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ORIGINAL ARTICLE

An efficient and universal *Agrobacterium*-mediated transformation protocol in rice

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Abstract For development of transgenic varieties of interest in rice, we have developed a simple, efficient and universal *Agrobacterium* mediated transformation protocol. Mature seeds of two *indica* (IR64 and Jaya), one each from *japonica* (AC41039) and *aromatic* (Basmati370) varieties were used as explants in the present study. *Agrobacterium tumefaciens* strain EHA 105 carrying Ti plasmid (pBI121) with the selectable marker npt^{II} along with the reporter gene uidA encoding β-glucuronidase (GUS) was successfully integrated with rice genome without use of acetosyringone. Sterile distilled water washing in place of cefotaxime in the elimination process has been used to control excess growth of *Agrobacterium*. All the material after transformation germinated in 2 to 3 days of co-cultivation on MS basal medium. Germinated seeds transferred to the selection medium i.e. plain MS medium with 50 mg/l of kanamycin, produced two to three primary tillers within 2 weeks. Mesocotyls from 2 week old in vitro grown plants were taken and cultured in the multiplication medium (MS supplemented with 0.5 mg/l BA and 50 mg/l Kanamycin) where within 6–8 days they produced 3–4 secondary tillers. All the five to six tiller shoots so produced in the process developed roots on plain MS and grew well when transferred to pots containing autoclaved soil and vermicompost in the proportion of 4:1. Transient expression of GUS was observed in all the tissues of recipient plants. Integration of the transgene was confirmed by employing southern blotting and real-time PCR technique. The transformation protocol developed can be efficiently used across the two major subspecies/ecotypes of the Asian rice cultivar *Oryza sativa*.

Keywords Rice · Mature seed · Mesocotyl · Acetosyringone · Transformation · Copy number

Abbreviations

CTAB	Hexadecyl-trimethyl-ammonium bromide
q-PCR	Quantitative PCR
T-DNA	Transfer DNA
C_T or Ct	Threshold cycle
RQ	Relative quantification

Introduction

Recombinant DNA technology is a potential tool to move genes of interest across sexually incompatible species and find thereby solution to problems that defy conventional breeding/selection approaches. Efficient protocols of tissue culture traits like regeneration and transformation are important prerequisites for successful development of transgenics of interest. Of them regeneration of plants by cell/tissue culture has been the challenging task across crop species in general and monocots in particular and rice is no exception. Despite efficient culture system already available for well responding model varieties such as Nipponbare (*japonica*) and Kasalath (*indica*), many of the popular varieties of both *indica* and *japonica* origin offer difficulty in both regeneration as well as transformation process. Regeneration efficiency is affected by a number of factors which include genotype, type and physiological status of explant,

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composition of culture medium, plant growth regulators and culture conditions (Rueb et al. 1994; Ge et al. 2006). Of them, genotype is the most important. As of now, many different protocols have been developed for *Agrobacterium*-mediated transformation in different sub-species of *O.sativa*. For instance, Ge et al. (2006) have developed variety specific regenaration-transformation protocols for different varieties of *indica* rice, while Nishimura et al. (2007) have developed *Agrobacterium*-mediated transformation protocol for elite *japonica* varieties. Likewise for *aromatic* varieties also many standardized protocols are available (Rashid et al. 2003; Rao and Rao 2007). Standardization of genotype specific protocols for so many varieties/subspecies is however quite laborious and time consuming, warranting thus the need and urgency for development of a universal protocol for use across genotypes.

Keeping in view the need for a transformation protocol usable across a varieties/subspecies the present study was undertaken with the sole objective of developing an efficient *Agrobacterium*-mediated transformation system. To represent fairly divergent germplasm, varieties belonging to the two major subspecies of *Oryza sativa* viz *indica* and *japonica* as well as a basmati type, which is considered to be different from both recognized sub species were used as test germplasm. The efficiency of the regeneration protocol was assessed from the frequency of multiple shoot formation while transformation by Real time PCR and Southern blot analysis for putative transformants. The real time PCR (TaqMan assay) analysis was done with reference to sucrose phosphate synthase (SPS), a single copy endogenous control gene (Ding et al. 2004; Wang et al. 2010 and Weng et al. 2004).

Materials and methods

Plant material and surface sterilization

Four rice genotypes viz., IR64 and Jaya (*indica*), AC41039 (*japonica*) and Basmati370 (*aromatic*) have been chosen to represent the rice sub-species/ecotype for transformation. Seeds of the genotypes were dehusked and after washing in SDW rinsed with Tween 20, a liquid detergent solution for 15–20 min followed by 0.1% (w/v) $HgCl_2$ for 7–8 min for surface sterilization. Finally, the seeds were thoroughly rinsed in sterile double distilled water for at least four times under aseptic conditions and used as the source material for both in vitro culture and transformation with *Agrobacterium*.

