The Effect of Different Antibiotics on the Elimination of *Agrobacterium* and High Frequency *Agrobacterium*-mediated Transformation of Indica Rice (*Oryza sativa* L.)

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Abstract: We report here the suitable explant with high efficiency of transformation and the positive effects of timentin over other antibiotics like carbenicillin and cefotaxime on the elimination of *Agrobacterium tumefaciens* during the genetic transformation of popular indica rice (*Oryza sativa* L.). The tissues assayed were embryogenic calli, embryos with endosperm contamination, intact seeds, leaf blades, leaf bases and coleoptiles. The frequency of transient β-glucuronidase (GUS) expression as revealed by histochemical assay was 90% for embryogenic calli, which was the highest among the explants used. On the basis of disc-diffusion assay, the maximum zone of inhibition (29 mm) at 250 mg/l was obtained for timentin. In tissue culture conditions the frequency of *Agrobacterium* recurrence after 20 days of infection was minimum (2.3%) at 200 mg/l of timentin. At 250 mg/l of timentin there was no *Agrobacterium* growth, besides, there were no negative effects on the callus growth unlike other antibiotics, hence it was selected as the optimum concentration for high frequency callus proliferation and regeneration. The effect of the parameters evaluated was determined by the callus proliferation during selection, reduction in browning, transient GUS expression and stable transformation efficiency (23.3%). The resultant plants were stable transformants as confirmed by a molecular analysis of the *gus* and *hpt* genes. The developed transformation protocol will be very helpful for the information on indica rice cultivars in general and on IR 64 in particular.

Keywords: *Agrobacterium*-mediated transformation; *Agrobacterium* elimination; *gus* reporter gene; hygromycin phosphotransferase selectable marker gene (*hpt*); rice

The indica rice, which is the most important crop in eastern Asia, provides the staple food for more than a half of the world population but indica rice yield is affected by both biotic and abiotic stresses. Conventional breeding approach cannot be applied as the limitations of conventional breeding are time-consuming, it allows crossing of only closely related species rather limiting the gene pool available for improvement (Kumria *et al*. 2000). Further, the screening methods and phenotypic characterization are inadequate for improving abiotic stress tolerance in rice through conventional breeding (Sarangi *et al.* 2011). Attempts to improve resistance to biotic and abiotic stresses by conventional breeding through introgression of traits have limited success owing to a lack of resistance germplasm in the wild relatives, therefore gene transfer from other sources can be used to make rice plants resistant or tolerant to stresses. In addition to this the per capita consumption of indica rice is high, therefore improvement of any of its traits is significant (Datta & Datta 2006).

The production of transgenic plants by genetic transformation has become an important tool for genetic engineering of agronomically important crop species and for elucidating mechanisms of

gene expression (Rosahl *et al.* 1987). Transformation has been reported but in selected rice varieties (Rashid *et al*. 1996; Khanna & Raina 1999) indicating that experimental parameters for reproducible rice transformation have not been fully optimized (Datta *et al*. 2006; Ozawa 2009). The indica rice cultivars were found to be recalcitrant to tissue culture whereas it is possible to obtain high frequency plant regeneration in japonica rice varieties (Kyozuka *et al*. 1988; Terada & Shimamoto 1993; Rance *et al*. 1994; Kumria *et al*. 2000). It is therefore essential to establish parameters for high efficiency regeneration and transformation of popular indica rice varieties (Visarada *et al*. 2002).

Agrobacterium-mediated transformation has several advantages such as high transformation efficiency compared to protoplast transfer (Rao *et al*. 2009), the ability to transfer large pieces of DNA, minimal re-arrangement of transferred DNA, and characteristic insertion into the recipient genome of a discrete segment of DNA at a low copy number (Kumar *et al*. 2005; Tyagi *et al*. 2007) compared to particle bombardment approach (Shrawat *et al*. 2006; Rao *et al.* 2009). Efficiency of *Agrobacterium*-mediated transformation and delivery of T-DNA into plant cells is influenced by several parameters including explant selection (Owens & Cress 1985; Hiei *et al*. 1994; Ishida *et al*. 1996). The advantage of transient gene expression (TGE) over the analysis of stable transformants is its simplicity and easy performance (Wydro *et al*. 2006) and the gene activity can be measured within hours or days after DNA introduction whereas it takes several weeks before stably transformed lines are available for detailed study. The frequency of false positive results is also lower as TGE assay is not confounded by influences exerted by chromosomal sequences adjacent to the sites of integration (Dekeyser *et al*. 1990) as the majority of the transferred DNA remains extrachromosomal during the analysis of TGE (Werr & Lorz 1986). As six different explants have been chosen for the study, the necessity to screen the explants after months of stable transformation has been replaced with TGE analysis.

