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SHORT COMMUNICATION

An improved *Agrobacterium*-mediated transformation of recalcitrant indica rice (*Oryza sativa* L.) cultivars

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Abstract *Agrobacterium*-mediated transformation of indica rice varieties has been quite difficult as these are recalcitrant to in vitro responses. In the present study, we established a high-efficiency *Agrobacterium tumefaciens*-mediated transformation system of rice (*Oryza sativa* L. ssp. *indica*) cv. IR-64, Lalat, and IET-4786. *Agrobacterium* strain EHA-101 harboring binary vector pIG121-Hm, containing a gene encoding for β-glucuronidase (GUS) and hygromycin resistance, was used in the transformation experiments. Manipulation of different concentrations of acetosyringone, days of co-culture period, bacterial suspension of different optical densities (ODs), and the concentrations of L-cysteine in liquid followed by solid co-culture medium was done for establishing the protocol. Among the different co-culture periods, 5 days of co-culture with bacterial cells ($OD_{600\ nm}$=0.5–0.8) promoted the highest frequency of transformation (83.04 %) in medium containing L-cysteine (400 mg l^{-1}). Putative transformed plants were analyzed for the presence of a transgene through genomic PCR and GUS histochemical analyses. Our results also suggest that different cultural conditions and the addition of L-cysteine in the co-culture medium improve the *Agrobacterium*-mediated transformation frequencies from an average of 12.82 % to 33.33 % in different indica rice cultivars.

Keywords *Agrobacterium* · L-Cysteine · Rice · Transformation · Recalcitrant · Indica cultivars

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Introduction

Genetic transformation using *Agrobacterium tumefaciens* has been extensively used for developing transgenic rice plants (Roy et al. 2000; Ignacimuthu et al. 2000). In rice, different types of explants are used for transformation, such as mature seed-derived callus (Sahoo and Tuteja 2012), immature embryo-derived calli (Hiei and Komari 2006), leaf-derived calli (Karthikeyan et al. 2011), and shoot apex (Park et al. 1996). Most of the studies suggest that the transformation efficiency of the indica rice varieties is unsatisfactory in comparison to japonica cultivars as majority of the indica rice varieties are recalcitrant to in vitro responses (Ge et al. 2006; Tie et al. 2012). However, Kumar et al. (2005) reported a high-efficiency transformation protocol for recalcitrant indica rice cultivars IR-64 and obtained a transformation efficiency of 4.6–5.5 %. Tie et al. (2012) reported that the stable transformation efficiency of the two indica cultivars—MH and ZS—ranged from 1.5 to 3.5 % after *Agrobacterium*-mediated transformation. Hiei and Komari (2006) developed a protocol for the transformation of indica rice varieties using freshly isolated immature embryos from plants grown in a greenhouse. The use of immature tissues as the starting material for in vitro regeneration to produce transgenic rice plants has several disadvantages (Lee et al. 2002). For developing a viable transformation protocol, the use of a mature embryo-derived callus is a prerequisite to avoid donor material from the greenhouse before starting the experiment. Though a few methods have been developed, the transformation efficiency using mature embryo-derived indica rice varieties is still unsatisfactory, and only a limited number of indica cultivars have been genetically manipulated efficiently through this method. In addition, the genotype remains the limiting factor restricting the successful transformation in

indica rice (Lin and Zang 2005), which limits the use of such protocols.

In plants, many factors are known to affect the efficiency of T-DNA delivery to the plant cell. These factors include the different explant types; the cell density of *Agrobacterium* for inoculation; the inoculation period; the co-culture medium; and the induction agent of *vir* genes, such as acetosyringone, in the inoculation and co-culture media, L-cysteine in the pre-culture or co-culture medium, etc. Therefore, there is a need to develop a protocol for *Agrobacterium*-mediated rice transformation which can be used in a genotype-independent manner for functional genomics and for developing transgenic rice varieties for value addition. Here, we report, the improvement of *Agrobacterium*-mediated transformation in three indica rice cultivars (IR-64, Lalat, and IET-4786) using standard binary vectors. We show that optimized culture conditions and the addition of L-cysteine into the co-culture medium improved the *Agrobacterium*-mediated stable transformation frequencies from an average of 12.85 % to 33.33 % in these indica rice cultivars in a genotype-independent manner.