Agrobacterium mediated transformation and subculturing

Surface sterilized dehusked rice seeds of the four varieties and the *Agrobacterium s*train EHA105 carrying a disarmed Ti plasmid (pBI121) with the selectable marker gene npt^{II} that confers resistance against kanamycin to transformed cells along with the reporter gene (*uidA*) that encodes β-glucoronidase (GUS) ware taken for transformation. The *Agrobacterium tumefaciens* strain grown on 30 ml of YEM [Yeast extract(0.4 g) + mannitol(10.0 g) + NaCl (0.1 g) + $MgSO_4.7H_2O$(0.2 g) + K_2HPO_4(0.5 g) in one litre of water] medium with kanamycin (50 mg/l) was set for shaking at 150 rpm at 28^0 C for 20 h by inoculating a single colony with a sterile loop.10 ml of overnight grown bacterial culture diluted to $1/5^{th}$ with fresh YEM medium (adjusted $O.D_{600}$.-0.3-0.6) poured in a petridish (90 mm) containing 10 seeds of each variety. Seeds were slightly pounced with a pointed needle mixed with the bacterial suspension by gentle swirling and immersed in the same for 35 min. Seeds were blot in sterile tissue paper and placed on the co-cultivation medium CCM Murashige and Skoog (1962) basal medium +3% sucrose + 0.8% agar(pH-5.8) for 2 days at 25 ± 2^0 C in a culture room for co-cultivation. For elimination of *Agrobacterium* the seeds were simply washed 2 to 3 times in sterile distilled water and blot dried with sterile filter paper. Later the seeds were placed on selection medium (CCM +50 mg/l Kanamycin). Mesocotyls (containing multiple meristems) from 2 week old in vitro grown plants derived from the selection medium were transferred onto multiplication medium (full strength MS medium with 0.5 mg/l of 6-benzyladenine with 50 mg/l kanamycin) for shoot proliferation. Then these shoots were transferred to MS basal medium + 3% Sucrose + 0.8% Agar (pH-5.8) for rooting. Then for hardening, all the plants of (10 of each variety) were transferred to pots containing 4:1 ratio of autoclaved soil and vermicompost.

Media preparation, culture condition and data analysis

MS (Murashige and Skoog 1962) salt composition, sucrose (30 g/l) and bacteriological grade agar-agar (8 g/l) were used throughout the study with or without incorporation of growth regulators and the pH was adjusted to 5.8. All the cultures were incubated in a culture room maintained at 25± 2°C under 16 h/8 h light/dark cycle, 45 μmol m^{-2} s^{-1} irradiance level was provided by cool white fluorescent tubes (Philips, India) and with 55–60% relative humidity. The experiment was repeated thrice with each treatment consisted of 10 explants. Duncan's multiple range test (DMRT) was applied for comparison of regeneration results in terms of number of shoots and shoot length for the four cultivars.

GUS assay

The plants obtained from the process of *Agrobacterium* transformation were placed in petriplates containing GUS assay solution consisting of 10 mg X-gluc (5-bromo-4-choloro-3-indolyl, β-D-glucuronic acid), 100 μl dimethyl formamide (DMF), 1 ml of 1 M sodium phosphate (pH 7.0) and 1 ml of the detergent Triton X-100. The volume was made up to 20 ml

with distilled water and the solution was stored in dark in refrigerator for the assay. A solution of X-Gluc was prepared, aliquoted, and stored frozen until needed. A positive control was included to monitor the extent of penetration of the substrate into the material. The plants were incubated at 37°C overnight and placed in 70% ethanol to bleach chlorophyll so as to make it easier to see the blue staining of the plant tissue. The plants were placed into fresh 70% ethanol at least three times till the plant tissues became light green.