The antibiotic timentin is a mixture of ticarcillin, a penicillin derivative, and clavulanic acid with activity against many gram-negative bacteria. Compared to carbenicillin, timentin was found to be beneficial irrespective of the explant type of *Nicotiana tabacum Havana* SRI (Nauerby *et al*. 1997) and it improved *in vitro* morphogenesis (Cheng *et al*. 1998; Ling *et al*. 1998).

In the present study we evaluate a suitable explant with high transformation efficiency and the effect of timentin on the elimination of *Agrobacterium* and callus proliferation in IR 64 transformed rice tissues to improve transformation efficiency.

MATERIAL AND METHODS

***In vitro* germination and callus induction**. Mature seeds of indica rice (*Oryza sativa* L.) cultivar IR 64 were the experimental material. The seeds were obtained from Tamil Nadu Agricultural University, Coimbatore, India. They were dehusked and then washed thoroughly in running tap water for 30 min followed by surface sterilization with 0.1% (w/v) mercuric chloride for 4 min and with 70% (v/v) ethyl alcohol for 1 min under aseptic conditions. The sterilized seeds were transferred to hormone-free half-strength MS medium. For callus induction, the seeds were placed on Petri dishes (9 cm diameter) containing a callus induction medium (CIM) which consisted of MS basal salts and B5 vitamins supplemented with 13.5μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.3μM kinetin (Kin) for induction of callus from the scutellar region of the seed. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 20 min. The cultures were maintained in the dark at 25–26°C for 3–4 weeks, after that the proliferating scutellar derived calli were sub-cultured onto the same fresh medium. For germination, the sterilized seeds inoculated on half-strength MS medium were maintained at 24 ± 2°C under a light-dark cycle of 16:8 h with a light intensity of 70 μmol/m^2/s.

Explant preparation. Two-months-old calli (5 mm), 3 days and 7 days old seedlings germinated in the dark were used for excision of leaf bases (4 mm) and coleoptiles (3 mm), respectively. Leaf blades were excised from 28-days-old seedlings (light grown). Dehusked seeds (with intact embryo) and embryo with endosperm contamination (embryo removed from the seed along with a part of the endosperm region) were used for transformation after surface sterilization.

Rice transformation. The binary plasmid pCAMBIA 1301 present in *Agrobacterium tumefaciens* EHA 105 harbours *hpt* (hygromycin phosphotransferase) and *gus-int* genes (β-glucuronidase)

in the T-DNA region and *nptII* (kanamycin phosphotransferase) gene outside the T-DNA region, as a bacterial selection marker. A single colony of the *Agrobacterium* strain EHA 105 harbouring pCAMBIA 1301 was inoculated into 2 ml of yeast extract peptone (YEP) containing 10 mg/l rifampicin and 100 mg/l kanamycin. 500 µl of this culture was inoculated into 50 ml of YEP containing the same antibiotics. The overnight culture was centrifuged at 3220 g for 10 min and the optical density (OD) was adjusted to 1 by resuspending the pellet with liquid co-cultivation medium, i.e. 3% MS medium containing 100µM acetosyringone, 1% polyvinylpyrrolidone (PVP), 10% coconut water, and pH 5.6. This bacterial suspension was used for co-cultivation of various explants such as calli, embryo with endosperm contamination, intact seeds, leaf bases, leaf blades and coleoptiles separately for 2 min. After blot drying, the co-cultivated explants were post incubated for 3 days in dark on a solid co-cultivation medium (MS salts, B5 vitamin, 30 g/l maltose, 10 g/l glucose, 100µM acetosyringone, 3.0 g/l phytagel (pH 5.6)) overlaid with Whatman #1 filter paper. At the end of incubation, the explants were washed with 5 ml of 3% MS supplemented with six different concentrations (50, 100, 150, 200, 250, 300 mg/l) of each antibiotic (cefotaxime, carbenicillin and timentin) separately and blot dried on sterile filter paper. From all the six different explants randomly selected explants from each type were used for histochemical GUS assay analysis. Since the frequency of TGE was maximum for scutellar derived calli, they were subjected to further selection on a selection medium, i.e. CIM with 50, 100, 150, 200, 250, 300 mg/l of cefotaxime, carbenicillin and timentin and 30 mg/l hygromycin. After two rounds of selection of 20 days each, healthy pieces of calli were transferred to a regeneration medium, i.e. MS supplemented with 13.3µM 6-benzylaminopurine (BAP) and 8µM α-naphthaleneacetic acid (NAA) along with 250 mg/l of timentin and 30 mg/l hygromycin and grown under 16/8 h photoperiod with 70 µmol/m^2/s light from cool, white fluorescent lamps. The regenerated shoots were transferred to half-strength MS for rooting. The rooted plantlets were acclimatized and they were actively growing in a shade house.

