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Development of Transgenic Rice (*Oryza sativa* L.) Plant Using Cadmium Tolerance Gene (*YCFI*) through *Agrobacterium* Mediated Transformation for Phytoremediation

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ABSTRACT

Cadmium has adverse effects on plant development and ultimately reduces production. The investigation was undertaken for successful gene transfer and phytoremediation through an efficient *Agrobacterium*-mediated genetic transformation method using cadmium tolerance gene (*YCF1*). Embryogenic calli induced after 20 days of highly regenerating rice cultivar BRRI dhan29 and *Agrobacterium* strain GV 3101 was transformed with binary vector pCAMBIA 1303-YCF1 which contained the hygromycin phosphotransferase (*HPT*) gene as a selectable marker and the yeast cadmium factor 1 (*YCF1*) gene, were used for genetic transformation in the experiment. The transformed colonies were selected on 15 mg L^{-1} hygromycin and 50 mg L^{-1} rifampicin to select hygromycin resistant shoots. Hygromycin-resistant shoots were subsequently rooted on root induction medium. Rooted plantlets were transferred to pot-soil, hardened and grown in a greenhouse until maturity and stable integration, expression of *YCF1* gene was also confirmed by using PCR analysis. The maximum transformation efficiency of 22% was obtained using 500 mg L^{-1} cefotaxime as a bacteriostatic agent to inhibit growth of *Agrobacterium* and 100 mM acetosyringone in co-cultivation medium. Southern blot analysis was performed to confirm that transgenes (*HPT* and *YCF1*) were stably integrated into the plant genome. All transgenic plants showed single-copy of transgene integration in the host genome. This transgenic rice plant will uptake cadmium from soil and will protect rice grain from cadmium and store into cell vacuoles of rice plants. As a result soil will be free from cadmium through phytoremediation process.

Key words: *Agrobacterium*, cadmium, *Oryza sativa*, phytoremediation, transformation

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops of Poaceae family cultivated for more than 10,000 years (Sasaki, 2005). Rice consumers are increasing at the rate of 1.8% every year. It is estimated that rice production has to be increased 50% by 2025 (Khush and Virk, 2000). But the rate of growth in rice production has slowed down due to different types of heavy metals such as Cu, Zn and Ni are necessary micronutrients compulsory for a variety of functions including electron transfer reactions and as cofactors in many proteins and enzymes, on the other hand other metals like Ar, Cd and Pb are considered non-necessary. Both types of metals are toxic above certain concentrations. Mainly, heavy metals are toxic because they cause DNA damage and their carcinogenic effects in animals and humans are probably caused by their mutagenic ability

(Knasmuller *et al*., 1998; Baudouin *et al*., 2002; Hooda, 2007). Heavy metals are not degradable without intervention stay in soil for centuries. As a result over recent decades an annual worldwide release of heavy metals reached 22,000 t for cadmium, 939,000 t for copper, 783,000 t for lead and 1,350,000 t for zinc (Singh *et al*., 2003).

The heavy metal cadmium (Cd) is a highly toxic pollutant which, apart from one exception (Lane *et al*., 2005), is not required by living organisms. Environmental contamination with heavy metals, including Cd, is an emerging global problem that severely endangers vegetation, human health and even completes ecosystems (Jarup, 2003). According to the American chemical Society (ACS), Bangladesh has topped the list of 12 countries where high concentrations of cadmium was found in rice samples during a recent study. The ACS study found 0.01 to 0.3 ppm cadmium per kilogram in Bangladesh rice and the quantity. Phytoremediation strategies offer a promising approach towards the remediation of such heavy metal-polluted environments but the plants to be used still require significant improvements in several critical processes, including metal uptake by their roots, translocation from roots to the above-ground tissues and enhanced mechanisms of detoxification (Cobbett and Goldsbrough, 2000; Clemens, 2006).

One suitable tool for plant molecular breeding and genetics is gene transformation by the *Agrobacterium*-mediated gene transfer system (Hiei *et al*., 1997). In contrast to the direct gene delivery systems such as polyethylene glycol (PEG)-mediated protoplast transformation (Peng *et al*., 1992), the electroporation method (Yamamoto *et al*., 1994) and the particle bombardment method (Wakita *et al*., 1998), with the *Agrobacterium*-mediated method it was easy to produce independent transformants in a single series of experiments without complicated manipulations in tissue culture and only one or a few copies of transgenes were integrated into the host genome. Although the *Agrobacterium* mediated method has been used to produce transgenic rice of some cultivars (Hiei *et al*., 1994), the frequency of transformation varies with the plant genotype and with the bacterial strains and vectors, since rice was not originally infected by *Agrobacterium* in the outfield conditions (Hiei *et al*., 1997). Therefore, it is premature to conclude that all cultivars of rice are transformable.

