Ind J Plant Physiol. (July–September 2017) 22(3):346–353
DOI 10.1007/s40502-017-0304-1

ORIGINAL ARTICLE

Development of an efficient protocol for Agrobacterium mediated transformation of some recalcitrant indica rice varieties

Ubaid Yaqoob[1,2] · Tanushri Kaul[3] · Irshad Ahmad Nawchoo[1]

Received: 12 October 2016 / Accepted: 20 July 2017 / Published online: 14 August 2017

Abstract Rice is a staple food whose productivity is affected by multiple environmental stresses. The use of biotechnological tools is the best possibility to develop rice varieties with increased productivity, exceptional grain quality and resistance to various stress. Several parameters in the genetic transformation (co-cultivation period, acetosyringone concentration, temperature of the co-cultivation medium, antibiotic concentration etc.) of rice varieties—Shalimar Rice 1, Jhelum and K 332 were optimized for the first time using β-glucuronidase (GUS) gene as a reporter. The GUS expression in presence of 100 μM acetosyringone at pH 5.2 was more at 22 °C as compared to 28 °C in all the rice varieties. The selection medium containing 250 mg l^{-1} of cefotaxime and carbenicillin prevented Agrobacterium overgrowth and the use of 50 mg l^{-1} hygromycin resulted in killing of the untransformed calli. The regeneration medium containing MS B5 medium supplemented with 4% sucrose, 3 g l^{-1} phytagel, 2.5 mg l^{-1} BAP, 1 mg l^{-1} zeatin, 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} TDZ allowed efficient regeneration of the rice calli. The overall regeneration frequency of Shalimar Rice 1, Jhelum and K 332 were 64, 75 and 77% respectively. Putative transformed plants were analyzed for the presence of the transgenes through GUS histochemical analyses and southern blotting. The present protocol provides an efficient and rapid embryogenic callus induction, transformation and regeneration system, which could be conveniently used for producing genetically modified plants and might help in the transformation of other related rice genotypes.

Keywords Agrobacterium · Hygromycin · *Oryza sativa* · Rice calli · Transformation

✉ Ubaid Yaqoob
ubaidyaqoob@yahoo.in

[1] Plant Reproductive Biology, Genetic Diversity and Phytochemistry Research Lab, Department of Botany, University of Kashmir, Srinagar, J&K 190006, India

[2] Present Address: Nutritional Improvement of Crops Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

[3] Nutritional Improvement of Crops, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

Introduction

Rice, the staple food of greater than half of the world's population (Hadiarto and Tran 2011) serves more than 90% of the Asian population (Khush and Brar 2001; Zeigler and Barclay 2008). *Indica* rice comprises 80% of cultivated rice in the world (Ramesh et al. 2009; Tie et al. 2012). Due to increase in population (1.6% $year^{-1}$) there is an everyday increasing demand of rice production and the area under rice cultivation is expected to reduce to 40 million ha in the next 15–20 years (Shobarani et al. 2010). To feed the 700 million Asians getting 60% of their daily calorific intake from rice, yields must be increased by at least 50% over the next 40 years (Hibberd et al. 2008). Due to abiotic factors, 52% of the global production of rice is lost annually, of which almost 21% is because of the attack of insect pests (Brookes and Barfoot 2003). According to Savary et al. (2000), 24–41% of the rice yield was lost annually because of pests, diseases and weeds. Furthermore, the nutritional improvement of rice can also help in decreasing the evil of malnutrition in the developing world (Bajaj and Mohanty 2005). Hence, the use of biotechnological tools is the novel, powerful and effective way to increase the yield and

productivity of rice varieties with exceptional grain quality and resistance to various biotic and abiotic stresses (Yaqoob et al. 2016).

Agrobacterium mediated genetic transformation appears more effective and is commonly used in rice (Lin et al. 2009; Xiao 2009). The embryogenic calli obtained from mature seed embryos are the best explants for *indica* rice transformation (Kant et al. 2007; Kumar et al. 2010; Sahoo et al. 2011; Sripriya et al. 2011; Endo et al. 2012). However, the genetic transformation of *indica* rice is a difficult task due to a low induction rate of embryogenic callus and plantlet regeneration (Kumar et al. 2005; Nishimura et al. 2006; Arockiasamy and Ignacimuthu 2007; Yookongkaew et al. 2007; Hiei and Komari 2008) and majority of the *indica* rice varieties are recalcitrant to *invitro* responses (Ge et al. 2006; Saika and Toki 2010; Tie et al. 2012). Furthermore, the transfer and integration of foreign DNA into plant genome are influenced by numerous factors like plant genotype, vector-plasmid design, phenolic compounds, bacterial strain, culture medium composition, the selectable marker genes and selection agents, temperature of co-cultivation medium and chemicals used in eliminating the *Agrobacterium* after co-cultivation (Dong et al. 1996; Hiei et al. 1997; Hellens et al. 2000; Cheng et al. 2004). Keeping all this in mind the present study has been devised for the first time to establish an efficient transformation protocol in rice varieties - Shalimar Rice 1, Jhelum and K332 using embryogenic callus cultures.