Histochemical analysis. The histochemical GUS assay was conducted as described by Jefferson (1987). After blot drying the explants which were co-cultivated for 3 days with *Agrobacterium* strain EHA 105 carrying the *gus* reporter gene were exposed to the freshly prepared and filter sterilized GUS assay buffer. The histochemical reaction was done overnight in dark at 37°C for 48 h. To improve the visualization in the case of leaf blades, the tissues were cleared through ethanol series to remove chlorophyll. The cross-sections of the tissues were observed under a microscope and photographed. For all explants, transformation experiments were repeated thrice and for each experiment 30 explants were used.

Disc diffusion method. The disc-diffusion assay was used to determine the growth inhibition of *Agrobacterium* by various antibiotics. Petri plates were prepared by pouring 25 ml of YEP agar and letting solidify. Sterile discs (6 mm in diameter) were placed on the surface of YEP agar plates seeded with overnight culture of EHA 105. A single antibiotic disc was tested per agar plate. To the sterile discs 50, 100, 150, 200, 250, 300 mg/l of antibiotics such as cefotaxime, carbenicillin and timentin were added. A sterile disc without antibiotic was used as positive control and with DMSO as negative control. The YEP agar assay plates used for testing bacterial susceptibility were incubated at 37°C for 24 h. The assessment of antibacterial activity was based on the measurement of diameter of the zone of inhibition formed around the disc. Three independent trials were conducted for each concentration of each antibiotic.

Effects of antibiotic concentration on the elimination of *Agrobacterium* in co-cultivated cultures. After 48 h the calli were washed in 3% MS containing various concentrations (50, 100, 150, 200, 250 and 300 mg/l) of cefotaxime, carbenicillin and timentin and the embryogenic clusters were transferred to the respective antibiotic-containing selection medium (SM) and observed for the presence of *Agrobacterium* every 5 days. Six treatments with these three different antibiotics were tested and compared for their ability to control the *Agrobacterium* growth on callus cultures.

Molecular analysis of putative transgenic plants. DNA isolation was carried out from fresh, young leaves of putatively transformed acclimatized plantlets and untreated control with the HiPurA kit (HiMEDIA, Mumbai, India) according to the manufacturer's instructions. The stable integration of *gus* and *hpt* genes was confirmed by PCR analysis of the putative T0 transgenic plants (primary transformants). The amplification of the introduced genes was performed using the gene specific primers (GUS-F-5'-GGT GGG