Materials and methods

Plant materials

Mature, dehusked seeds of the rice varieties IR-64, Lalat, and IET-4786 (*Oryza sativa* L. ssp. *indica*) were used for callus induction. The calli were induced on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962); supplemented with 2,4-dichlorophenoxyacetic acid (2 mg l^{-1}), maltose (30 gl^{-1}), casein hydrolysate (0.3 g l^{-1}), proline (0.5 gl^{-1}), and Phytagel (4 gl^{-1}); and incubated at 26 °C in the dark (Chakrabarty et al. 2010). After 3–4 weeks, the proliferating calli were sub-cultured onto the same medium and cultured for another 3–4 weeks. White friable embryogenic calli appear at this stage. The embryogenic calli were sub-cultured onto the same medium 10 days before infection with *Agrobacterium*.

Agrobacterium-mediated transformation

A supervirulent *Agrobacterium* strain, EHA-101, was transformed with a binary vector, pIG121-Hm. *Agrobacterium* EHA-101 (pIG121-Hm) was grown to different optical densities (ODs), i.e., 0.5, 0.8, and 1.2, at 600 nm in AB medium (Chilton et al. 1974) containing hygromycin (20 mg l^{-1}), kanamycin (50 mg l^{-1}), and rifampicin (25 mg l^{-1}). The culture was centrifuged at 3,000×*g* for 5 min and the pellet resuspended in an equal volume of the AA-AS medium [AA medium containing sucrose (20 gl^{-1}), glucose (10 gl^{-1}), casein hydrolysate (0.3 gl^{-1}), and different concentrations of acetosyringone (0, 100, and 200 μM)] (pH 5.6). pIG121-Hm contains β-glucuronidase (GUS) as the reporter gene and a hygromycin resistance gene (*hptII*) as the selectable marker. The calli were immersed in bacterial suspension for 30 min and the excess bacterial suspension removed by blotting on sterile tissue paper. Infected calli were transferred onto an MS co-cultivation medium [MS callus induction medium containing different concentrations of acetosyringone (0, 100, and 200 μM)]. After co-cultivation, infected calli were washed twice with sterile distilled water and, finally, once with aqueous solution containing cefotaxime (250 mg l^{-1}) and carbenicillin (250 mg l^{-1}); blotted on sterile tissue paper; and transferred to MS selection medium [MS callus induction medium containing hygromycin (40 mg l^{-1}), kanamycin (50 mg l^{-1}), and refampicin (25 mg l^{-1})] and sub-cultured after every 15 days. Different concentrations of L-cysteine (0, 100, 200, 400, and 600 mg l^{-1}) both in liquid and following solid co-culture medium were also used for co-culture. All initial experiments were done in indica variety Lalat. The transformation frequency was evaluated after the fifth round of selection on the selective medium as the number of blue spots (GUS-expressing cell clusters, visible under a stereomicroscope at ×50 magnification) per total number of inoculated explants.

After five rounds of selection (sub-cultured after every 15 days), actively growing pieces of calli were transferred to the MS regeneration medium [MS basal medium supplemented with 1-naphthaleneacetic acid (1 mg l^{-1}), 6-benzyl adenine (3 mg l^{-1}), thidiazuron (1 mg l^{-1}), casein hydrolysate (3 gl^{-1}), maltose (30 gl^{-1}), and hygromycin (40 mg l^{-1})] and kept under a 16-h light (110–130 μE m^{-2} s^{-1}) and 8-h dark photoperiod (Chakrabarty et al. 2010). Shoot regeneration was observed after 4 weeks. The regenerated shoots were transferred to the rooting medium (half-strength MS medium without a growth regulator containing 40 mg l^{-1} hygromycin). After 4 weeks, rooted plants were transferred in soilrite and grown for 4 weeks for hardening. The hardened plants were transferred into plastic pots and kept in the transgenic greenhouse for flowering and fruiting. Leaf pieces from the regenerated plants and germinated seeds from T1 generation were tested histochemically for GUS expression as described below.

Assay for GUS activity

GUS activity was assayed following an improved histochemical staining procedure (Jefferson et al. 1987). In brief, after five rounds of selection in the selection media, embryo-derived calli were incubated in X-Gluc solution containing 5-bromo-4-chloro-3-indolyl β-D-glucuronidase (1 mg l^{-1}), triton X-100 (0.06 %), ferricyanide (500 μM), EDTA (10 mM), and sodium phosphate buffer (100 mM, pH 7.0). The reaction mixture was incubated at 37 °C for 2–3 days. The calli were examined under a stereomicroscope and photographed.