Materials and methods

Collection of plant material and callus induction

The experiment was conducted on the mature seeds of three high yielding commercial rice varieties - Shalimar Rice 1 (SR1), Jhelum and K 332 commonly grown in J&K obtained from the SKUAST-K, J&K, India. The research work was carried out in the plant tissue culture laboratory, ICGEB, New Delhi, India. The dehusked mature seeds of all the rice varieties were surface sterilized with 70% alcohol for 1 min under laminar air flow cabinet and washed thrice with double distilled water. These seeds were then surface sterilized in 4% sodium hypochlorite with Tween 20 (one drop per 50 ml volume) for 10 min with intermittent shaking, followed by five rinses in sterile water (3 min each). The seeds were dried on sterilized Whatman sheet No. 1 and then plated on callus induction medium (15 seeds per plate) incubated in darkness at a temperature of 25 ± 2 °C and relative humidity of 50–60%. The composition of the callus induction media used is shown in Table 1. The medium was prepared from stock solutions and pH was adjusted to 5.8, prior to addition of the gelrite. Embryogenic calli from 8 days old seeds were dissected with forceps and then sub-cultured onto fresh plates. The plates were incubated in dark for another 3 d prior to transformation.

Agrobacterium tumefaciens strain and transformation

The binary plasmid pCAMBIA1301 having *gus* and *hpt* as reporter and plant selection marker genes respectively, driven by CaMV 35S promoter and *npt* II gene outside the T-DNA region as bacterial selection marker was mobilized into *Agrobacterium* strain EHA105. Primary culture was initiated by inoculating a single colony in 10 ml YEM overnight incubated at 28 °C with appropriate antibiotics like 15 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin on a rotatory incubator shaker (Kuhner, Switzerland) at 200 rpm. About 1% of this primary culture was inoculated in a 250 ml flask containing 100 ml YEM broth. The culture was then grown at 28 °C till $O.D_{600}$ reached up to 1. The cells were pelleted down by centrifugation at 4000 rpm for 30 min at 4 °C. After decanting the supernatant, the pellet was dissolved in 1–2 ml of the liquid infection medium (Table 1) using a micropipette. Using liquid infection medium, the cells were diluted to desired final $O.D_{600}$ of 0.5.The bacterial pellet was suspended in 100 ml of infection medium at room temperature along with 100 µM of acetosyringone. The 11 days old embryonic calli (~100) were placed in the 500 ml Erlenmeyer flask containing 100 ml bacterial suspension for 30 min with 20 min intermittent shaking followed by 10 min 25 Hg vacuum (Millipore). The embryonic calli of all the rice varieties were then dried on sterile tissue paper and co-cultured on plates containing co-cultivation medium (Table 1). To standardize the optimum requirement, 100–200 µM of acetosyringone was added to the co-cultivation medium. The plates were incubated in dark for 48–72 h at 22–28 °C. After different experimental durations, co-cultured calli were washed with 100 ml of double distilled water containing cefotaxime (250 mg l^{-1}) and carbenicillin (250 mg l^{-1}) for 4–5 times, 12 min for each wash. The embryonic calli were then dried on sterile tissue paper and cultured on plates containing resting media (Table 1). The plates were incubated again in the dark for 5–7 days at 25 °C.

Selection and regeneration

The calli were then transferred to plates containing selection medium (Table 1). The plates were incubated under continuous dark conditions at 25 °C for 10 days; the

Table 1 Composition of the different media used

Medium	Constituents (per litre)
Callus induction medium	MS B5 medium 4.4 g, 30 g maltose, 500 mg L-proline, 500 mg casein hydrolysate, 5 mg L-glutamine, 40 mg cystiene, 5 mg L-asparagine, 100 mg ascorbic acid, 4 mg $AgNO_3$, 600 mg $MgCl_2$, 0.2 mg BAP, 2.5 mg 2, 4-D, 4 g gelrite
Liquid infection medium	MS B5 medium 4.4 g, 30 g glucose, 500 mg L-proline, 500 mg casein hydrolysate,100 μM acetosyringone, pH 5.2
Co-cultivation medium	MS B5 medium 4.4 g, 30 g glucose, 500 mg L-proline, 500 mg casein hydrolysate,40 mg cystiene, 2.5 mg 2, 4-D, 100–200 μM acetosyringone, pH 5.2, 4 g gelrite
Resting medium	MS B5 medium 4.4 g, 30 g sucrose, 500 mg L-proline, 500 mg casein hydrolysate, 5 mg L-glutamine, 40 mg cystiene, 2.5 mg 2, 4-D, 250 mg cefotaxime, 250 mg carbenicillin, 4 g gelrite
Selection medium	MS B5 medium 4.4 g, 30 g sucrose, 2 mg dicamba, 1 mg picrolam, 250 mg cefotaxime, 250 mg carbenicillin, 50 mg hygromycin, 4 g gelrite
Regeneration medium	MS B5 medium 4.4 g, 40 g sucrose, 500 mg carbenicillin, 2.5 mg BAP, 1 mg zeatin, 0.2 mg NAA, 0.5 mg TDZ, 40 mg hygromycin, 3 g phytagel
Rooting medium	MS B5 medium 2.2 g, 10 g sucrose, 30 mg hygromycin, 250 mg cefotaxime, 4 g gelrite

resistant calli was sub-cultured for an additional 10 days in the same medium. The hygromycin resistant calli, which survived after two rounds of selection were transferred to regeneration medium (Table 1) kept in the dark for one week. The regenerated rice shoots were separated and transferred to fresh tubes containing half MS B5 media supplemented with 1% sucrose. For rooting, the explants were transferred to the rooting medium after which the plantlets were hardened and transferred to controlled glasshouse conditions. These plants were used for further transgenic analysis.

