



Research Article

Standardization of *Agrobacterium* mediated genetic transformation in *Indica* rice cv BPT-5204

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Abstract: The BPT-5204 genotype from *Indica* rice cultivar (*cv*) is recalcitrant and showed low transformation frequency compare to *japonica* rice *cv*. Here we have optimized the efficient transformation protocol to minimize the time scale and enhance the transformation frequency by altering the key parameters like, acetosyringone (AS) concentration, optical density of bacterial culture and co-cultivation time. Highly proliferated 21 days old Scutellum derived embryogenic calli were infected with *Agrobacterium tumefaciens* harbouring pCAMBIA2300-*Ds*-*En-Bar* binary vector. T-DNA contains tetrameric CaMV35S enhancer elements along with *bar* gene which embedded between the transposable *Ds* element. At 0.5 OD_{600nm} of *Agrobacterium* culture and co-cultivation for 2 days on MS co-cultivation medium containing 100 µM acetosyringone proved to be optimal and attained 6.2 % of transformation efficiency. The transformed calli and regenerated plantlets were proliferated on Murashige and Skoog (MS) medium containing phosphinothricin (PPT). Polymerase chain reaction (PCR) confirmed that intact T-DNA was successfully integrated in the rice genome. This protocol can be employed to develop transgenic rice plants with gain of functional mutagenesis.

Keywords: *Agrobacterium tumefaciens*; BPT-5204; Embryogenic calli; Activation tagging

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crop and a primary food source for half of the world's population. The increasing demand for rice provide chances for biotechnologists to develop efficient, quick and reproducible transformation protocols to meet the demand. Major constraints in rice production are biotic and abiotic stress causing significant yield losses up to 10 and 30% in rice growing countries (Krishnaiah and Varma 2012). Genetic engineering is alternative to overcome these problems associated with narrow genetic base and tissue culture is the pre requisite event for generation of transgenic plants. More sustained production of rice is essential which in turn requires an efficient protocol for its regeneration and transformation. Various gene transfer protocols were developed for rice among them *Agrobacterium tumefaciens* genetic transformation is a preferred method due to stable and transfer of larger DNA segments and low integration copy number of transfer-DNA (T-DNA) into the plant genome (Gelvin 2000). Different types of explants are used for transformation, such as mature seed derived calli, immature embryo-derived calli, leaf base derived calli and shoot apex. Among these explants, calli derived from mature seeds showed high transformation efficiency (Hiei and Komari 2008; Rance *et al.*, 1994; Manimaran *et al.*, 2013). In rice, many factors are influencing the efficiency of

T-DNA delivery to plant cell such as type of explants, optical density of *Agrobacterium*, infection time, co-cultivation medium with acetosyringone for *vir* gene induction. An excessive number of bacteria can stress plant cells and affect their regeneration potential, whereas low concentration can reduce the frequency of T-DNA transfer (Fitch *et al.*, 2011; Zhao *et al.*, 2010). In general, co-cultivation time of 2-3 days is standard for most transformation systems (Mourgues *et al.*, 1996; Cervera *et al.*, 1998). Phenolic compounds like acetosyringone are recommended for enhancing plant genetic transformation (Kumlehn *et al.*, 2006; Kavitha *et al.*, 2010). Rice became a model crop for genetic and functional genomic studies among monocots (Jung *et al.*, 2008). In recent years, considerable progress has been made in the improvement of important agronomic traits of rice through biotechnological approaches (Hao *et al.*, 2009; Skamnioti and Gurr 2009). Insertional mutagenesis is one of the most useful methods for analyzing gene function (Upadhyaya *et al.*, 2011). Activation tagging has become an important tool in targeted improvement and gene function studies of *indica* rice (Manimaran *et al.*, 2017). Hence, the present investigation was undertaken with the objective of developing an efficient *Agrobacterium*-mediated genetic transformation method and subsequent transgenic plant recovery system using

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embryogenic calli of *Indica* rice cv BPT-5204. Various factors influencing the transformation frequency such as optical density (OD), co-cultivation duration and acetosyringone concentration were evaluated.

Materials and Methods

Plant material and methods

Embryogenic calli derived from mature rice seeds of *Oryza sativa* L. subsp. *Indica* cv BPT-5204. Surface sterilization of dehusked seeds with autoclaved double distilled water for four times for 1 min each followed by ethanol (70%) for 2 min, mercuric chloride (0.1%) for 3-4 min and then washed with double distilled water for three times. These seeds were dried on sterile filter paper.

Agrobacterium strain and plasmid construct

Agrobacterium tumefaciens EHA105 harbouring the binary vector pCAMBIA2300-Ds-En-Bar. The T-DNA region contains the 4XCaMV35S enhancer elements transcriptional activation of flanking plant genes, *Bar* gene under control of CaMV35S promoter were embedded in between transposable element Ds 5' to 3' (Fig. 1). Culture was maintained in glycerol stocks at -80°C.



