respectively infected by Agrobacterium strain LBA4404 (pBI121CIPK), Lane 1 and 4, PCR product of non-transformed variety BRRI dhan 47 and BRRI dhan 53 (control).

IV. Conclusion

The purpose of this study was to transformation of PsCBL and PsCIPK gene to the BRRI dhan 47 and BRRI dhan 53 cultivars for future studies on improvement of abiotic stress tolerance to increase rice production which is an increasing demand of present world due to the rapid growth of population and environmental changes. In this study, highest callus induction 85% and 71% respectively was observed on MS medium supplemented with 2 mg/l 2,4-D, 1 mg/l BA and 0.5 mg/l NAA from the mature seeds of BRRI dhan 47 and BRRI dhan 53. The embryogenic calli derived from mature seeds of the rice cultivars, therefore, were subjected to preculture of three days for enhancing number of competent cells in the calli for the better transformation. The best plant regeneration frequency of BRRI dhan 47 and BRRI dhan 53 was 64% and 74% respectively observed on MS medium supplemented with 1 mg/l BA, 0.5 mg/l Kinetin, 0.5 mg/l NAA and kanamycin. In both varieties, selected amplified DNA bands were found against GUS primer. This result confirms the stable transformation of the transgenes and successful integration in the rice genome.

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