GUS histochemical staining assay

The histochemical assay for *gus* gene expression was performed with the putative transformant calli of the three rice varieties according to Jefferson (1987) using 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-Gluc - Duchefa, The Netherlands) as the substrate. The GUS assay buffer contained 0.1 M sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, 10 mM EDTA, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide and 0.1 M X-Gluc. The samples were incubated overnight at 37 °C and cleared in 50% ethanol for 4 h to clean the tissue before observation. The transient transformation frequency was calculated by the blue spots which were observed 3 day's post-transformation. The frequency of transient transformation is expressed as the percentage of calluses showing GUS spots over the total number of callus kept for staining (calculated following De Clercq et al. 2002). To determine the optimum conditions for transformation, the conditions were changed each time and the effects on the percentage transient *gus* expression was calculated. The calli were observed under a stereo microscope (Stereo Discovery V8 - Carl Zeiss) and were photographed by the attached digital camera (Carl Zeiss Axio Cam).

Genomic DNA isolation and Southern blot analysis

Genomic DNA was isolated from young leaves of the putative transgenic plants following the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol described by Doyle and Dickson (1987). 15 μg of genomic DNA from putative transgenic plants and negative controls (non-transformed plants) were digested by *Xho* I for *hpt* gene confirmation and by *Hind* III for identifying the independent transformation events in southern positive plants by overnight incubation at 37 °C. The digested samples were run on 0.8% agarose gel prepared in 1X TBE overnight at 30 V and transferred onto positively charged nylon membrane (Hybond N^+, Amersham Pharmacia) by capillary blotting. The membrane was UV cross-linked by exposure to ultraviolet light using Stratalinker UV crosslinker (Stratagene, UK). A 1.1 kb *Xho* I digested fragment of *hpt* gene was used as probe. Probe was prepared as the manufacturer's instructions (Roche Diagnostics Inc., Mannheim, Germany). Hybridization was carried out at 55 °C. All other steps like hybridization, washing, detection of the blot etc. were carried out according to the manufacturer's instructions.

Data analysis

All data were subjected to analysis of variance (ANOVA) and caparisons of the mean were made with the Tukey's least significant difference test at $P \leq 0.05$.

Results and discussion

Callus induction

The optimum conditions for the callus induction and plant regeneration from mature seeds in the three rice varieties -

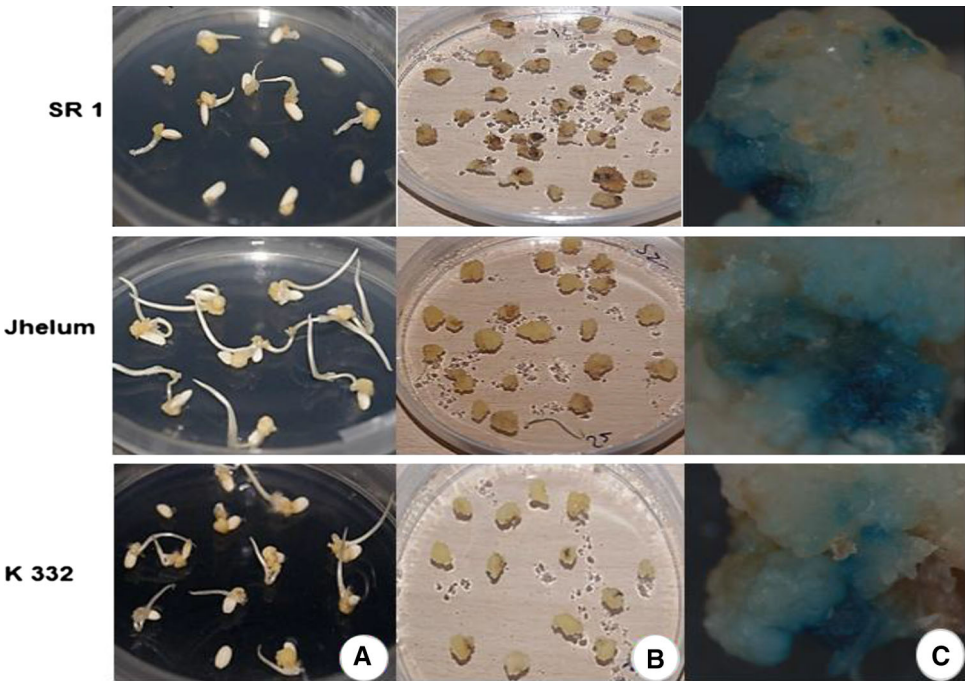

Fig. 1 Callus induction, transformation and regeneration of transgenic rice. Three rice varieties, VIZ., SR 1, Jhelum and K 332 were used: (**A**) 6 days old scutellar-derived calli with seed, (**B**) calli on the co-cultivation medium, (**C**) blue spots showing transient GUS expression