PCR analysis

Presence of the transgene in transformed and non-transformed plants was confirmed by PCR analysis. Genomic DNA was extracted from leaves using modified CTAB method (Cheng et al. 2003). Presence of GUS and *npt*^{II} was confirmed by amplification of PCR specific primer sequences 5′TTCGCGTCGGCATCCGCTCAGTGGCA3′ and 3′ GCGGACGGGTATCCGGTTCGTTGGCA5′ for uidA (GUS) 5′GAGGCTATTCGGCTATGACTG3′and 3′ ATCGGGAGGGGCGATACCGTA5′ for *npt*^{II}. Each PCR reaction was performed in 25 µl (total volume) of reaction mixture consisting of approximately 50 ng of genomic DNA obtained from the transgenics showing Gus expression, 1 X PCR buffer, 50 µM dNTPs, 1.5 mM $MgCl_2$, 5 pmoles of each primer and 1 unit of Taq DNA polymerase. PCR was carried out in a thermal cycler (ABI Veriti) using the following conditions: 94°C for 4 min as preheating, 35 cycles of 94°C denaturing for 1 min, 58°C (for *uidA* g)/55°C (for *npt*^{II}) annealing for 1 min and 72°C extension for 1 min and 10 min at 72°C final extension. Amplified DNA fragments were electrophoresed on 1.2% agarose gel detected by ethidium bromide staining and photographed under ultraviolet light of Bio-Rad Molecular Imager Gel Doc XR System.

Real-Time PCR analysis

Real time PCR (RT-PCR) was carried out in Applied Boisystems 7500 Real-Time PCR System with Software v2.0. The corresponding nucleotide sequence of the SPS F-5′-TTG CGC CCT GAA CGG ATA T-3′;SPS R- 5′-CGG TTG ATC TTT TCG GGA TG-3′; SPS P - FAM-TCC GAG CCG TCC GTG GGT/GB and GUS F-5′TTA ACT ATG CCG GAA TCC ATC GC-3′; GUS R- 5′AAC GCT GAC ATC ACC ATT GGC-3′ GUS P- FAM-ATG CTC TAC ACC ACG CCG AAC/GB were taken for amplification. All the primers and fluorogenic probes were synthesized and taken from Integrated DNA technologies, RFCL Limited. The SPS and GUS probes were labeled with FAM™ reporter dye at 5′ end and Quencher NFQ-MGB (non fluorescent quencher—minor groove binder) at 3′ end. For q-PCR each reaction mixture of 25 µl containing 12.5 µl of TaqMan universal PCR master mix of 2.0X (P/N 4304437—It contains AmpliTaq Gold® DNA, AmpErase Uracil-N-glycosylase, dNTPs with dUTPs, optimized buffer components, and Passive Reference dye); 50 ng of cDNA sample synthesized from RNA isolated from individual transgenic lines (by Promega Kit), 750nM of each SPSF/R primers with 300nM flurogenic SPSP probe and 500nM GUSF/R primer and 150nM GUSP probe. The amplification conditions were 50°C for 2 min(holding stage-UNG incubation),95°C for 10 min(holding stage for AmpII TaqGold activation),cycling stage of 40 cycles for 30s at 94°C,annealing at 56°C of 30s and extension of 30s at 72°C. Each sample was quantified in triplicate.

Calculation of relative gene expression level

To analyze data from real-time quantitative PCR experiments relative quantification (RQ) was done. The $2^{-\Delta\Delta Ct}$ method was used being a convenient way to analyze relative changes in gene expression from real-time quantitative PCR experiments (Pfaffl 2001; Pfaffl et al. 2002; Livak and Schmittgen 2001; Yuan et al. 2006). The experiment involved control and treatment sample. For each sample, a target gene and a reference gene for internal control were included for PCR amplification. The threshold cycle C_T (Ct) is the cycle number from the amplification curves, at which the amount of amplified target gene reaches a fixed threshold in a real-time PCR assay.

For relative quantification (RQ) comparative Ct method was adopted and CV, SD was calculated for validation of reliability and reproducibility of the results.

Southern blot analysis

Ten micrograms of each DNA of four transgenic rice varieties and plasmid DNA were completely digested with *HindIII*. The digested DNA resolved in 0.8% agarose gel and then transferred into nitrocellulose membrane. A 800 bp *npt*^{II} (amplified PCR product) DNA fragment was used as the hybridized probe by using Amersham ECL Direct Nucleic Acid Labeling and Detection Systems (GE healthcare).Hybridization was performed at 42°C for 24 h and the filter was washed at room temperature with 2x SSC/0.1%SDS and 1xSSC/0.1%SDS for 10 min each and at 42°C with 0.2x SSC/0.1% SDS for 30 min. DNA markers (λ DNA digested with *Hind III*) were run on the same gel.