AAA GCG CGT TAC AAG-3' and GUS-R-5'-GTT TAC GCG TTG CTT CCG CCA-3') and (HPT-F- 5'-GCC TGA ACT CAC CGC GAC G-3' and HPT-R-5'-CAG CCA TCG GTC CAG ACG-3'). Each PCR reaction was performed in a 25 µl reaction volume consisting of 2.5 µl of 10X PCR amplification buffer, 2mM $MgCl_2$, 200µM each of dNTPs, 20 pm each of forward and reverse primer, 1 U Taq DNA polymerase (MBI Fermentas, Burlington, Ontario, Canada), and 50 ng of total genomic DNA. Amplification was performed in a programmable thermal cycler (Eppendorf, Hamburg, Germany). Initial denaturation was done at 95°C for 7 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 1 min at 62°C, primer extension for 1 min at 72°C, the cycles were followed by a final extension period of 10 min at 72°C. 100 bp and 1 Kb ladder (MBI Fermentas, Burlington, Ontario, Canada) were used as standard molecular weight markers. The PCR product was resolved using 0.8% agarose gel, stained with ethidium bromide (0.5µg/l). The banding patterns were visualized and photographed using the Gel Documentation System (Gel Doc XR+, BIO RAD, Quarry Bay, Hong Kong).

RESULTS

Agrobacterium-mediated transformation

The general distribution and different levels of GUS activity obtained in different tissues of rice explants indicate the potential of pCAMBIA 1301 vector for transformation. Among the various explants such as calli, embryos with endosperm contamination, intact seeds, leaf bases, leaf blades and coleoptiles used for transformation, the maximum GUS expression was observed in the calli. Thus it is evident from the present study that the starting material for *Agrobacterium*-mediated transformation is the most crucial factor for high efficiency of transformation. Two-months-old hard friable calli were used since the age of the callus is essential to withstand *Agrobacterium* infection efficiently. It was observed that the use of (1 OD) *Agrobacterium* culture suspended in 3% MS containing 1% PVP and 10% coconut water as co-cultivation medium reduced the browning of calli after co-cultivation. A possible reason may be the reduced damage to explants during *Agrobacterium* infection which resulted in lower phenolic production and better recovery of callus during selection. The shoot bud induction was observed after 10 days of inoculation on the selection regeneration medium (Figure 1h, i). One-month-old plantlets (Figure 1j) transferred to half-strength MS medium for root induction were acclimatized and transferred to a shade house (Figure 1l).

Gus expression analysis

Several explants were used to study the effects of explant source on transformation efficiency. The effective gene delivery was analysed by GUS expression analysis after co-cultivation. The high percentage expression of 90% (Table 1) was found in the embryogenic calli, they also had the high capability for regeneration indicating that this is the best tissue for transformation, whereas the lowest expression of 51% was observed in the leaf blade. Next to callus the transient GUS expression was high (84.3%) in leaf base explants (Figure1f). The percentage expression of GUS in coleoptiles, leaf blades, intact seeds and embryos with endosperm contamination was 71.6, 51, 83 and 69.3%, respectively (Table 1) (Figures 1b—e).

Table 1. Frequency of GUS positive explants

Explants	Percentage of GUS positive explants
Embryogenic calli	90 ± 1.0**
Embryos with endosperm contamination	71.6 ± 0.5*
Seeds	83 ± 1.0*
Leaf bases	84.3 ± 0.5*
Leaf blades	51 ± 1.0*
Coleoptiles	69.3 ± 0.5**