Fig. 1 Linear map of the gene construct pCAMBIA2300-5'-Ds-En-Bar-Ds-3'. 35S: CaMV35S promoter, 3' and 5'Ds: Ds element, 4xEn: Four copies of CaMV35S enhancer, Bar: Bar gene cassette, The T-DNA is in the backbone of pCAMBIA 2300

Agrobacterium mediated genetic transformation

Surface sterilized seeds were inoculated on to the callus induction (CI) medium (Fig 2 A) [Murashige and skoog (MS) salts supplemented with 2,4-D 2.5 mg/L, kinetin 0.5 mg/L, proline 500 mg/L, casein enzymatic Hydrolysate 500 mg/L and maltose 30 g/L, pH of the medium was adjusted to 5.8] solidified with 0.4% phytigel and autoclaved at 121°C for 15 min at 15psi pressure. The calli derived from Scutellum region of the rice seeds were subculture and incubated on fresh CI medium for 5 days (Fig. 2B).

Preparation of primary *Agrobacterium* culture

Agrobacterium culture was prepared by inoculating single colony from a freshly streaked plate, in a 250 ml baffled conical flask containing 50 ml of autoclaved liquid YEB medium (5 g/L Beaf extract, 1 g/L yeast extract, 5 g/L peptone and 5 g/L sucrose and 1.5% agar, pH 7.0) supplemented with rifampicin 10 mg/L, kanamycin 50 mg/L. The culture was incubated at 220 rpm in dark at 28°C varying optical density (OD_{600nm}) between 0.1 to 0.7 and these *Agrobacterium* cells were pelleted by centrifugation at 3100 rpm for 15 min at 28°C.

Agrobacterium infection and selection of transformed calli

The harvested *Agrobacterium* cells were resuspended in MS resuspension liquid (MSL) medium containing 50 to 200 μM of acetosyringone (sigma-Aldrich, St Louis, USA) (MS basal salts, 68 g/L sucrose, 36 g/L glucose, 100mg/L myo-inositol, 876 mg/L L-glutamin, 3 g/L KCl, 4 g/L MgCl₂, pH 5.2) and incubated for 28°C for 1 hr. Scutellum derived embryogenic calli (Fig. 2 C) were infected with these various concentration (0.1 to 0.7 of OD_{600nm}) *Agrobacterium* culture for 30 min with intermittent gentle shaking at 100 rpm. The infected embryogenic calli were transfer on sterile filter paper for 3 min to remove excess bacterial culture. Infected calli were transfer on to the co-cultivation (COC) medium (CI medium with containing 10 mg/L glucose, 0 to 150 μM acetosyringone, pH 5.2 solidified with 0.4% phytigel) for 1 to 3 days of co-cultivation time at 28°C in dark. The co-cultivated calli were washed with sterile liquid medium containing 250 mg/L cefotaxime to remove *Agrobacterium*. The washed calli blot dried on filter paper and transferred on to first selection medium (CI medium containing 250 mg/l cefotaxime and 5mg/L phosphinothricin) and incubated for 3 weeks at 28°C in dark. The resistance calli were further selected through two rounds of selection cycles for 15 days each (Fig. 2D). Newly proliferated micro calli generated on selection medium were transfer to the regeneration medium (MS basal salts, 2.5 mg/L kinetin, 0.5 mg/L naphthyl acetic acid (NAA), 30mg/L sucrose, 20mg/L D-sorbitol and pH 5.8 solidified with 0.4% phytigel). The cultures were maintained in dark for 1 week then shifted to photo period of 16-hrs light/8 hrs dark for 3 weeks. For roots development, the regenerated shoots buds were transfer to rooting medium (half strength MS basal salts 15 g/L sucrose, pH 5.8 and 0.3% phytigel) and maintained at 28°C in light for two weeks (Fig. 2 E). Fully rooted plants were transferred to pots and maintained at transgenic glass house (Fig. 2 F).

Molecular analysis of putative transgenic plants

Confirm the presence of transgene in putative transformed and non-transformed plantlets through PCR analysis. Genomic DNA from various independent transgenic lines was extracted by the method described by Dellaporta *et al.*, (1983). Following primers pair was used for PCR analysis: En-F (5'-CAAAGGGTAATATCGGGAAACC-3') and Bar-R (5'-CATCAGATCTCGGTGACG-3'). The reaction mixture of 20 μl (total volume) containing 50ng good quality template DNA, 1X Taq buffer (with 25 mM MgCl₂), 0.125mM dNTPS, 5pM primers and 1U Taq DNA polymerase and the profile was as follows: initial denaturation 95°C for 5 min followed by 32 cycles; denaturation 95°C for 30 sec; annealing 58°C for 30 sec; elongation 72°C for 1 min with final extension 72°C for 5 min. The

amplified PCR product was resolved on 1.2% agarose gel electrophoresis.