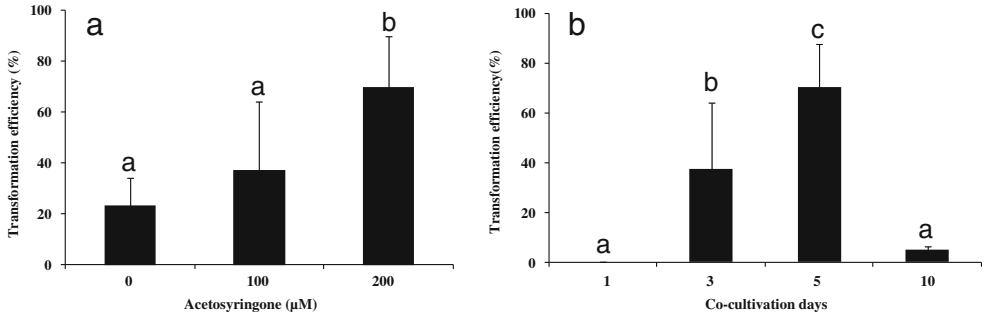

Fig. 1 Effect of the different concentrations of acetosyringone (**a**) on the transformation of rice calli (Lalat) as determined by the transient expression of the *gusA* gene. Effect of the different co-cultivation days (**b**) on the transformation of rice calli as determined by transient expression of the *gusA* gene. *Values marked with similar letters* are not significantly (Duncan's test: $p<0.05$) different

PCR confirmation

Genomic DNA was extracted from young leaves of transgenic and non-transgenic plant lines at the adult stage using a DNeasy Plant Mini Kit (Qiagen, USA). A pair of primers was designed for PCR analysis according to the *hptII* gene sequence: *hpt-II*-F (5′–CTATTTCTTTGCCCTCGGACG-3′) and *hptII*-R (5′-ATGAAAAAGCCTGAACTCACCG-3′). A 20-µl reaction mixture containing 30 ng of template DNA, 1 U Taq polymerase (Genei, India), 2.0 µl 10X buffer, 1.0 µl 2 mM dNTP, 1.5 µl 25 mM $MgCl_2$, and 0.4 µl each of 10 mM primers was prepared for the PCR assay. The PCR reaction proceeded at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 15 s, and 72 °C for 1.5 min, with a final extension of 72 °C for 5 min. The amplification product (1,026 bp) was checked on 0.8 % agarose gel and photographed.

Statistical analysis

All experiments were carried out in triplicate and repeated three times ($n=9$). Data obtained from the experiments were subjected to one-way analysis of variance. Statistical significance was determined using Duncan's multiple range test for a multi-comparison of means (Gomez and Gomez 1984). Significance levels were compared at $p<0.05$. Statistical analyses were performed using SPSS 16.0 software.

Results and discussion

For efficient plant transformation, the selection of actively growing regenerable calli and the optimization of the culture conditions for the co-cultivation of rice calli with *Agrobacterium* are the most important factors. The addition of acetosyringone in the co-cultivation media, which acts as an inducing agent for T-DNA transfer into the host cell, significantly increases the efficiency of rice transformation. To establish an efficient transformation protocol, modulation in the concentration of acetosyringone, co-culture period, and in bacterial growth needs to be optimized.

To evaluate the effects of acetosyringone concentration on T-DNA delivery, callus regeneration, and the final transformation efficiency, different concentrations of acetosyringone (0, 100, and 200 µM) were used at the time of co-culture. In our experiment, the transformation efficiency was enhanced with increasing concentrations of acetosyringone, with the maximum transformation efficiency (69.98 %) at 200 µM of acetosyringone (Fig. 1a). Our results are in agreement with Amoah et al. (2001) who reported a final concentration of 200 µM for the enhanced transformation efficiency in wheat. Similar to our data, Terada et al. (2004) reported maximum rice transformation efficiency at 200 µM concentration. In dicot tissues, the exudation of phenolic compounds during wounding is known to activate the *VIR* genes present in Ti plasmids. Similar to dicots, monocots may also produce these compounds that are insufficient to serve as a signal for *VIR* gene induction. Therefore, the addition of acetosyringone is essential for successful and higher frequency transformation, but the concentration of acetosyringone in the co-culture medium may vary between different plants.