Results and discussion

Standardisation of germination and multiplication protocol

Of different medium with various hormone composition tried for in vitro seed germination and plant multiplication of mesocotyl explants derived from germinated rice seeds. MS medium without any hormone supplement was found to

be the best. Mesocotyls from germinated seeds of Basmati 370, Jaya, IR64 and AC41039 when cultured on MS medium with BA and KN alone or in combination, the medium containing 0.5 mg/l BA produced three to five shoots. Among the four varieties tried, Basmati 370 responded comparatively with higher shoot multiplication than other three varieties (Table 1). As use of plain MS and MS with BA supplement proved effective and rewarding in terms of shoot formation in the standardization process, so this medium composition was used for transformation/regeneration in the respective protocol.

Agrobacterium infection and shoot multiplication

Dehusked seeds of the four rice varieties punctured and co-cultivated with the *Agrobacterium s*train EHA 105 carrying the disarmed Ti plasmid (pBI121) with the selectable marker (npt^{II}) and the reporter gene (uidA) germinated on co-cultivation medium (CCM) (Table 2 and Fig. 1a) and produced 2 to 3 primary tillers after 1 week of culturing on selection medium (SM) (Fig. 1b). After 2 weeks, the primary tillers were cut leaving the mesocotyls with multiple meristems and placed in the rooting medium. The mesocotyls when raised on the multiplication medium (selection medium with 0.5 mg/l of BA) for its secondary passage resulted again in 2–4 secondary tillers with well developed roots (Table 3 and Fig. 1c). The individual shoots derived from the primary and secondary tillers when cut at the base and grown on the same MS medium for rooting produced 2–3 well developed roots (Fig. 1d). As against many an attempt made earlier to develop efficient transformation protocol in rice through indirect organogenesis, the present finding is the first successful direct method (Tyagi et al. 2007; Rao and Rao 2007).

Effect of acetosyringone, cefotaxime and kanamycin concentration on transformation and regeneration efficiency

In the absence of acetosyringone, in the co-cultivation medium all the four genotypes responded with 100% transformation as they were growing well on the selection medium (Fig. 1a). Nontransformed seeds either failed to germinate or showed rudimentary growth and died in the selection medium.

Use of acetosyringone in co-cultivation possibly accelerated the pace of *Agrobacterium* growth which was not easy to control with cefotaxime treatment. Acetosyringone is a phenolic compound secreted from wounded tissues of dicotyledons. Monocot species which lack *vir* inducing compounds though impair *Agrobacterium* mediated transformation in them. Importance of acetosyringone in *Agrobacterium* mediated transformation of monocot species has thus seen emphasized by various workers viz in rice (Chan et al. 1993; Hiei et al. 1994), maize (Gould et al. 1991), wheat (Cheng et al. 1997) and sorghum (Pandey et al. 2010).As against the belief that addition of acetosyringone in the transformation and co-cultivation medium is important for to improving *Agrobacterium*-mediated transformation efficiency, in the present study, without using acetosyringone, 100% transformation frequency could be observed significantly. Only distilled water washing controlled the bacterial growth in the cocultivation medium. Addition of acetosyringone did not show any significant difference in the vector mediated transformation. Its use rather created a problem in controlling the growth of the bacterium during the co-cultivation process. The present findings were in conformity with those of Rao and Rao (2007) who have reported that in the absence of acetosyringone at bacterial pre-induction or co-cultivation or both the stages showed transient expression of transgenes in *indica* rice.

Table 1 Effects of various hormones on shoot multiplication of mesocotyl explants of different rice cultivars

Medium	Mesocotyls	Number of shoots	Shoot length	Number of Leaves
MS	Basmati370	4.5±0.008 a	3.9±0.05 a	8.0±0.30 b
	IR64	3.5±0.005 d	2.1±0.003e	7.1±0.26 c
	AC41039	3.8±0.005 b	2.1±0.003e	5.5±0.33 d
	Jaya	3.2±0.005 c	3.4±0.003 b	7.1±0.26 c
MS +0.5BA	Basmati370	4.5±0.008 a	3.9±0.05 a	10.±0.30a
	IR64	3.5±0.005 d	3.4±0.003 b	10.±0.30a
	AC41039	4.5±0.008 a	3.4±0.005 b	8.0±0.30 b
	Jaya	4.5±0.008 a	3.4±0.003 b	10.±0.30a
MS +0.5KN	Basmati370	4.5±0.008 a	2.8±0.005 c	7.1±0.26 c
	IR64	3.7±0.005 c	2.8±0.005 c	7.1±0.26 c
	AC41039	4.5±0.008 a	2.3±0.005 c	2.0±0.03 g
	Jaya	3.5±0.005 d	2.1±0.003 e	5.5±0.05 d
MS +0.5KN + 0.5BA	Basmati370	2.4±0.005 e	3.4±0.003 b	4.7±0.13 e
	IR64	2.4±0.005 e	3.4±0.003 b	4.7±0.13 e
	AC41039	2.4±0.005 e	2.7±0.005 d	4.3±0.08 e
	Jaya	2.4±0.005 e	2.8±0.005 c	3.3±0.03 f