In most recent studies, either immature embryos or calli induced from mature seeds have been used as the starting material for *Agrobacterium*-mediated transformation in rice. Immature embryos seem to be most efficiently transformed; however, mature seeds are more convenient because they can be obtained in quantity and kept viable for a long time on laboratory shelves. There are several reports on rice (*Oryza sativa* L.) transformation with the respect to the introduction of various new traits such as modified oil composition (Das *et al*., 2006), pod shatter-resistance (Ostergaard *et al*., 2006), salt tolerance (Prasad *et al*., 2000), herbicide tolerance (Bisht *et al*., 2004), insect resistance (Cao *et al*., 2008) and heavy metal resistance (Gasic and Korban, 2007; Zhu *et al*., 1999). The aim of this study was to develop cadmium tolerance transgenic rice plant using the yeast cadmium factor 1 (*YCF1*) gene through *Agrobacterium* mediated genetic transformation and to study phytoremediation.

MATERIALS AND METHODS

Seed sterilization: Seeds of rice cultivars BRRI dhan29 were collected from the Regional Research Station of Bangladesh Rice Research Institute (BRRI), Rajshahi, Bangladesh. The seeds were dehusked manually to preserve the embryos from mechanical damage. The dehusked seeds were surface sterilized in 70% ethanol for 1 min and then shaken for 30 min on a gyratory shaker at

200 rpm in 2.6% w/v sodium hypochlorite (50% Clorox) containing 3 drops of Tween 20 per 100 mL Clorox solution. The seeds were rinsed in sterile distilled water and cultured on callus induction media.

Culture media and culture conditions: The Basic Medium (BM) was composed of MS (Murashige and Skoog, 1962) salts and organic compounds, 30 g L^{-1} sucrose and 8 g L^{-1} agar. The pH was adjusted at 5.7 before adding the gelling agent and the media were autoclaved for 20 min at 121°C and 1.07 kg cm^{-2}. Petri dishes with 25 mL of medium and sealed with Parafilm were used.

Callus induction: Ten mature embryos from isolated sterilized seeds were placed individually in each Petri dish containing 25 mL of modified MS with various concentrations of 2, 4-D singly and 2, 4-D with NAA. The seeds were incubated in the dark at 25±2°C. Only embryogenic calli were used for genetic transformation. The percentage (%) of Callus Induction Frequency (CIF) for each group was calculated using the following formula:

$$\text{CIF (\%)} = \frac{\text{Total No. of mature embryo that produced callus}}{\text{Total No. of mature embryo plated}} \times 100$$

Selection of embryogenic callus: Embryogenic callus of indica rice (*Oryza sativa* L.) cultivars namely BRRI dhan29 can be described as yellowish and granular callus, compact, greenish-yellow, granular with smaller cells and very dense cytoplasm callus. These types of embryogenic callus were selected for genetic transformation.

Bacterial strain and plasmid vector construction: The recombinant plasmid vector pCAMBIA 1303-*YCF1* containing hygromycin phosphotransferase (*HPT*) and yeast cadmium factor 1 (*YCF1*) genes was introduced into *Agrobacterium tumefaciens* strain GV 3101 by *Agrobacterium* mediated transformation by heat-shock method. The *HPT* gene confers resistance to the antibiotic hygromycin as plant selection marker and the *YCF1* gene was introduced into the vector as a target gene with the aim of enhancing heavy metal tolerance and accumulation.

***Agrobacterium* strain culture and infection:** *Agrobacterium* strain GV 3101 was cultured on liquid YEP medium containing kanamycin (50 mg L^{-1}), rifampicin (50 mg L^{-1}) and agar (8 g L^{-1}) for 3 days at 27°C in the dark. The bacteria were collected and suspended in medium containing acetosyringone (100, 200, 400, 600 and 1000 ppm). For *Agrobacterium* infection, the density of the bacteria was adjusted (OD_{600} = 1.2, 1.1, 0.9, 0.8 and 0.6) and the rice calli were immersed in a bacterial suspension for 25 min. Excess bacteria were removed by blotting the calli on filter paper. The calli were transferred to Petri dish containing MS medium. The plates were sealed with parafilm to prevent evaporation of the medium and submitted to 3 days of co-cultivation at 25±2°C in the dark. Calli were then washed twice in sterile water to remove *Agrobacterium*. The co-cultured calli were blotted dry on filter paper and plated on MS medium supplemented with rifampicin (50 mg L^{-1}) and hygromycin (0 to 30 mg L^{-1}). The plates were sealed with surgical tape and incubated at 25±2°C using a 16 h light. Proliferating hygromycin resistant calli were

transferred to the same fresh medium. After shoots formation from hygromycin resistant calli and transferred on selection medium. Explants were sub cultured every three weeks followed by harvest of shoots which appeared.

Suitable concentration of selective agent (hygromycin) for transformant selection: To determine the effect of hygromycin concentrations on shoot regeneration, 20 days old were placed on shoot induction medium (MS medium supplemented with 2.0 mg L^{-1} BAP, 1.0 mg L^{-1} NAA and 1.5 mg KIN^{-1}) with hygromycin (0, 5, 10, 15, 20 and 30 mg L^{-1}) in Petri dishes and the cultures were maintained at previously described conditions. The regeneration response was evaluated under the selection conditions after 3 weeks of culture *in vitro*.