Result and Discussion

Modified protocol for callus induction and transformation

Elite *Indica* rice cultivar BPT-5204 was used for initial standardization of various parameters for enhancement of embryogenic callus induction and regeneration. This optimized protocol was used for

further experiments to enhance transformation as well as regeneration efficiency. Maximum callus proliferation was observed after 19-21 days (fig 2 C). The callus induction percentage was found to be 85%. The embryogenic calli formed on the CI medium were creamy white, compact and globular structure. Pre-cultured embryogenic calli (for 5 days) on CI medium (2.5 mg/L 2, 4-D, 0.5 mg /L kinetin) was used as an explants for transformation.

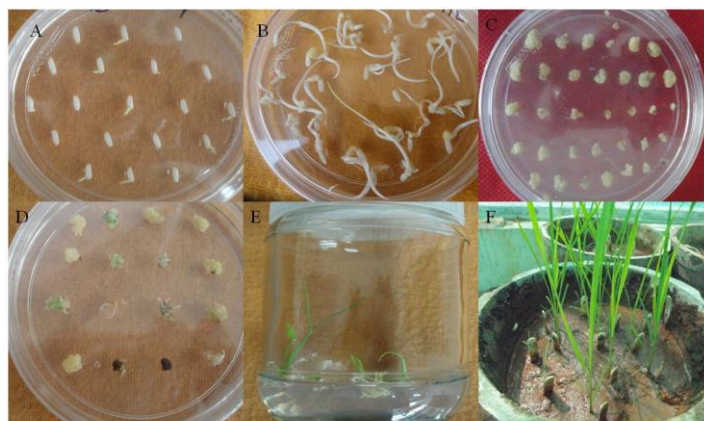


Figure 2: A) Rice seed inoculated on CI medium. B) callus induction in rice from Scutellum. C) 21 days embryogenic calli. D) co-cultivated calli on PPT(5mg/L) selection medium. E) Regenerated plants in root induction medium. F) Established plants in green house

Effect of optical density (OD) on transformation frequency

Pre-cultured embryogenic calli were co-cultivated with different optical density (OD_{600nm}) values (0.1, 0.3, 0.5 and 0.7) of *Agrobacterium* culture with 1 hr of infection time. It was observed that the increase of *Agrobacterium* OD was directly proportional to increase in the percentage of survival calli. Transformation event was not detected at lower bacterial density (0.1). Embryogenic calli infected with *Agrobacterium* culture of an (OD_{600nm}) of 0.5 were observed to give the best transformation frequency (16%) (Table 1). However, increasing the *Agrobacterium* concentration to (OD_{600nm}) 0.7 which negatively affects the transformation frequency. The (OD_{600nm}) of 0.5 value might be representing the active log phase of *Agrobacterium* growth and thus very effective for transformation. Previous reports indicated that the OD_{600nm} of the bacterial density in a range from 0.3 to 1 is suitable for transformation (Aananthi et al., 2010; Baskaran et al., 2012). In the present study, OD_{600nm} of 0.5 proved to be the best concentration of *Agrobacterium* for successful transformation in *Indica* rice cultivar BPT-5204.

Table.1: Influence of concentration of *Agrobacterium* on transformation frequency

<i>Agrobacterium</i> concentration (OD_{600nm})	Phosphinothricin resistant calli %
0.1	0
0.3	7±1.7
0.5	16±1.9
0.7	6±1.5

Pre-cultured embryogenic calli were infected for 1hr with *Agrobacterium* strain EHA105 containing pCAMBIA 2300-*Ds-En-bar* and co-cultivation for 2 days on MS basal medium, 100 μ M concentration of acetosyringone containing respective *Agrobacterium* concentration (OD). Transformed calli were transferred to selection medium (phosphinothricin 5 mg/L). The mean of three independent experiments (\pm standard error) are provided.

Influence of Addition of acetosyringone on transformation frequency

Acetosyringone is a phenolic compound produced during plant cell wounding that induces the transcription of *Agrobacterium tumifaciens* virulence genes that regulate the processing and transfer of T-DNA from *A.tumifaciens* to plant cells (Shimoda et al., 1990; Gelvin 2003; Tripathi et al., 2010). Higher transformation frequency is achieved by addition of acetosyringone in co-cultivation medium. In present study, embryogenic calli were co-cultivated with *Agrobacterium* cultures (EHA105: pCAMBIA2300-*Ds-En-Bar* strain) which supplemented with different concentrations of acetosyringone (AS) (0, 50, 100 and 150 μ M) to identify the optimized concentration (Table 2). Embryogenic calli infected with *Agrobacterium* OD_{600nm} of 0.5 and with addition of 100 μ M acetosyringone showed better response (regenerated calli after two sub-cultures on phosphinothricin selection media) of 18.7% when compared with other concentrations.