Data (mean ± SE) of three independent experiments each with 10 replicates. Means followed by the same letter within the column are not significantly different ($p<0.05$) as tested by the multiple range test of Duncan (1955). Explants responded only to callus formation in 2,4D. Different conc. of BA and KN did not show any remarkable difference. (Data not shown). Shoots measuring <0.5 cm not taken into account for calculation of shoot length

Table 2 Results of germination from seeds of rice cultivars after infection with *Agrobacterium* on plain MS (CCM) for co-cultivation and shoot formation on selection medium(SM)

Seeds on CCM			Germinated seeds on SM		
MS without growth regulators	Response (%)	Days to germination	Mean number of shoots(primary tillers) (after 1 week)	Shoot length	Number of leaves
Jaya	100 ±0.000a	3.00 ±0.577a	1.53 ±0.033a	1.71 ±0.005b	3.97 ±0.005b
Basmati370	100 ±0.00a	3.00±0.577a	1.56±0.181a	2.9±0.057a	4.05±0.0288a
IR64	100±0.00a	2.90±0.577a	1.56±0.033a	1.72±0.005b	3.22±0.005c
AC41039	100 ±0.000a	3.21 ±0.577a	1.53 ±0.033a	1.71 ±0.005b	3.97 ±0.005b

Data (mean ± SE) of three independent experiments each with 10 replicates. Means followed by the same letter within the column are not significantly different ($P<0.05$) as tested by the multiple range test of Duncan (1955) Leaflets measuring <2 cm is not taken into account

Use of cefotaxime even at a low concentration of 5 mg/l in CCM medium or its wash before cocultivation for control of bacterial growth was found to cause a severe necrotic and detrimental effect resulting in inhibited regeneration of transformed explants. Instead of cefotaxime only distilled water washing was found to optimize the transformants growth in selection medium.

Use of kanamycin in different concentrations as a selectable marker as well was observed to affect the efficiency of plant transformation. The antibiotic used as 50 mg/l completely blocked regeneration process in untransformed seeds and thus

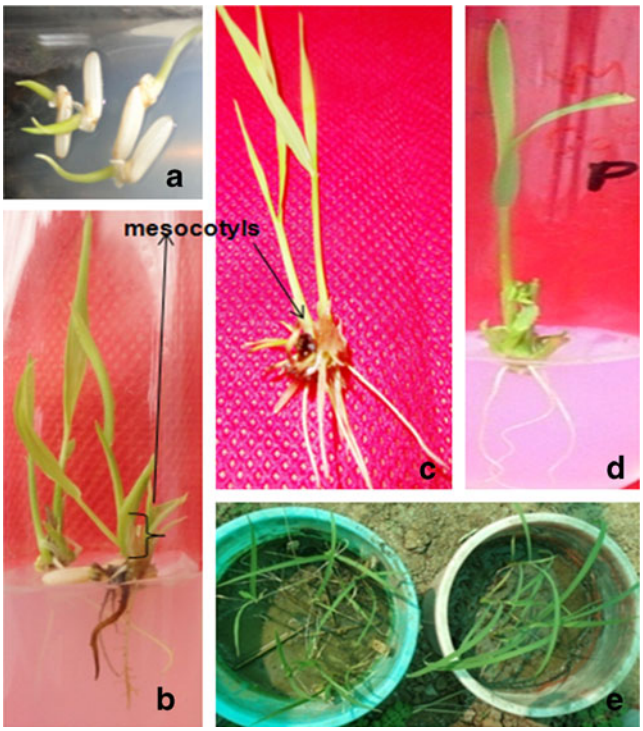

Fig. 1 In vitro germination and multiplication of transgenic shoots from mesocotyls of different rice varieties. a Germination of seeds on selection medium after infected with *Agrobacterium* (4 days old) **b** Clusters of primary shoots(from Basmati370 seeds on plane MS medium) **c** Secondary tillers from mesocotyls (Jaya mesocotyls on MS + BA 0.5 mg/l) **d** Shootlets rooted on MS basal media without any growth regulators (2 weeks old) **e** Hardened plants (5 weeks old)

was found useful to select transformed seedlings. This finding is in agreement with those reported of Indian mulberry, (*Moruus indica)* (Bhatnagar and Khurana 2003) and grasspea, (*Lathyrus sativus* L.) (Barik et al. 2005).