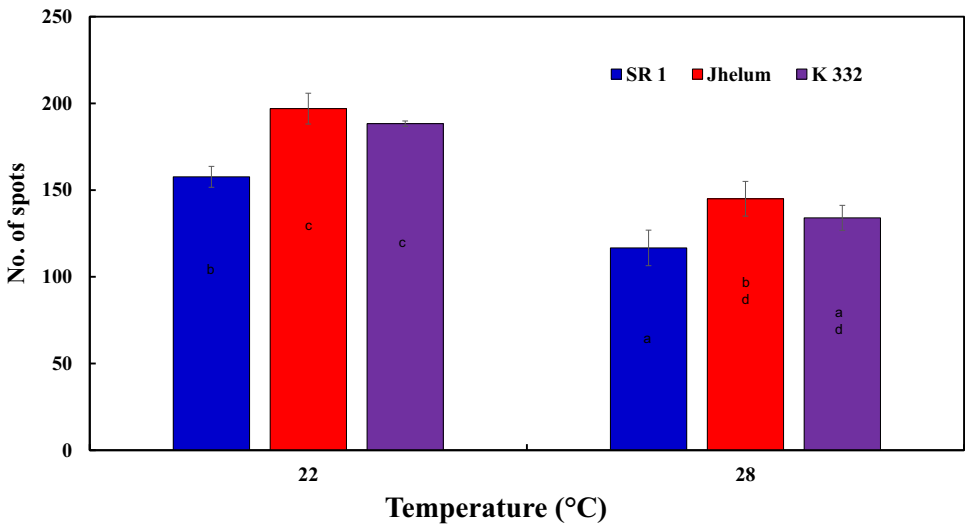

Fig. 2 Effect of temperature during co-cultivation period on transient GUS expression. The experiments were carried out with 50 calli and repeated three times in all the rice varieties. *Different small letters* mean that they significantly differ from each other at $P = 0.05$. *Vertical bars* indicate standard deviation

Shalimar Rice 1, Jhelum and K 332 have been already standardised in our lab (Fig. 1A). Maximum callus induction was observed in MS B5 media containing 3% maltose, 500 mg l^{-1} L-proline, 500 mg l^{-1} casein hydrolysate, 5 mg l^{-1} L-glutamine, 40 mg l^{-1} cystiene, 5 mg l^{-1} L-asparagine, 100 mg l^{-1} ascorbic acid, 4 mg l^{-1} $AgNO_3$, 600 mg l^{-1} $MgCl_2$ supplemented with 4 g l^{-1} gelrite, 0.2 mg l^{-1} BAP and 2.5 mg l^{-1} 2,4-D. The initiation of callus formation started just after 5–7 days on the scutellum region. In rice, maltose as a carbon source has been recommended for the embryogenic calli induction (Lin and Zhang 2005; Zaidi et al. 2006; Jadhav et al. 2011). 2,4-dichlorophenoxyacetic acid (2,4-D) is very effective in callus induction (Shahsavari et al. 2010) and L-proline has a positive impact on calli and its regeneration ability (Shahsavari 2010).

Agrobacterium mediated transformation

Since co-cultivation with Agrobacterium decreases embryogenesis and regeneration frequency (He et al. 2010), we optimized the conditions of co-cultivation. The embryonic calli dried on sterile tissue paper were co-cultured on plates containing co-cultivation medium (Fig. 1B). The plates were incubated in dark for 48–72 h at 22–28 °C. The effect of different concentrations of acetosyringone and temperature

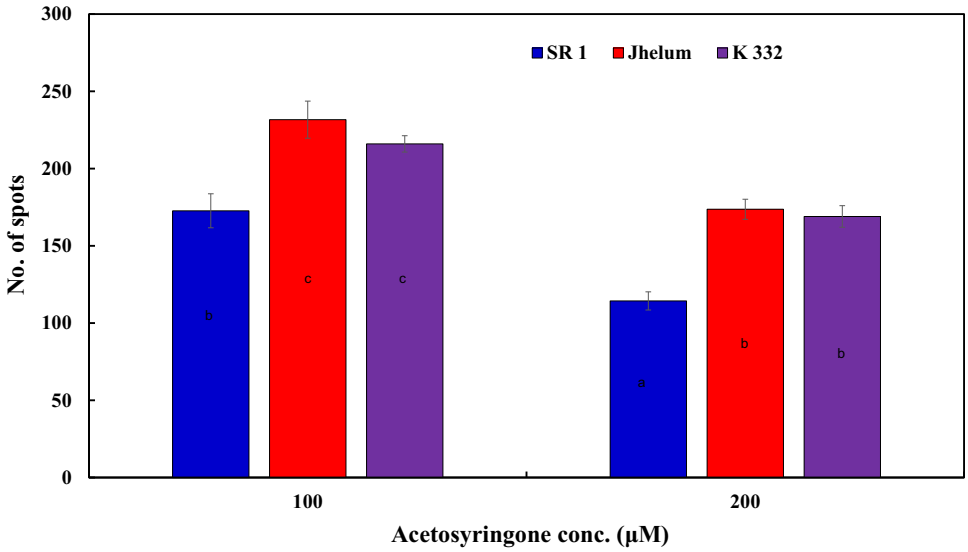

Fig. 3 Effect of different concentrations of acetosyringone during co-cultivation period on transient GUS expression. The experiments were carried out with 50 calli and repeated three times in all the rice varieties. *Different small letters* mean that they significantly differ from each other at $P = 0.05$. *Vertical bars* indicate standard deviation