Regeneration: Proliferating Hygromycin resistant calli were transferred to the same fresh medium. Additionally, non- infected by *Agrobacterium* embryogenic calli were included as controls. The number of shoots and *in vitro* plants per embryogenic calli were determined after ten weeks of culture on regeneration medium that consisted of MS mineral salts supplemented 2.0 mg L^{-1} 6-benzylaminopurine (BAP), 1.5 mg L^{-1} Naphthalene Acetic Acid (NAA) and 1 mg L^{-1} kinetin, 30 g L^{-1} sucrose and 6 g L^{-1} agar. The explants were cultured in the dark at 26±1°C. The percentage of calli with shoots and regeneration rate were calculated using the following formula:

$$\text{Embryogenic calli with shoots (\%)} = \frac{\text{No. of calli with shoots}}{\text{Total of embryogenic calli}} \times 100$$

$$\text{Regeneration (\%)} = \frac{\text{No. of } \textit{in vitro} \text{ plants}}{\text{Total of embryogenic calli}} \times 100$$

PCR analysis of transformation: Genomic DNA was isolated from transformed shoots. The PCR analyses were carried out by using *YCF1* gene two primers namely, forward 5'TAC CGA GGA ACT TTA GTA GTG3' and reverse 5' TGG CAT CAT AAT AAC TAG TAT 3' for amplification of *YCF1* gene transformants and 5' CAT GTG TAT CACTGG CAA ACT GT 3' (forward) and 5' GTA CTT CTA CACAGC CAT CGG TC 3' (reverse) for the *HPT* gene. The reaction mixture (20 μL) of PCR composed of 1.0 μL DNA template, 2.0 μL 10x 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 TaqDNA polymerase and ddH_2O 13 μL. Reaction procedures were carried out at 94°C for 4 min followed by 25 cycles at 94°C for 1 min, 56°C for 45 sec 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 0.5x TBE buffer.

Southern blot analysis of the regenerated plants: Leaves of the fully-grown putative transgenic rice plants were used to extract total genomic DNA by the methods described by McCouch *et al*. (1988). Southern blot analysis was performed to confirm the stable integration of *YCF1* genes in to the transgenic rice. DNA samples (5 μg) were digested with restriction endonuclease *Eco*RV and then fractioned on 0.8% agarose gels and transferred to a Hybond-N membrane according to manufactures instructions. The 500 bp PCR amplified *HPT* gene and 700 bp PCR amplified *YCF1* gene were labeled with a-32P dCTP using the Rediprime II random

prime abeling system and used as hybridization probes. The probes were labelled with α-32P dCTP using rediprime labelling kit. Membranes were washed twice at room temperature in 2x SSPE/1% Sodium Dodecyle Sulphate (SDS) for 10 min and at 65°C in 1x and 0.1 x SSPE/0.1% SDS for 15 min each time and then autoradiograph

RESULTS AND DISCUSSION

Callus induction: Callus induction of dehusked rice seeds of elite rice cultivar, namely BRRI dhan29 was used in this investigation. Calli are produced by using different types of hormone singly or combination (Fig. 1). The highest induction of callus was recorded in BRRI dhan29 as 92.0% in MS having 2.0 mg L^{-1} 2, 4-D + 1.0 mg L^{-1} NAA among four combinations 2, 4-D and NAA (Fig. 2a) when 80.0% in MS having 2 mg L^{-1} 2, 4-D was used singly (Fig. 2b). With the increase of concentration of 2, 4-D above 2.0 mg L^{-1} the callus induction efficiency was reduce in all cultivars. This indicate that the use of low concentration of 2, 4-D was enough for production of high amount of callus in rice. Similar results in rice were also reported by others (Wang *et al*., 1987; Rashid *et al*., 2003; Roly *et al*., 2014). Callus induction was found more effective in BRRI dhan29 when 2.0 mg L^{-1} of 2, 4-D was supplemented with 0.5 of NAA produce higher amount of callus but higher than single use of 2.0 mg L^{-1} of 2, 4-D. However, required days of callus initiation were decreased. In combination of 2, 4-D with NAA treatment high amount of embryogenic callus produced (Xing *et al*., 1996; Islam *et al*., 2014b). The result is conformity with similar findings reported by some researchers (Sripichitt and Cheewasestatham, 1994; Islam *et al*., 2013) Calli was