Different days of co-culture period were also used as a parameter to investigate the transformation efficiency. No transformation was observed on day 1 of co-cultivation. The

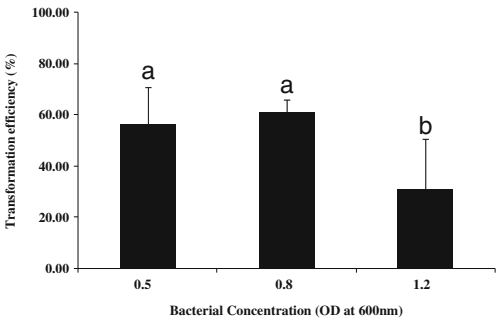

Fig. 2 Effect of bacterial culture density on the transformation of rice calli (Lalat) as determined by the transient expression of the *gusA* gene. *Values marked with similar letters* are not significantly (Duncan's test: $p<0.05$) different

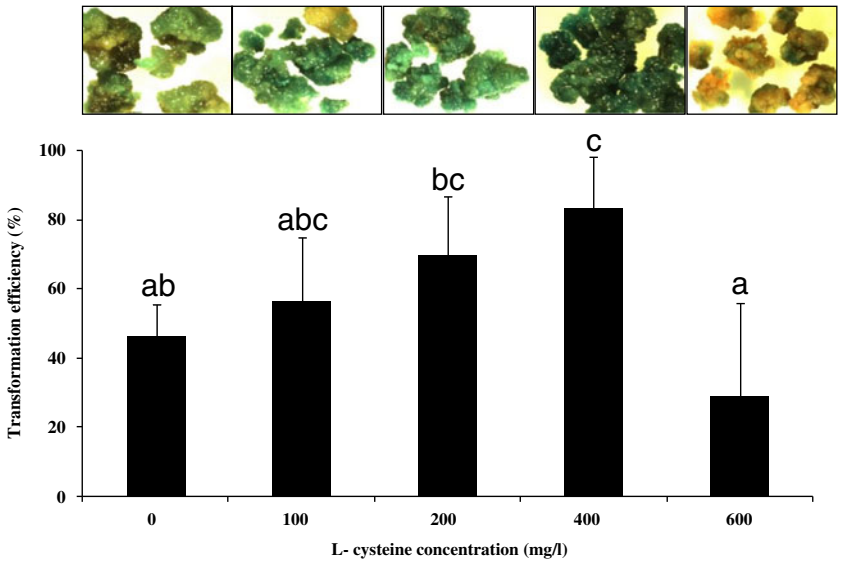

Fig. 3 Effect of L-cysteine on T-DNA delivery into transgenic rice embryogenic calli (Lalat) as determined by the transient expression of the *gusA* gene. *Values marked with similar letters* are not significantly (Duncan's test: $p<0.05$) different

maximum transformation efficiency (70.44 %) was observed after 5 days of co-cultivation in the co-cultivation plates (Fig. 1b).

Bacterial cultures of ODs, i.e., 0.5, 0.8, and 1.2, at 600 nm were analyzed in the present study to study its effect on the transformation efficiency of rice cv. Lalat. The maximum transformation efficiency (64.06 %) was observed at $OD_{600\,nm}$=0.5–0.8 (Fig. 2). Similar results were reported by several other groups working with monocot transformation (Ignacimuthu et al. 2000).

Commonly, co-culture proceeds in darkness at laboratory temperature (25–27 °C). We performed several experiments with different light regimes similar to the study of De Clercq et al. (2002). Continuous darkness (D24), 16-h/8-h (light/dark) photoperiod, and continuous light (L24) were compared. In the absence of acetosyringone (basal experiment), the highest transformation efficiency was observed under the 24L regime, followed by 24D and 16L/8D, but the differences were not statistically significant. At the same time, no correlation between the transformation efficiency with 200 μM acetosyringone and the light regime could be established (data not shown). A similar observation was made by Kondo et al. (2000) in garlic where no significant differences between the cultures in light and dark conditions were observed for the transformation.