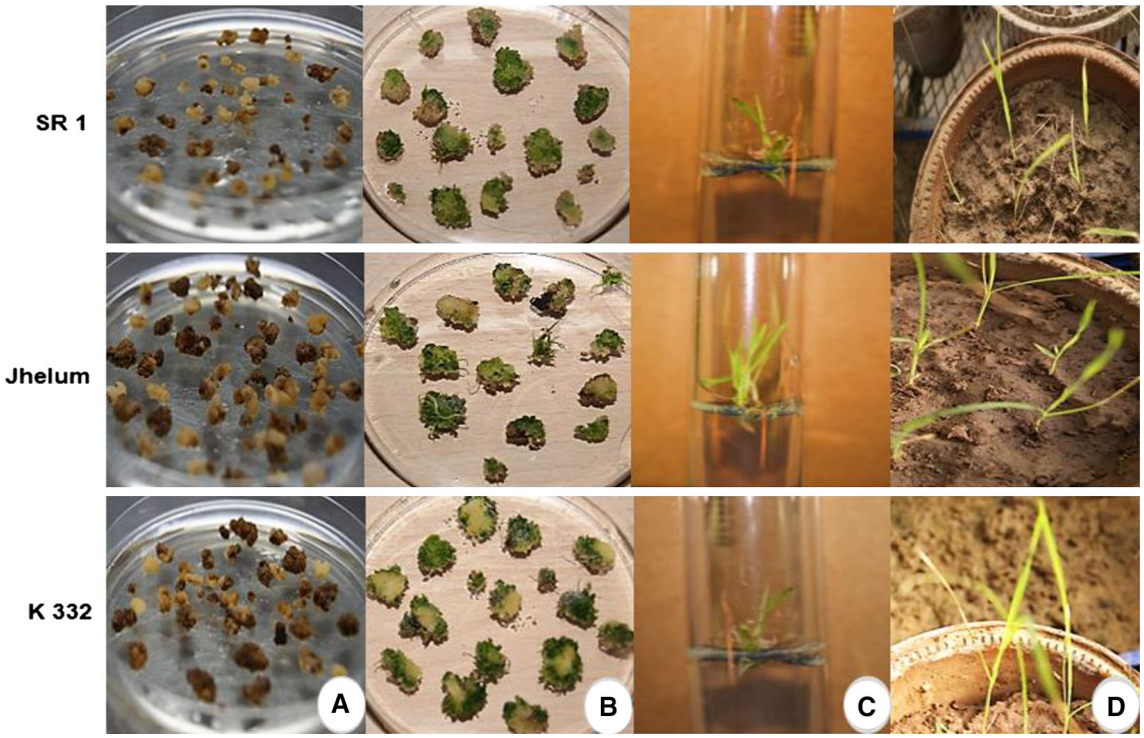

Fig. 4 (**A**) Calli on the selection medium containing 50 mg l^{-1} hygromycin, (**B**) calli showing initiation of regeneration of shoots, (**C**) plantlets in the glass tubes for elongation and rooting, (**D**) plants regenerated in the green house

during co-cultivation period on transient GUS expression was observed (Fig. 1C). The GUS expression was more at 22 °C as compared to 28 °C in all the rice varieties (Fig. 2). The Agrobacterium overgrowth in the calli kept at 22 °C and 48 h was less as compared to co-cultivation at 28 °C and 72 h in all the rice varieties. Furthermore, co-cultivation at 28 °C and 72 h resulted in necrosis of the calli. The overall transformation efficiency of Shalimar Rice 1, Jhelum and K 332 were 5.7, 9.25 and 7.69% respectively. Temperature during co-cultivation affects the T-DNA transfer during *Agrobacterium*-mediated transformation (Subramaniam and Rathinam 2010). Co-cultivation in presence of 100 µM acetosyringone resulted in substantial GUS expression at pH 5.2 in all the rice varieties (Fig. 3). Similar results have been reported by several workers in the genetic transformation of *indica* rice cultivars (Kumar et al. 2005; Švábová and Griga 2008; Karthikeyan et al. 2012).

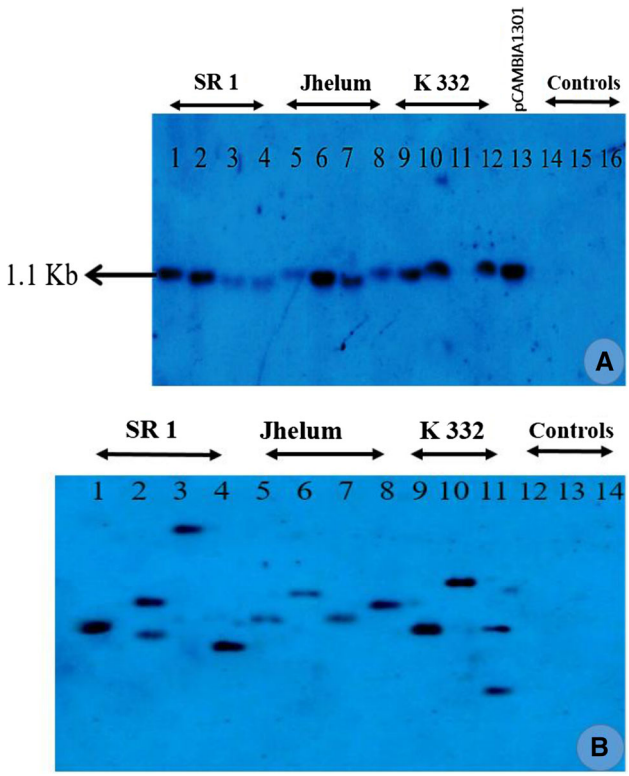

Fig. 5 Southern hybridization of transgenic plants. About 15 µg of genomic DNA from transformed and non-transformed plants was digested with (**A**) *Xho* I; *Lanes 1 to 4*—SR 1, *5 to 8*—Jhelum, *9 to 12*—K 332, *13*—Plasmid pCAMBIA1301 and *14 to 16*—Non-transformed control DNAs of SR 1, Jhelum and K 332 respectively and (**B**) *Hind* III; *1 to 4*—SR 1, *5 to 8*—Jhelum, *9 to 11*—K 332 and *12 to 14*—Non-transformed control DNAs of SR 1, Jhelum and K 332 respectively

Addition of acetosyringone in the co-cultivation medium has been reported to induce vir genes, extend host range to Agrobacterium strains and was found to be essential for rice transformation (Mohanty et al. 1999; Hoque et al. 2005; Tyagi et al. 2007). The induction of *vir* genes and initiation of T-DNA transfer during *Agrobacterium*-mediated transformation occur better at acidic pH of co-cultivation medium (Huang and Wei 2005). The use of reductants such as ascorbic acid or L-cysteine in the co-cultivation medium increased the frequency of transformation (Enríquez-Obregón et al. 1999; Olhoft and Somers 2001).