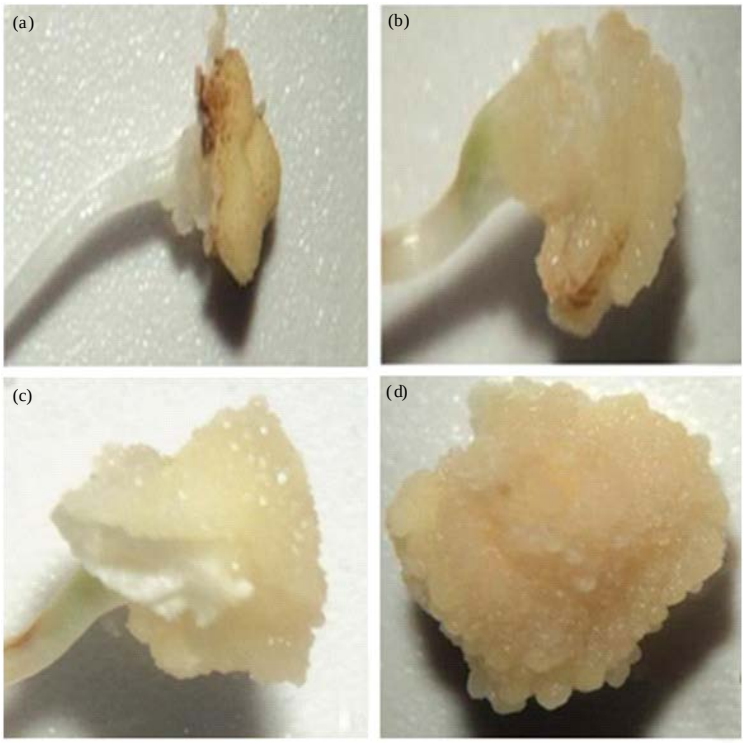

Fig. 1(a-d): Different types of calli from mature embryo in elite rice cultivar BRRI dhan29, (a) Five days old callus, (b-c) Developed proembryogenic callus and (d) Developed embryogenic callus derived from mature embryo on callus induction medium

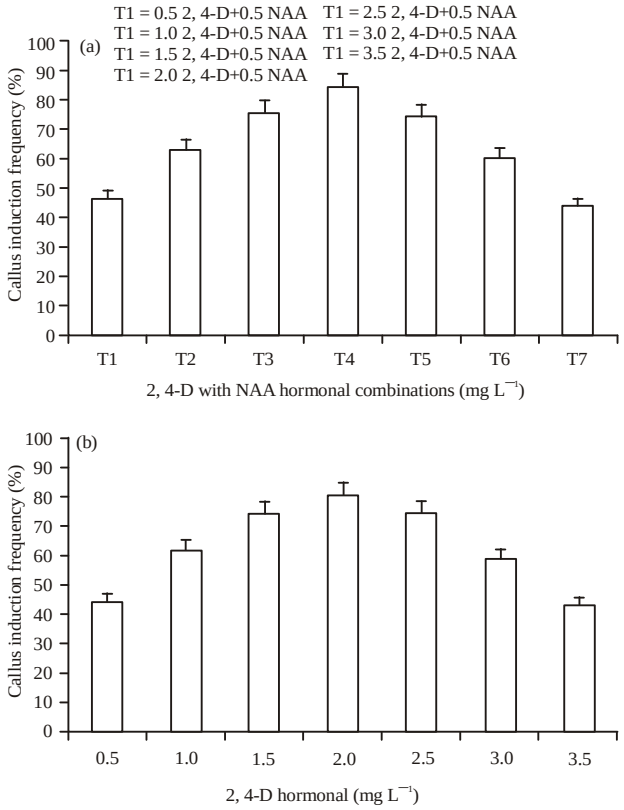

Fig. 2(a-b): Effect of different kinds and concentrations of (a) 2, 4-D with NAA for callus induction frequency (%) from mature embryo of elite rice cultivar BRRI dhan29 using MS medium and (b) 2, 4-D for callus induction frequency (%) from mature embryo of elite rice cultivar BRRI dhan29 using MS medium

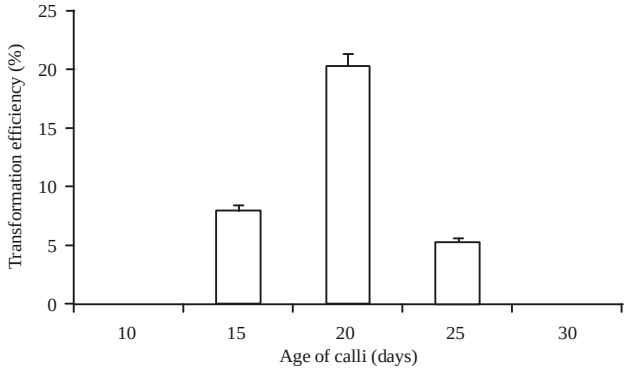

Fig. 3: Transformation efficiency (%) of BRRI dhan29 calli at 10, 15, 20, 25 and 30 days old

developed within 10 days of inoculation. Different days (10, 15, 20, 25 and 30 days) of calli were tested as a suitable for transgenic rice BRRI dhan29. Twenty days of old calli are suitable for transgenic rice BRRI dhan29 (Fig. 3). A reporter also obtained same result in rice (Islam *et al*., 2013, 2014a; Roly *et al*., 2014).