Values represent means ± standard deviation; *$P < 0.5$; **$P < 0.05$

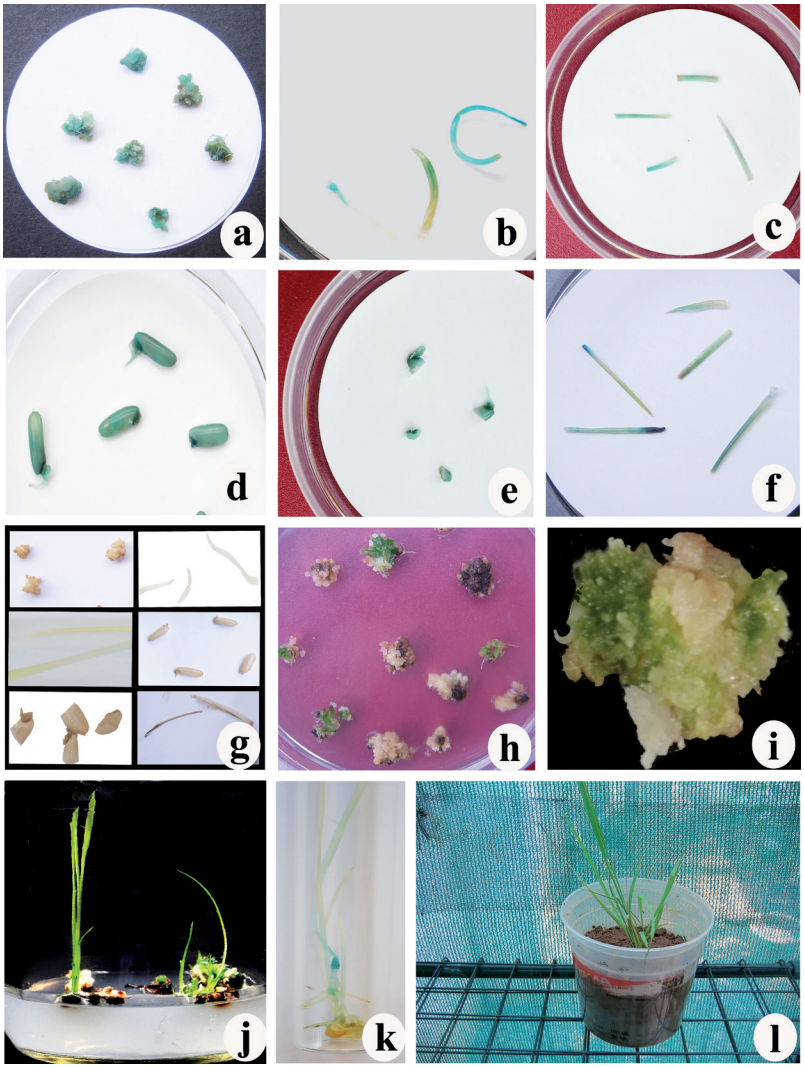

Figure 1. GUS expression pattern in different co-cultivated explants of *indica* rice cv. IR 64 and development of hygromycin resistant plants: (a) calli, (b) coleoptiles, (c) leaf blades, (d) seeds, (e) embryos with endosperm contamination, (f) 3-days-old seedling derived leaf bases, (g) controls of a, b, c, d, e, f, (h, i) greening of calli in regeneration medium, (j) 1-month-old hygromycin resistant plantlet, (k) GUS expression of whole plantlets, (l) hardened IR 64 plant in the shade house

None of the control explants developed visible blue colour after 48 h of co-cultivation (Figure 1g). The frequency of transient transformation is expressed as the ratio between the number of calli showing GUS expression and the total number of transformed calli. For qualitative assay, the area of GUS expression and the intensity of blue colour were given priority to analyse the effects of the various parameters tested. The area of blue colour > 1 mm was observed in the calli, as well as the intensity was strong. Larger spots (> 1 mm in diameter) representing complete GUS^+ cell clusters were reported by Rao and Rao (2007). On the basis of the intensity of blue colour scores were assigned: 0 (no GUS expression), 0.5–1.0 (slight expression), 1–2 (moderate expression), 2–3 (strong expression). The maximum score of 3 was observed for embryogenic calli and leaf bases. The minimum of 0.6 was found out for leaf blades. The scores for embryos with endosperm contamination, seeds and coleoptiles were 2, 1.6 and 2.3, respectively. The transient GUS expressions in the tissues were confirmed by cross-section analysis using a light microscope (Nikon H550L, Nikon, Tokyo, Japan) (Figures 2a–c). Finally the GUS expression analysis of the whole plantlets was carried out (Figure 1k). Based on both quantitative and qualitative assays, the efficacy of TGE was evaluated.

Table 2. Mean diameter of the zone of inhibition using different antibiotics against *A. tumefacians* (mm)

	Concentration of antibiotics (mg/l)					
	50	100	150	200	250	300
Cefotaxime	4.6 ± 0.5**	7.3 ± 0.5**	20 ± 1*	24 ± 1	24 ± 1*	27.6 ± 1*
Carbenicillin	7.6 ± 0.5*	9.6 ± 0.5*	21 ± 1**	24 ± 1**	25 ± 1**	26.6 ± 0.5**
Timentin	21 ± 0.1**	24 ± 0.1**	25.3 ± 0.5**	27 ± 0.5**	29 ± 0.5**	31 ± 0.5**