The co-cultivated calli at 22 °C were then washed with 100 ml of double distilled water containing 250 mg l^{-1} cefotaxime and carbenicillin for 4–5 times. Similar concentration of cefotaxime and carbenicillin (250 mg l^{-1}) proved effective in preventing Agrobacterium overgrowth in rice (Kumar et al. 2005). 200–500 mg l^{-1} cefotaxime has been used for genetic transformation of indica rice cultivars (Kumria and Rajam 2002; Nazim-Ud-Dowla et al. 2008). Carbenicillin possesses auxin-like structural features thereby increasing the regeneration potential of the explants (Nauerby et al. 1997; Ling et al. 1998).

Selection and regeneration

The selection media containing 250 mg l^{-1} of cefotaxime and carbenicillin prevented Agrobacterium overgrowth and the use of 50 mg l^{-1} hygromycin resulted in killing of the untransformed calli (Fig. 4A). The hygromycin resistant calli, which survived after two rounds of selection were transferred to the regeneration medium containing MS B5 medium supplemented with 4% sucrose, 3 g l^{-1} phytagel, 2.5 mg l^{-1} BAP, 1 mg l^{-1} zeatin, 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} TDZ. BAP and NAA were found essential to facilitate regeneration in rice callus cultures (Rashid et al. 2004; Karthikeyan et al. 2009; Hussain et al. 2010; Karthikeyan et al. 2012). Thidiazuron (TDZ) has been reported to induce high-frequency somatic embryogenesis and organogenesis in monocots (Ganeshan et al. 2006; Cheruvathur et al. 2010; Deroles et al. 2010). After 15–25 days, the calli started to turn green in colour and the appearance of shoot primordial was noticed (Fig. 4B). The regenerated rice shoots were separated and transferred to glass tubes containing half MS B5 medium supplemented with 1% sucrose for elongation and rooting (Fig. 4C). After rooting, the plants were transferred to the pots containing 50% vermiculate mixed with soil for hardening. Pots were kept in the humidity chamber for 3–5 days and then transferred to green house (Fig. 4D).

Molecular analysis of the transgenic plants

DNA from the regenerated transgenic plants and control plants in all the three rice varieties were digested with *Xho* I enzyme to confirm for the *hpt* gene (1.1 kb) by Southern hybridization (Fig. 5A). DNA of the southern positive plants were subsequently digested by single cutter enzyme (*Hind* III) to identify the independent transformation events (Fig. 5B). Most of the plants had single copy T-DNA insertion but a few had multiple copy T-DNA insertions. All the confirmed transgenic plants were fertile and set seed normally. The overall regeneration frequency of Shalimar Rice 1, Jhelum and K 332 were 64, 75 and 77% respectively. This is the first report on Agrobacterium mediated transformation in these rice varieties. This protocol can be used further for genetic modifications in these rice varieties and might help in the transformation of other related rice genotypes.

Conclusions

The present protocol provides an efficient and rapid transformation system, which could be conveniently used for producing genetically modified plants.

Acknowledgements The authors acknowledge SKUAST-K for providing the plant material. The first author is grateful to Council of Scientific and Industrial Research (CSIR) for providing financial assistance as SRF during this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Arockiasamy, S., & Ignacimuthu, S. (2007). Regeneration of transgenic plants from two indica rice (*Oryza sativa* L.) cultivars using shoot apex explants. *Plant Cell Reports, 26*(10), 1745–1753.

Bajaj, S., & Mohanty, A. (2005). Recent advances in rice biotechnology—towards genetically superior transgenic rice. *Plant Biotechnology Journal, 3*(3), 275–307.

Brookes, G., & Barfoot, P. (2003). GM Rice: Will this lead the way for global acceptance of GM crop technology?. International Service for the Acquisition of Agri-Biotech Applications (ISAAA) (no. 28, p. 53). Ithaca, NY: ISAAA. ISBN:1-892456-33-8.

Cheng, M., Lowe, B. A., Spencer, T. M., Ye, X., & Armstrong, C. L. (2004). Factors influencing Agrobacterium-mediated transformation of monocotyledonous species. *Vitro Cellular and Developmental Biology-Plant, 40*(1), 31–45.

Cheruvathur, M. K., Abraham, J., Mani, B., & Thomas, T. D. (2010). Adventitious shoot induction from cultured internodal explants of *Malaxis acuminata* D. Don, a valuable terrestrial medicinal orchid. *Plant Cell, Tissue and Organ Culture (PCTOC), 101*(2), 163–170.

De Clercq, J., Zambre, M., Van Montagu, M., Dillen, W., & Angenon, G. (2002). An optimized Agrobacterium-mediated transformation procedure for *Phaseolus acutifolious* A. Gray. *Plant Cell Reports, 21,* 333–340.

Deroles, S. C., Seelye, J. F., Javellana, J., & Mullan, A. C. (2010). In vitro propagation of *Sandersonia aurantiaca* Hook using thidiazuron. *Plant Cell, Tissue and Organ Culture (PCTOC), 102*(1), 115–119.

Dong, J., Teng, W., Buchholz, W. G., & Hall, T. C. (1996). Agrobacterium-mediated transformation of Javanica rice. *Molecular Breeding, 2*(3), 267–276.

Doyle, J. J., & Dickson, E. E. (1987). Preservation of plant samples for DNA restriction endonuclease analysis. *Taxon, 36*, 715–722.