Determination of the optimal hygromycin concentration: Among the various selectable marker genes, hygromycine phosphotransferase is one of the widely used antibiotic resistant marker gene transformation of japonica and indica rice varieties (Zaidi *et al*., 2006; Pipatpanukul *et al*., 2004). The concentration of hygromycine is a crucial factor for the selection of transformed cells because it is amino glycoside antibiotic which cause harmful death to plant cells by inhibiting transcription and translation. At the higher concentration levels of hygromycine the transformed cells could not grow normally and leads to death. An optimized concentration of a suitable selection agent can efficiently inhibit growth of non-transformed tissues and increase the acquisition of transgenic plants. In present investigation, hygromycin was tested as a selective agent for transgenic rice BRRI dhan29. Infected calli from mature seeds of BRRI dhan29 were cultured on shoot induction medium containing various concentrations (0, 5, 10, 15, 20 and 30 mg L^{-1}) of hygromycin. Shoot regeneration was greatly inhibited by the increased concentration of hygromycin (Fig. 4). Only 15.0% of the explants regenerated shoots in the presence of 15 mg L^{-1} hygromycin and there was no shoot regeneration with 20 mg L^{-1} or greater concentrations of hygromycin. Therefore, 15 mg L^{-1} hygromycin was used for the primary selection of transgenic shoots and subsequently was increased to 20 mg L^{-1} for the secondary sub-culturing steps. To eliminate false-transgenic shoots, hygromycin concentrations were further increased to 30 mg L^{-1} for shoot-elongation and root-induction steps. The hygromycine levels was found to be genotype specific and it is first pre requisite for using tissue culture based transformation system (Datta *et al*., 2004). Other workers have reported a range 30 to 100 mg L^{-1} hygromycine for selection of rice transformants (Rafique *et al*., 2010; Kumar *et al*., 2010).

Effects of incubation period on transformation: The effects of different incubation periods (10, 15, 20, 25, 30, 35 and 40 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 30%transformation efficiency was found with 30 min incubation period. On the other hand, the lower transformation efficiency result was found below and above 20 min incubation period (Fig. 5). Some researchers found same result (Islam *et al*., 2014a).

Effect of Optical Density (OD): There are many reports describing *Agrobacterium* mediated transformation using varying concentrations of *Agrobacterium* from 0.5 to 1.5 OD_{600} (Ali *et al*., 2007;

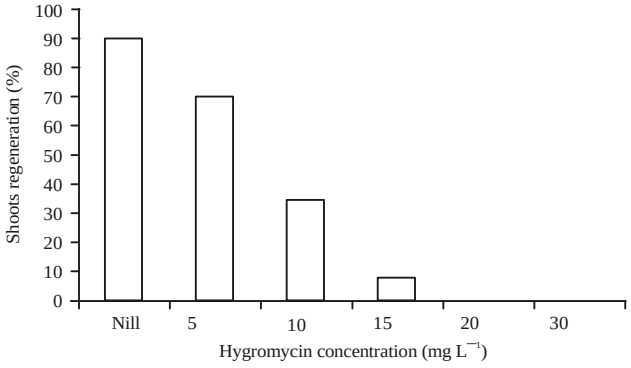

Fig. 4: Effect of hygromycin concentrations (nill, 5, 10, 15, 20 and 30 mg L^{-1}) on shoot regeneration from calli of mature seed of elite rice cultivar BRRI dhan29

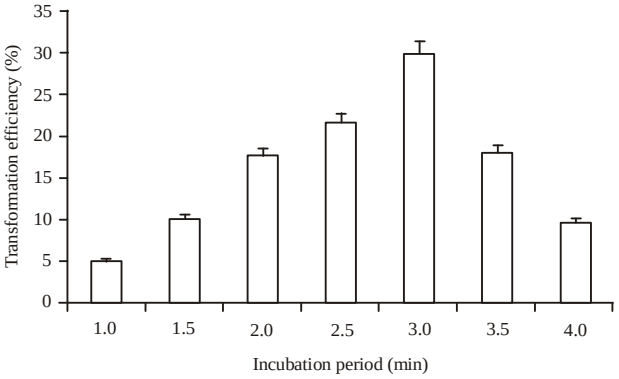

Fig. 5: Transformation efficiency (%) of the 3 week old calli of BRRI dhan29 incubation time with *Agrobacterium* strain GV 3101 harbouring pCAMBIA 1303 plasmid at 10, 15, 20, 25, 30, 35 or 40 min determined by *YCF1* gene expression

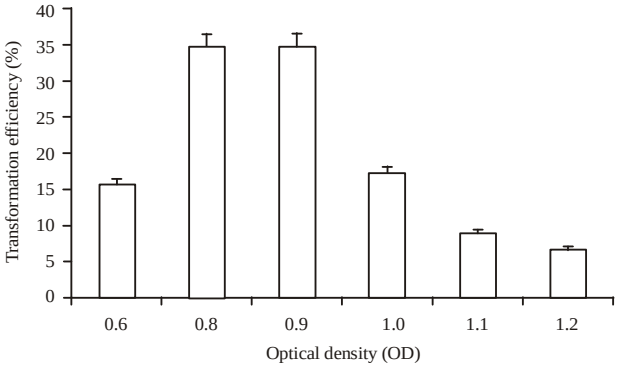

Fig. 6: Transformation efficiency (%) of the 3 weeks old calli of BRRI dhan29 co-cultivated with *Agrobacterium* strain GV 3101 harbouring pCAMBIA 1303 plasmid at 0.6, 0.8, 0.9, 1.0, 1.1 or 1.2 OD_{600} determined by *YCF1* gene expression

Nishimura *et al*., 2007; Hiei and Komari, 2008; Lin *et al*., 2009). The optimum concentration of *Agrobacterium* was determined by co-cultivation of the calli with different concentrations (0.6, 0.8, 1.0 or 1.2 OD_{600}) of *Agrobacterium* for three days. Optical density of *Agrobacterium* is an important factor for genetic transformation. In this study, five different optical density levels were tested viz., 1.2, 1.1, 0.9, 0.8 and 0.6. The highest number of transformation efficiency was observed at 0.8 to 9.0 OD_{600} were significantly higher than those at 1.2, 1.1 and 0.6 OD_{600}, respectively (Fig. 6). It was clearly demonstrated that the bacterial strain GV 3101 showed highest peak of performance in lower range of OD_{600} and it gradually decreases with the increase of OD_{600} taken.