For all the transformation process as incubation period was 35 min while co-cultivation period 2 to 4 days. For selection of transformed plants 50 mg/l of kanamycin was used in the selection medium (MS with and without BA 0.5 mg/l). As for the differential response of genotypes and multiplication medium as summarized in Tables 2 and 3, Basmati370 to be the best in comparison to other varieties as and MS with 0.5 mg/l BA to be the best medium for multiplication. The mesocotyls with multiple meristems when cultured in MM produced 2–3 secondary tillers with well developed roots (Fig. 1c) within 6 to 8 days and every tiller when cut at the base and when cultured on plain MS medium produced roots (Fig. 1d) after 1 week. Regenerated transformants established well when transferred to plastic cups then to buckets (Fig. 1e).

GUS expression

To confirm the transformation, leaf samples drawn from both primary and secondary tillers, as well as from whole plants were subjected to GUS assay. All the plant parts and leaves drawn from both primary and secondary tiller shoots showed diffused green fluorescent spots appearing as patches or scattered individual spots (Fig. 2a and b) confirming them to be transformed. Use of the widely used GUS assay as a rapid way to detect the presence of *uidA* gene (GUS) in the putative transformants as described by Jefferson et al.(1987) revealed the *Agrobacterium* strain EHA105 mediated transformation to be highly efficient irrespective of genotype and to be in agreement with earlier reports (Yi et al. 2001; Jeoung 2001; and Pandey et al. 2010).

Molecular analysis of transgenic plants

Aside GUS assays PCR analysis as well done to confirm the presence and integration of T-DNA revealed all the transformed

Table 3 Results of shoot regeneration from seeds of rice cultivars after infection with *Agrobacterium* on MS +50 mg/l Kanamycin (SM) and SM + BA (MM)

Germinated seeds on Selection Medium	Mean primary tillers per responding explants(After 2 weeks)	Average shoot length (cm)
Jaya	2.8±0.17a	3.1 ±0.05a
Basmati370	3.2±0.05a	3.1 ±0.05a
IR64	2.3±0.75a	3.0 ±1.18a
AC41039	3.0±0.17a	2.9±1.27a
Mesocotyls from 2 week old seedlings (secondary passage) onSM +0.5 mg/lBA(MM)	Mean of tillers per responding explants(After 2 weeks)	Average shoot length (cm)
Jaya	5.3±0.33b	1.6±0.12c
Basmati370	7.6±0.33a	2.3±0.05a
IR64	5.3±0.33b	2.0±0.05b
AC41039	4.6±0.33b	1.6±0.05c

Data (mean ± SE) of three independent experiments each with 10 replicates. Means followed by the same letter within the column are not significantly different ($P<0.05$) as tested by the multiple range test of Duncan (1955) Shootlets measuring <2 cm is not taken into account

explants to show amplification at 0.53 kb (Fig. 2c) suggesting integration of GUS gene. The results of PCR analysis were consistent with the X-gluc assay results of the GUS activity, thereby confirming the stable integration of the transgenes into the rice genome. For PCR confirmation npt^{II} primers were used with both positive and negative control. All the putative transgenic plants showed the npt^{II} DNA fragment amplification at 0.8 kb (Fig. 2d and e) and as expected, no npt^{II} *PCR* product was seen in untransformed control plants.

RT-PCR and Southern blot techniques were employed further to confirm the transgenicity of putative transformants. The techniques used to eliminate *Agrobacterium* contamination and confirmation through RT-PCR and Southern blot, although successful, they involve more processing time and require various resources (Nain et al. 2005). The present protocol is similar to that of the improved procedure developed by Zou et al. (2008) in trifoliate orange (*Poncirus trifoliate* L. Raf.) wherein the limitations of southern blot and RT-PCR could be overcome by employing modified protocol for