Endo, M., Shimizu, T., & Toki, S. (2012). *Selection of transgenic rice plants using a herbicide tolerant form of the acetolactate synthase gene* (pp. 59–66). Transgenic Plants: Methods and Protocols.

Enríquez-Obregón, G. A., Prieto-Samsónov, D. L., Gustavo, A., Pérez, M., Selman-Housein, G., & Vázquez-Padrón, R. I. (1999). Agrobacterium-mediated Japonica rice transformation: a procedure assisted by an antinecrotic treatment. *Plant Cell, Tissue and Organ Culture, 59*(3), 159–168.

Ganeshan, S., Chodaparambil, S. V., Båga, M., Fowler, D. B., Hucl, P., Rossnagel, B. G., et al. (2006). In vitro regeneration of cereals based on multiple shoot induction from mature embryos in response to thidiazuron. *Plant Cell, Tissue and Organ Culture, 85*(1), 63–73.

Ge, X., Chu, Z., Lin, Y., & Wang, S. (2006). A tissue culture system for different germplasms of indica rice. *Plant Cell Reports, 25*(5), 392–402.

Hadiarto, T., & Tran, L. S. P. (2011). Progress studies of drought-responsive genes in rice. *Plant Cell Reports, 30*(3), 297–310.

He, Y., Jones, H. D., Chen, S., Chen, X. M., Wang, D. W., Li, K. X., et al. (2010). Agrobacterium-mediated transformation of durum wheat (*Triticum turgidum* L. var. durum cv Stewart) with improved efficiency. *Journal of Experimental Botany, 61*(6), 1567–1581.

Hellens, R., Mullineaux, P., & Klee, H. (2000). Technical focus: a guide to Agrobacterium binary Ti vectors. *Trends in Plant Science, 5*(10), 446–451.

Hibberd, J. M., Sheehy, J. E., & Langdale, J. A. (2008). Using C 4 photosynthesis to increase the yield of rice—rationale and feasibility. *Current Opinion in Plant Biology, 11*(2), 228–231.

Hiei, Y., & Komari, T. (2008). Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nature Protocols, 3*(5), 824–834.

Hiei, Y., Komari, T., & Kubo, T. (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Molecular Biology, 35*(1–2), 205–218.

Hoque, M. E., Mansfield, J. W., & Bennett, M. H. (2005). Agrobacterium-mediated transformation of Indica rice genotypes: an assessment of factors affecting the transformation efficiency. *Plant Cell, Tissue and Organ Culture, 82*(1), 45–55.

Huang, X., & Wei, Z. (2005). Successful Agrobacterium-mediated genetic transformation of maize elite inbred lines. *Plant Cell, Tissue and Organ Culture, 83*(2), 187–200.

Hussain, Z., Khan, M. H., Bano, R., Rashid, H., & Chaudhry, Z. (2010). Protocol optimization for efficient callus induction and regeneration in three Pakistani rice cultivars. *Pakistan Journal of Botany, 42*(2), 879–887.

Jadhav, P. V., Mahendra, D., Saluja, T., Ak, S., Girish, C., & Ravi, S. (2011). Assessment of critical factors influencing callus induction, in vitro regeneration and selection of bombarded indica rice genotypes. *Journal of Cell and Plant Sciences, 2*(2), 24–42.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant molecular biology reporter, 5*(4), 387–405.

Kant, P., Kant, S., Jain, R. K., & Chaudhury, V. K. (2007). Agrobacterium-mediated high frequency transformation in dwarf recalcitrant rice cultivars. *Biologia Plantarum, 51*(1), 61–68.

Karthikeyan, A., Pandian, S. T. K., & Ramesh, M. (2009). High frequency plant regeneration from embryogenic callus of a popular indica rice (*Oryza sativa* L.). *Physiology and Molecular Biology of Plants, 15*(4), 371–375.

Karthikeyan, A., Shilpha, J., Pandian, S. K., & Ramesh, M. (2012). *Agrobacterium*-mediated transformation of *indica* rice cv. ADT 43. *Plant Cell Tiss Organ Cult, 109,* 153–165.

Khush, G. S., & Brar, D. S. (2001). Rice genetics from Mendel to functional genomics. In: G. S. Khush, D. S. Brar, B. Hardy (Eds.), *Rice genetics IV: proceedings of the fourth international rice genetics symposium* (pp. 3–25). World Scientific Publishing Co. Pte. Ltd. http://www.worldscibooks.com/lifesci/6842.

Kumar, K. K., Maruthasalam, S., Loganathan, M., Sudhakar, D., & Balasubramanian, P. (2005). An improved Agrobacterium-mediated transformation protocol for recalcitrant elite indica rice cultivars. *Plant Molecular Biology Reporter, 23*(1), 67–73.

Kumar, V., Shriram, V., Kishor, P. K., Jawali, N., & Shitole, M. G. (2010). Enhanced proline accumulation and salt stress tolerance of transgenic indica rice by over-expressing P5CSF129A gene. *Plant Biotechnology Reports, 4*(1), 37–48.

Kumria, R., & Rajam, M. V. (2002). Alteration in polyamine titres during Agrobacterium-mediated transformation of indica rice with ornithine decarboxylase gene affects plant regeneration potential. *Plant Science, 162*(5), 769–777.

Lin, Y. J., & Zhang, Q. (2005). Optimising the tissue culture conditions for high efficiency transformation of indica rice. *Plant Cell Reports, 23*(8), 540–547.