Transformation and proof of stable integration of foreign gene: After inoculation with *Agrobacterium*, the explants were co-cultivated for 3 days on normal MS media (Fig. 7a, b). The explants were then subcultured on regeneration medium (BAP 2.0 mg L^{-1} +1.0 mg L^{-1} NAA+1.5 mg L^{-1} KIN) containing carbenicillin. With 2-3 weeks of culture, the calli developed

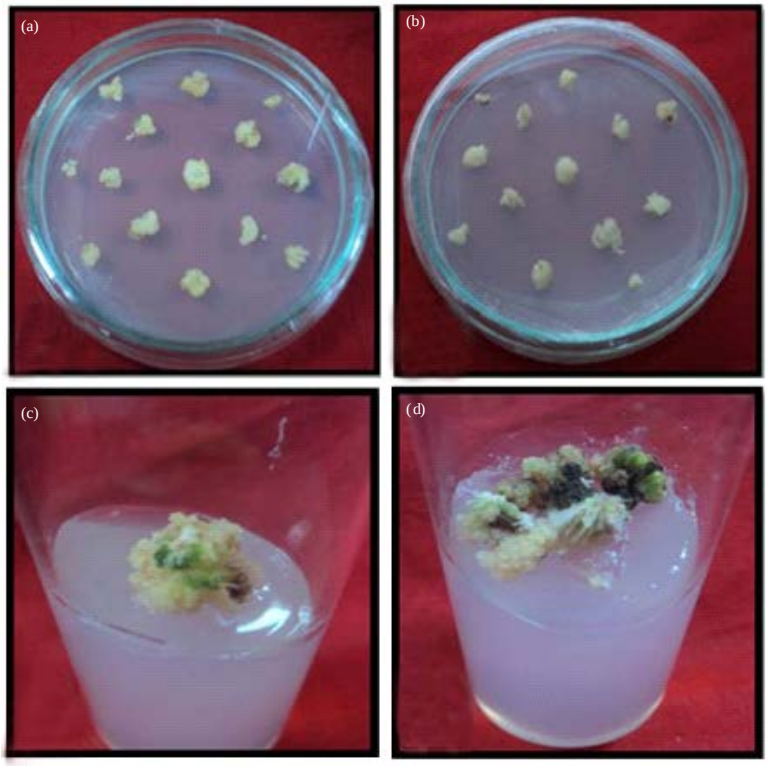

Fig. 7(a-d): (a and b) Infected calli of BRRI dhan29 cultivar in co-cultivation medium and (c and d) Shoot bud development in regeneration medium (BAP 2.0+NAA 1.0+KIN 1.5) containing 100 mg L^{-1} carbenicillin

shoot buds (Fig. 7c, d) and were sub-cultured on the same medium for shoot development (Fig. 8a, b). This positive effect of delayed exposure of the explants to selective agent recommended that delayed selection permitted the division of transformed cells which conferred greater protection against the selective agent. Visser *et al.* (1989) also reported that delayed application of the selective agent in potato enhanced transformation efficiency significantly. We projected that this strategy could be useful for plant species that tend to regenerate quickly under tissue culture conditions. To enhance the transformation efficiency, we adopted a three-stage hygromycin selection process. Initially, we exposed the explants to a stumpy concentration of hygromycin (5 mg L^{-1}) and then, in the next sub-culturing step in shoot-induction medium, the hygromycin level was increased. The initial low levels of hygromycin would potentially allow shoot regeneration of both transformed and untransformed explants. However, the higher concentration of hygromycin (0-30 mg L^{-1}) in subsequent steps retarded the division of untransformed cells and increased the division of transformed cells. After proliferation, shoot were transferred on selection medium with kanamycin and hygromycin and the same hormonal combination first cycle and second cycle selection (Fig. 9c, d), respectively. At the same time control plants were subsequently cultured on selection medium first cycle and second cycle selection and 90% shoots died after 25 days. Regenerated shoots harvested from selection medium and transfer in the normal MS medium for root induction (Fig. 9a) and micro plant transfer in pot for establishment (Fig. 9b).