Table 2: Influence of concentration of acetosyringone transformation frequency

Acetosyringone concentration	Phosphinothricin resistant calli %
0 μ M	5 \pm 0.6
50 μ M	9 \pm 0.5
100 μ M	18 \pm 0.7
150 μ M	7 \pm 0.8

Pre-cultured embryogenic calli were infected for 30 min with *Agrobacterium* strain EHA 105 containing pCAMBIA 2300-*D_s*-En-*bar* OD₆₀₀ of 0.5 and co-cultivation for 2 days on MS basal medium containing the respective concentration of acetosyringone. Transformed calli were transferred to selection medium (phosphinothricin 5 mg/L). The mean of three independent experiments (\pm standard error) are provided.

Influence of co-cultivation time on transformation frequency

Transformation frequency mainly depends on the duration of co-cultivation of the infected explants with *Agrobacterium*. The co-cultivation time duration varied with type of explants, genotype of the plant, *Agrobacterium* strain. Different co-cultivation periods (0, 1, 2 and 3 days) were investigated. The infected calli without co-cultivation period not show any transformation frequency (Table 3). One day co-cultivation period gives 10% transformation frequency, two days co-cultivation periods showed the highest percentage of transformation frequency by 19% whereas the transformation frequency was decreased dramatically in the case of using three days as a co-cultivation period (8%). Therefore, two day co cultivation period was considered as optimized and further prolonged exposure of cells (3days) to *Agrobacterium* may adversely affect the calli which causes the cell death or overgrowth of bacteria. Previous reports indicated embryogenic calli infected with *Agrobacterium* with two to three days of co-cultivation period resulted in the highest transformation frequency without overgrowth of *Agrobacterium* as longer time will cause bacterial overgrowth (Rashid et al., 1996; Urushibara et al., 2001; Zhang et al., 2015).

Table 3: Influence of co-cultivation time (days) on transformation frequency

co-cultivation time (days)	Phosphinothricin resistant calli %
0	0
1	10 \pm 1.4
2	19 \pm 2.1
3	8 \pm 0.8

Pre-cultured embryogenic calli were infected for 1hr with *Agrobacterium* strain EHA105 containing pCAMBIA 2300-*D_s*-En-*bar* OD_{600nm} of 0.5, MS basal medium containing the 100 μ M concentration of acetosyringone and respective co-cultivation time (days). Transformed calli were inoculated on selection medium (phosphinothricin 5 mg/L). The

mean of three independent experiments (\pm standard error) are provided.

Molecular confirmation of the transgenic plants of regenerated plants

The developed plants were analyzed for transgene through molecular technique. PCR is a sensitive technique allowing genes to be amplified and extracted out of a complex mixture of genomic sequences. Amplified DNA is visualized as distinct bands on agarose gels. Therefore, PCR was utilized to confirm the integration of T-DNA fragments in plantlets. The primers used for PCR were designed to amplify a 900bp fragment of *bar* gene. Expected fragment was observed in the 27 T₀ plantlets. Non transformed control BPT-5204 plantlets did not show any amplification with gene specific primers.

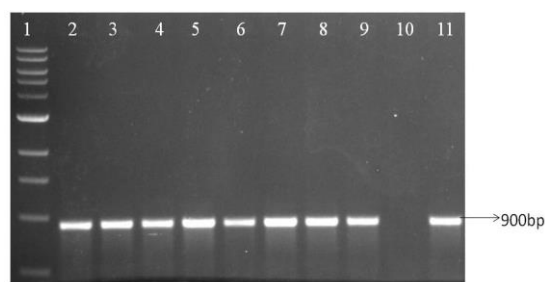


Figure 3 PCR verification of T₀ putative transgenics in rice variety BPT-5204 using *Bar* primers. Lane 1: 1kb DNA ladder marker, 2-9: Putative transgenic plants, 10: Negative Control, 11: Positive Control

We have developed a protocol for the genetic transformation of *Indica* rice (BPT-5204). By using *Agrobacterium* culture with 0.5 (OD_{600nm}) for co-cultivation, 2 days of co-cultivation on 100 μ M acetosyringone supplemented medium, high transformation efficiency (6.2%) was achieved.

Conclusion

The result obtained in the present study gives that the optical density of the *Agrobacterium*, acetosyringone concentration and co-cultivation time is critical parameters that influence the transformation in *indica* rice (BPT-5204). The genetic transformation method developed in our study is used to developed T-DNA based activation tagging lines, which involved in develop the gain of functional mutations in recalcitrant *indica* rice cv BPT-5204.


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