Lin, J., Zhou, B., Yang, Y., Mei, J., Zhao, X., Guo, X., et al. (2009). Piercing and vacuum infiltration of the mature embryo: a simplified method for Agrobacterium-mediated transformation of indica rice. *Plant Cell Reports, 28*(7), 1065–1074.

Ling, H. Q., Kriseleit, D., & Ganal, M. W. (1998). Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in Agrobacterium-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). *Plant Cell Reports, 17*(11), 843–847.

Mohanty, A., Sarma, N. P., & Tyagi, A. K. (1999). Agrobacterium-mediated high frequency transformation of an elite indica rice variety Pusa Basmati 1 and transmission of the transgenes to R2 progeny. *Plant Science, 147*(2), 127–137.

Nauerby, B., Billing, K., & Wyndaele, R. (1997). Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of Agrobacterium tumefaciens. *Plant Science, 123*(1), 169–177.

Nazim-Ud-Dowla, M. A. N., Ahmed, N. U., & Hassan, L. (2008). Optimization of Agrobacterium-mediated genetic transformation in indica rice. *Thai Journal of Agricultural Science, 41,* 127–133.

Nishimura, A., Aichi, I., & Matsuoka, M. (2006). A protocol for Agrobacterium-mediated transformation in rice. *Nature Protocols, 1*(6), 2796–2802.

Olhoft, P., & Somers, D. (2001). L-Cysteine increases Agrobacterium-mediated T-DNA delivery into soybean cotyledonary-node cells. *Plant Cell Reports, 20*(8), 706–711.

Ramesh, M., Murugiah, V., & Gupta, A. K. (2009). Efficient in vitro plant regeneration via leaf base segments of indica rice (*Oryza sativa* L.). *Indian Journal of Experimental Biology, 47,* 68–74.

Rashid, H., Saleem, M., Chaudhry, Z., Gilani, S. T., & Qureshi, A. S. (2004). Studies on developing a high regeneration from seed derived calli of rice (*Oryza sativa* L.) Cv Super basmati. *Pakistan Journal of Biological Sciences, 7,* 273–276.

Sahoo, K. K., Tripathi, A. K., Pareek, A., Sopory, S. K., & Singla-Pareek, S. L. (2011). An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. *Plant methods, 7*(1), 1.

Saika, H., & Toki, S. (2010). Mature seed-derived callus of the model indica rice variety Kasalath is highly competent in Agrobacterium-mediated transformation. *Plant Cell Reports, 29*(12), 1351–1364.

Savary, S., Willocquet, L., Elazegui, F. A., Castilla, N. P., & Teng, P. S. (2000). Rice pest constraints in tropical Asia: quantification of yield losses due to rice pests in a range of production situations. *Plant Disease, 84*(3), 357–369.

Shahsavari, E. (2010). Evaluation and optimizations of media on the tissue culture system of upland rice. *International Journal of Agriculture and Biology, 12*(4), 537–540.

Shahsavari, E., Maheran, A. A., Akmar, A. S. N., & Hanafi, M. M. (2010). The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice. *African Journal of Biotechnology, 9*(14), 2089–2094.

Shobarani, N., Prasad, G. S. V., Prasad, A. S. R., Sailaja, B., Muthuraman, P., Numeera, S., Viraktamath, B. C. (2010). Rice Almanac–India. In *DRR technical bulletin No. 5* (pp. 6–7) Directorate of Rice Research, Rajendranagar, Hyderabad.

Sripriya, R., Sangeetha, M., Parameswari, C., Veluthambi, B., & Veluthambi, K. (2011). Improved Agrobacterium-mediated co-transformation and selectable marker elimination in transgenic rice by using a high copy number pBin19-derived binary vector. *Plant Science, 180*(6), 766–774.

Subramaniam, S., & Rathinam, X. (2010). Emerging factors that influence efficiency of T-DNA gene transfer into Phalaenopsis violacea orchid via Agrobacterium tumefaciens-mediated transformation system. *International Journal of Biology, 2*(2), 64.

Švábová, L., & Griga, M. (2008). The effect of cocultivation treatments on transformation efficiency in pea (*Pisum sativum* L.). *Plant Cell, Tissue and Organ Culture, 95*(3), 293–304.

Tie, W., Zhou, F., Wang, L., Xie, W., Chen, H., Li, X., et al. (2012). Reasons for lower transformation efficiency in indica rice using Agrobacterium tumefaciens-mediated transformation: lessons from transformation assays and genome-wide expression profiling. *Plant Molecular Biology, 78*(1–2), 1–18.

Tyagi, H., Rajasubramaniam, S., & Dasgupta, I. (2007). Regeneration and Agrobacterium-mediated transformation of a popular indica rice variety, ADT39. *Current Science, 93*(5), 678–683.

Xiao, G. Y. (2009). Recent advances in development of herbicide resistant transgenic hybrid rice in China. *Rice Science, 16*(3), 235–239.

Yaqoob, U., Jan, B., Kaul, T., & Nawchoo, I. A. (2016). Tissue culture of rice: Problems, progress and prospects. *IJISET, 3*(6), 581–613.

Yookongkaew, N., Srivatanakul, M., & Narangajavana, J. (2007). Development of genotype-independent regeneration system for transformation of rice (Oryza sativa ssp. indica). *Journal of Plant Research, 120*(2), 237–245.

Zaidi, M. A., Narayanan, M., Sardana, R., Taga, I., Postel, S., Johns, R., et al. (2006). Optimizing tissue culture media for efficient transformation of different indica rice genotypes. *Agron Res, 4*(2), 563–575.

Zeigler, R. S., & Barclay, A. (2008). The relevance of rice. *Rice, 1*(1), 3–10.