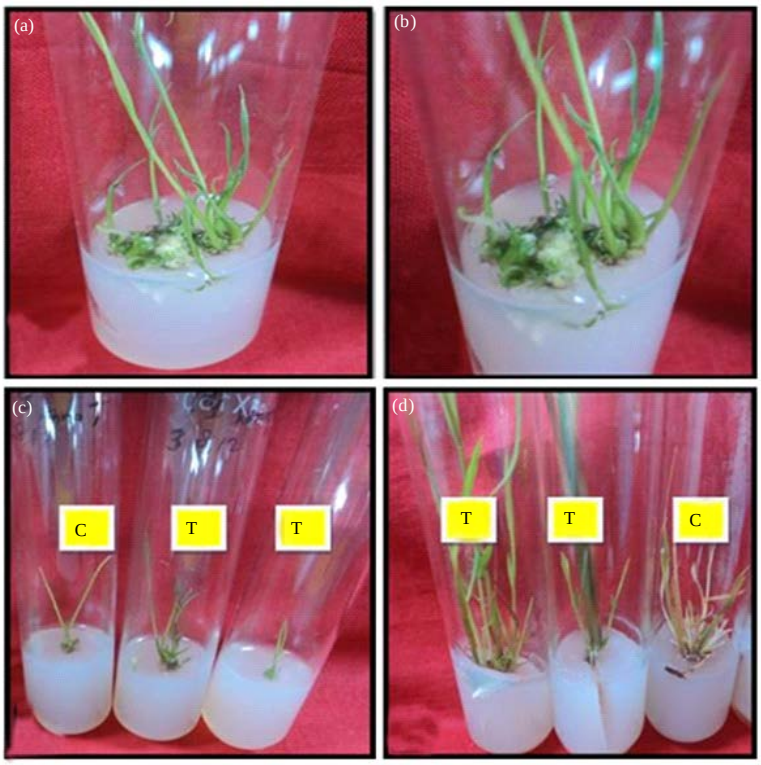

Fig. 8(a-d): (a and b) Shoot initiation and shoot elongation from transform calli in regeneration medium (BAP 2.0 + NAA 1.0 +KIN 1.5) containing 100 mg L^{-1} carbenicillin, (c) First cycle selection; shoots of transformed calli and control in selection medium with 50 mg L^{-1} kanamycin and 10 mg L^{-1} hygromycin and (d) Second cycle selection; subcultured of shoots after first cycle selection in medium with 100 mg L^{-1} kanamycin and 15 mg L^{-1} hygromycin.; transformed shoots and control (C = control), (T = transformed)

Table 1: Effect of delayed exposure the explants to selective agent (15 mg L^{-1} hygromycin) on transformation efficiency of rice cultivar BRRI dhan29

Period (day)	Total No. of explants tested	No. of explants regenerated shoot	Transformation efficiency (%)
0	60	0	
1	72	0	0.00
2	80	3	5.60
3	99	34	22.00
4	66	4	7.78
5	80	1	2.20

Longer period of co-cultivation (more than 3 days) proved to be detrimental as the plant tissue died resulting in no or transgenic shoots. An interesting result found in this study was that delaying the transfer of explants into selection media after co-cultivation until certain periods (2 to 5 days) dramatically enhanced the transformation efficiency (Table 1). A total of 34 putative transgenic shoots (transformation efficiency of 22%) were obtained. Putative transformants were transferred to soil and acclimatized for 5 days at controlled environment and subsequently transferred to the greenhouse.

Fig. 9(a-d): (a) Root induction in selection medium, (b) Establishment of regenerated plants on soil, (c) Tillering stage of transgenic rice plant and (d) Ripening stage of transgenic rice plant

Confirmation of the presence of transgene by PCR and Southern blot analyses: All hygromycin-resistant plants were subjected to PCR analysis with the primers specific for *YCF1* and *HPT* genes to confirm the insertion of transgenes into the BRRI dhan29 host genome. Expectedly, all transgenic lines showed 645-bp band representing the *YCF1* fragment and a 501 bp band representing the *HPT* fragment (Fig. 10a). No PCR band was observed in the control. Transgenic plants obtained by *Agrobacterium* mediated transformation could have been contaminated by latent *Agrobacteria* even with the use of high concentrations of *Agrobacterium*-eliminating antibiotics (Shackelford and Chlan, 1996; Ogawa and Mii, 2007). The presence of *Agrobacterium* in plant tissues often leads to misleading results including environmental hazards. Therefore, getting transgenic plants without *Agrobacterium*-contamination are highly desirable. The PCR is the most commonly used technique to monitor putative transformants whether they contain *Agrobacterium*. Therefore, we tried to check transgenic lines by PCR using *Agrobacterium*-specific primers. All transgenic lines were free of *Agrobacterium* contamination as they did not show any bands by primer specific PCR reaction. Randomly selected four PCR-positive plants were subjected to Southern blot analysis using *YCF1* and *HPT* probes to confirm the integration of the transgene and estimating the transgene copy number into the BRRI dhan29 genome (Fig. 10b). Fascinatingly, all transgenic lines showed a single locus for *HPT* and *YCF1* gene integration in the host genome which is considered most desirable for any *Agrobacterium* mediated genetic transformation. Although transgenic lines were obtained from independent transformation events, some of these