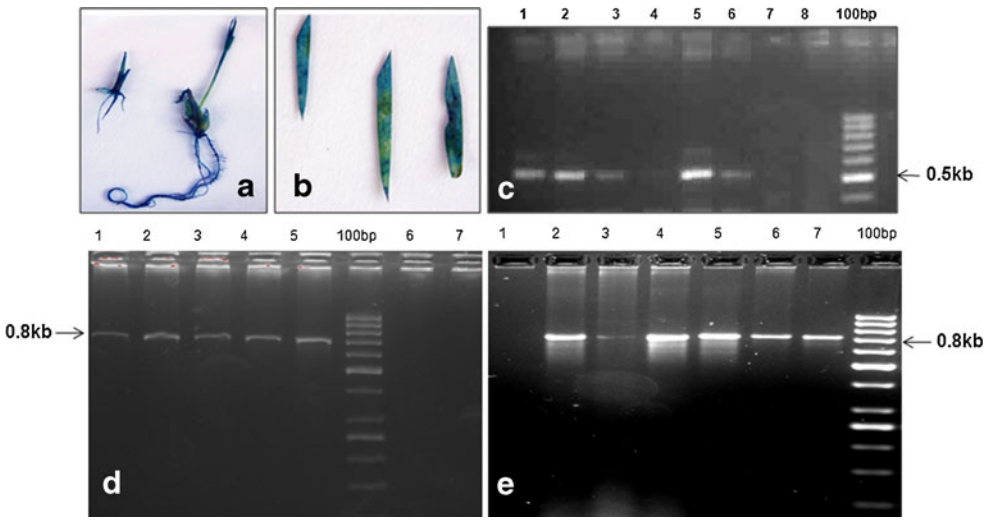

Fig. 2 Confirmation of transgenics by GUS staining and PCR analysis. **a** & **b** Transient GUS expression in transformed plants and leaves. **c** PCR analysis of transgenic shoots by amplication of the *uidA* (GUS) genes from total plant DNA extracts. Lane 1, 2, 3 & 6. AC41039, Jaya, Basmati370 and IR64 transformed genomic DNA; 4, 7 & 8. Non-transgenic DNA sample; 5. Positive control of plasmid DNA showing GUS reporter genes at 0.53 kb pairs. **d** PCR analysis of transformed plants showing npt^{II} marker gene amplification Lane 1,2,3,4 and 5 of AC41039,Jaya,Basmati370,IR64 transformed genomic DNA and Positive control of plasmid DNA respectively; 6 and 7 non transferred plant genomic DNA(negative control) showing amplification of npt^{II} marker genes at 0.8 kb pair. **e** All tiller shoots of Basmati370 derived in the process of transformation showing npt^{II} marker gene amplification. 1-negative control (DNA of non transformed plant) and 2,3,4,5 & 6-Genomic DNA from transformed primary and secondary tillers of Basmati370; Lane 7- Positive control of plasmid DNA showing amplification of npt^{II} marker genes at 0.8 kb pairs

DNA isolation from the transgenic plants that avoids *Agrobacterium* contamination.

Selection of single-copy endogenous gene for comparison and quantification

To make the transgenics detection more reliable and precise, a reference gene is indispensible. The reference gene should fulfill three requirements viz species specific, low copy number and low heterogeneity among cultivars. Based on the study (Ding et al. 2004; Wang et al. 2010) of single copy the endogenous gene Sucrose Phosphate Synthase (SPS) was selected for quantitative PCR amplification in transformed plants. The amplification curve analysis (Figure ESM_1 in online resource) done with c-DNA isolated of RNA extracted from four varieties exhibited Ct values to vary from 24.29 to 30.45 for SPS with coefficient of variation (CV%) with range of 0.69–2.04 in respect of SPS and from 27.29 to 29.14 with coefficient of variation (CV%) between 0.085 and 0.665 for GUS indicating the copy number of the SPS gene to be similar and without allelic variation in all the test cultivars. The range of variability of Ct values derived from these tests was relatively small suggesting that the quantitative PCR detection system worked stably and reliably.

As evident from the restriction map of T-DNA of pBI121 (Fig. 3a), the restriction digestion with *HindIII* separates GUS from npt^{II} genes. The DNA from transformed plant after digestion with *HindIII* hybridized with npt^{II} probe for confirmation by southern blotting, which showed npt^{II} gene to occur in two to three copy numbers (Fig. 3b). The results from RT-PCR analysis confirmed three to four copy number (Table ESM_2 in online resource) suggest some rearrangements in GUS and *nptII* insertions. The rearrangement perhaps happened during transformation process as explained as due to the possibility that the insertion of two T-DNA copies in an inverted orientation was cut in one fragment and yielded a single hybridization band. In southern blotting, multiple insertion, rearrangements, partial digestion and subjective evaluation of bands on the films are the most important reasons for affecting estimation of copy numbers (Yang et al. 2005). Because the real time PCR TaqMan assay being specific to a small sequence of the gene, rearrangement could be only factor that influence the true result (Mason et al. 2002).