We used different concentrations of L-cysteine (100, 200, 400, and 600 mg l^{-1}) in our experiment both in liquid and following solid co-culture medium and observed that

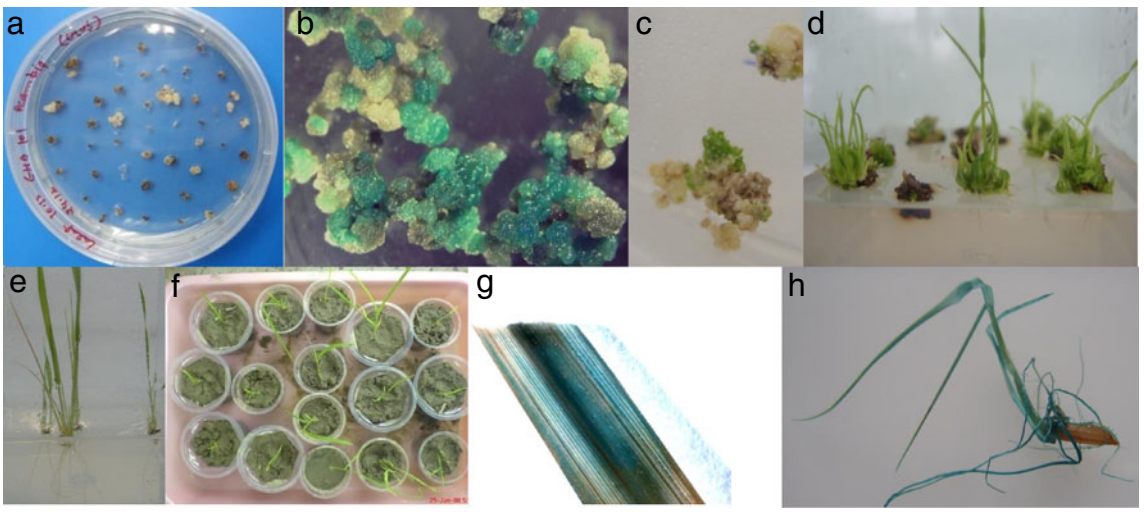

Fig. 4 Regeneration of transgenic rice lines (Lalat) from mature scutelar-derived embryogenic calli. **a** Callus formation on the selection medium after 45 days. **b** GUS-positive calli after five rounds of selection. **c** Shoot regeneration from transgenic calli. **d** Shoot elongation on MS medium without growth regulator. **e** Rooting of putative transgenic plants in half-strength MS medium without growth regulator. **f** Growth of transgenic plantlets in pots in a greenhouse. **g** *GUS* expression of a leaf of a putative transgenic plant. **h** *GUS* expression of germinating T1 seed

Table 1 *Agrobacterium*-mediated transformation frequency of three different indica rice cultivars

Genotype	Total no. of callus inoculated	Hygromycin-resistant calli after the 5th cycle	Putative transgenic plants	Transformation frequency (%)
IR-64	39	31	5	12.820
Lalat	57	31	8	14.035
IET-4786	64	50	20	33.333

400 mg l^{-1} L-cysteine is the optimum concentration for the transformation efficiency (Fig. 3). The addition of L-cysteine has significantly enhanced Hi-II maize transformation (Frame et al. 2002; Vega et al. 2008), three maize inbred lines (Frame et al. 2006), and soybean (Olhoft and Somers 2001). L-Cysteine is a compound that may increase the frequency of transformed cells either by acting as a nutritional supplement or by acting through its thiol group as an antioxidant or inhibitor of wound and pathogen defense responses. Generally, it belongs to the thiol compounds which inhibit wound and plant pathogen-induced responses. Similarly, it limits enzymatic browning and necrosis of wounded (in vitro cultured) and *Agrobacterium*-infected explants (Olhoft and Somers 2001; Olhoft et al. 2001).

In the present study, we observed that the use of a lower concentration (OD=0.5–0.8) of *Agrobacterium* culture suspended in 400 mg l^{-1} L-cysteine medium and 5 days of co-culture on media reduced the browning of callus after co-culture. It may possibly be because of the reduced damage to explants during *Agrobacterium* infection, which results in less phenolic production and better recovery of callus during the selection. Similar to our study, the influence of the concentrations of acetosyringone, co-culture period, cell density, and thiolic compounds on the efficiency of T-DNA transfer has been studied in the transformation of many plant species (Kondo et al. 2000, Olhoft and Somers 2001; Olhoft et al. 2001; Suzuki and Nakano 2002; Frame et al. 2002; Liping and Manzhu 2004; Vega et al. 2008). These studies reported that 2–5 days of co-culture period and OD of 0.5–1.0 at 600 nm are optimum for transformation. Our results were in agreement with these studies and suggest that these factors have a strong effect on bacteria growth and, subsequently, T-DNA transfer to the host plant.