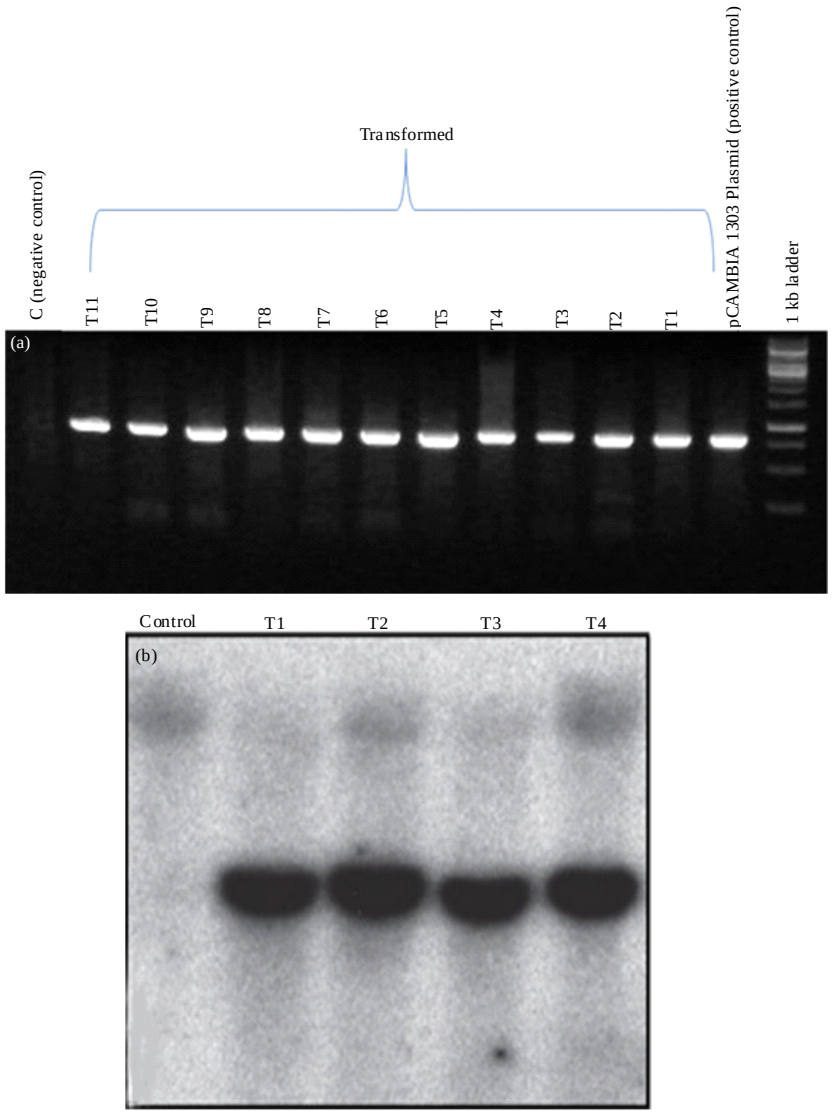

Fig. 10(a-b): Confirmation of *YCF1* gene expression (a) Confirmation of the introduction of *YCF1* gene through PCR and (b) Southern blot of EcoRV digested total DNA probed with *YCF1* gene (T = transgenic line)

lines (particularly T1, T2, T3 and T4) showed similar sizes of bands. Some researchers (Babic *et al*., 1998; Leelavathi *et al*., 2004) also reported alike findings of same size bands of transgenes in different transgenic plants in Southern blot analysis, even transgenic plants were obtained from different transformation events of *Agrobacterium*-mediated genetic transformation of *B. carinata* and *Gossypium hirsutum*.

Segregation of T1 progeny: The putative transgenic plants were grown in an environmentally-controlled greenhouse until seed setting. The T1 seeds were collected and grown on 1/2MS agar medium containing 15 mg L^{-1} hygromycin. In the presence of hygromycin, these

Table 2: Segregation of hygromycin-resistant and sensitive plants in the self-pollinated T1 progeny of transformed elite rice cultivar BRRI dhan29 with the expected Mendelian ratio of 3:1

		No. of seedling	
Plant line	No. of seeds tested	Resistant	Sensitive
C (control)	60	0	60
YCF1-1	72	50	22
YCF1-2	70	48	22
YCF1-3	65	45	20
YCF1-4	69	46	23
YCF1-5	70	47	23

seeds showed initiation of germination by the fourth but subsequently day, turned yellow and dried up, whereas the transformed seeds continued to develop as green seedlings. All transgenic lines presented 3:1 Mendelian ratio on hygromycin containing media (Table 2) which further demonstrated that a single copy of T-DNA was integrated in all transgenic plants. In the present transformation protocol using mature embryos, we got relatively high (22%) transformation efficiencies as compared with previous reports. This high-through put transformation method should facilitate the use of this plant species for studies in gene manipulation and expression. We are currently using the transgenic the BRRI dhan29 lines for heavy metal phytoremediation purposes. A researcher has got same results in *Brassica juncea* (Bhuiyan *et al*., 2011).

CONCLUSION

By using *Agrobacterium tumefaciens* strain GV 3101 carrying plasmid pCAMBIA 1303 harbouring *YCF1* gene, obtained several transgenic rice lines of elite rice cultivar BRRI dhan29. They were highly resistant to heavy metals especially cadmium. The presence and expression of the *YCF1* genes were confirmed by qualitative and molecular analyses. The obtained transgenic lines could be useful for phytoremediation and rice breeding in the world.

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