In addition, the exact copy number of transgenes in T_0 transformants can be estimated by the real time PCR method. After selfing, the segregation of transgenes in T_1 plants and their corresponding number of copies cannot be estimated by traditional southern analysis. However, hemizygotes or homozygotes for the transgene in T_1 plants can be identified directly by quantitative analysis method. Thus homozygous transgenic plants obtainable in the T_1 generation itself can greatly reduce the time period taken for selecting ideal transgenic plants (Weng et al. 2004).

The transformation protocol developed by us has many advantages. It enables producing 6–8 transformed plants from a single seed explant within 6 weeks (Schematic representation ESM_3 in online resource). The four genotypes representing three major subspecies of rice, used in the present study, showing no significant difference in their tissue culture response suggest that this protocol to be of universal kind and can be used across subspecies and ecotypes for effective transformation. Also it is proved most efficient by ensuring by 100% transformation and regeneration efficiency in addition to being a best method for large scale propagation of transgenic plants.

For effective transfer of alien gene(s) of interest by rDNA technology, efficient transformation and regeneration protocols are the prerequisites. Efficiency of transformation depends on many factors which primarily include type of explant, compo-

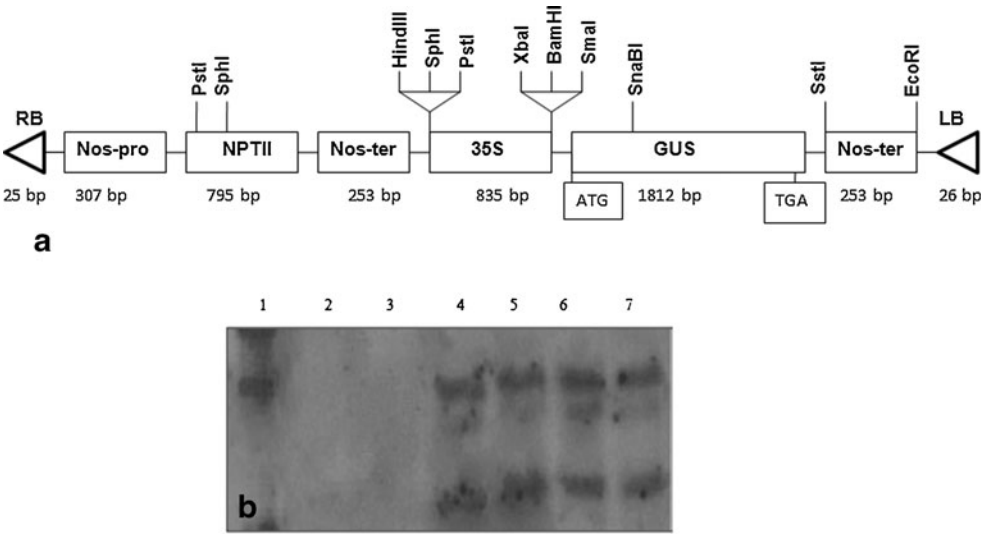

Fig. 3 Restriction site of T-DNA and Southern Blot. **a** Diagrammatic representation of the T-DNA region of plasmid pBI121 **b** Southern Blot: Lane 1- plasmid; 2 & 3 non-transformed plant; 4,5,6 & 7 transformed plant of 4 rice varieties showing hybridized DNA band confirming integration of *nptII* gene by 2–3 copy number

sition of culture medium and genotype. As of now, many efficient *Agrobacterium* mediated protocols have been developed for a monocot like rice. Experience shows that response to transformation—regeneration is subspecies and variety specific. Whereas japonicas are reported to be more responsive as compared to *indicas*, within subspecies only variety like Taipai 309 in *japonica* (Visarada and Sarma 2002) and Pusa Basmati and IR64 in *indica* relatively are more responsive (Hussain et al. 2010), though in general, transformation efficiency is low in rice. This technological constraint makes genetic engineering research relatively more difficult unlike in other crop species. Also, the need to develop variety specific protocols makes rDNA technology all the more difficult. Thus the need for *Agrobacterium* protocols applicable across varieties within and between subspecies has been felt since long. Generally, for realizing high response in rice tissue culture, immature embryos are used as explants to induce embryogenic calli for transformation. Preparation of high number of immature embryos is not only quite laborious and also their availability is season bound. Hence, the present effort has been to identify right explants and develop efficient protocols that can be used universally across the subspecies and varieties within. Whereas mature seeds identified as best explants for development of improved transformation-mediated regeneration protocols for efficient gene transfer, usable across a variety of genotypes through in vitro clonal propagation/shoot bud multiplication from mesocotyls avoiding the widely depended on callus—embryogenic route in transgenic research.

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