In our study, the callus turned brown after the second round of selection (30 days), and new callus growth appeared after 45 days (Fig. 4a). After five rounds of selection (75 days), the actively growing callus in the selection media were found to be positive to the GUS histochemical assay (Fig. 4b). After selection in the hygromycin-containing selection media, the calli were transferred to the regeneration medium. Multiple green spots were visible on the embryogenic calli 10 days after transfer to the shoot initiation media. The green sectors further differentiated into shoot meristems (Fig. 4c). Multiple shoots obtained on the shoot initiation medium did not elongate on the same medium. However, after transfer to the half-strength MS medium without a growth regulator, rapid shoot elongation and rooting was observed (Fig. 4d, e). The regenerated plants did not show any detectable abnormality in morphology or growth characteristics (Fig. 4f), flowered normally, and set viable seeds. The leaves of the regenerated plants showed GUS-positive results (Fig. 4g). Seeds of the T1 generation were germinated, and the GUS histochemical assay also showed positive results (Fig. 4h). In the present study, we obtained transformation frequencies of 12.82, 14.03, and 33.33 % in IR-64, Lalat, and IET-4786, respectively (Table 1). The presence of transgene (*hptII*) in all putative transformants was determined by genomic PCR analysis. PCR products of the expected size (1,026 bp) corresponding

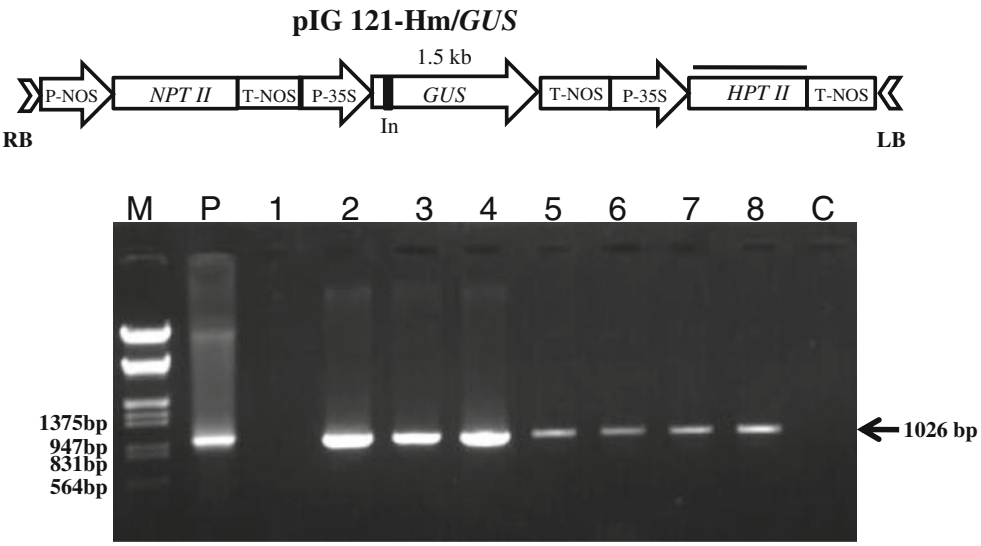

Fig. 5 Plasmid map and validation of transgenic lines through genomic PCR. *Vertical line above* the *hptII* gene in the plasmid represents the region selected for PCR amplification. *P* plasmid containing the *hptII* gene (positive control), *C* non-transgenic plant (negative control). *Lane M*, Lambda DNA/*Eco*RI+*Hind*III marker (Fermentas, USA). *Lanes 1–8*, different transgenic lines. Amplification products were separated on 0.8 % agarose gel

to *hptII* were amplified from most of the hygromycin-resistant plants, whereas DNA samples from non-transformed plants did not yield such PCR products (Fig. 5).

In the present study, we developed an *A. tumefaciens*-mediated efficient transformation protocol of three indica rice varieties. Furthermore, it was observed that by modulation of the different factors like the acetosyringone concentration in the co-culture medium, the co-culture period, the bacteria density, and L-cysteine concentration in the medium, efficient transformation can be achieved. The developed method may be useful to transform other recalcitrant indica rice varieties.

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Conflict of interest The authors declare that they have no conflict of interest associated with this work.

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