Values represent means ± standard deviation; *$P < 0.5$; **$P < 0.05$ (significance per rows)

Disc-diffusion assay

The results shown in Table 2 represent the antibacterial activity of the antibiotics. It was observed that timentin possessed maximum antibacterial activity manifested by the maximum zone of inhibition (29 mm at 250 mg/l and 31 mm at 300 mg/l). An increase in the concentration of the antibiotics enlarged the zone of inhibition. At 50 mg/l of cefotaxime the zone of inhibition was 4.6 mm whereas it was 7.6 mm and 21 mm in the case of carbenicillin and timentin, respectively. Similarly, at 250 mg/l the maximum zone of inhibition was observed in timentin (29 mm) and in the case of cefotaxime and carbenicillin it was 24 mm and 25 mm, respectively.

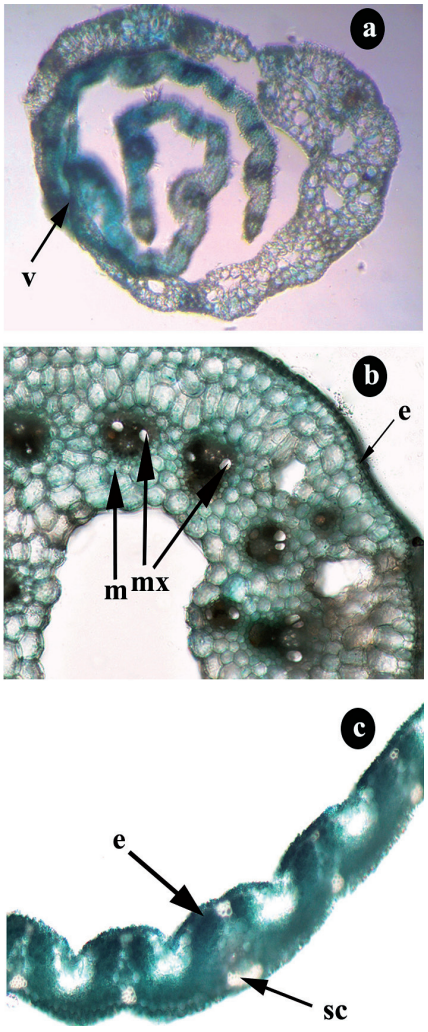

Figure 2. Histochemical localization of GUS expression: (a) cross-section of leaf base, (b) cross-section of coleoptile, (c) cross-section of leaf blade
v – vascular tissue, m – mesophyll, mx – metaxylem, e – epidermis, s – sclerenchyma

Effects of antibiotic concentration on the elimination of *Agrobacterium* in co-cultivation cultures

In order to determine the ability of the antibiotics in suppressing the growth of *Agrobacterium* during the transformation of IR 64 cultures, various concentrations (50, 100, 150, 200, 250 and 300 mg/l) of cefotaxime, carbenicillin and timentin were added to both the washing media and the solid co-cultivation media. It was observed that 250 mg/l of timentin was found to be the optimum concentration for eliminating *Agrobacterium*. Cefotaxime and carbenicillin did eliminate *Agrobacterium* at 250 mg/l but they caused severe necrosis in the callus tissues. Increasing concentrations of timentin at 300 mg/l resulted in moderate browning of callus and the level of embryogenic callus formation was low. Overgrowth of *Agrobacterium* could be observed in the control after 5 days. The effect of timentin on the elimination of *Agrobacterium* is shown in Table 3.

Molecular analysis of putatively transformed plants

The stable integration of *gus* and *hpt* genes into seven putatively transformed T0 plants was

Table 3. Effect of different antibiotic treatments on suppression of *A. tumefaciens* on IR 64 embryogenic callus clusters

Concentration of antibiotics (mg/l)	Percentage of callus with recurrence of *Agrobacterium* during treatment period (in days)					
	cefotaxime		carbenicillin		timentin	
	10	20	10	20	10	20
50	28.3 ± 0.3*	31.6 ± 0.9**	13.9 ± 0.8*	25.4 ± 0.4**	18.7 ± 0.2**	22.2 ± 0.3*
100	6.2 ± 0.6*	11.4 ± 0.8**	11.1 ± 0.3*	14.3 ± 0.4**	6.2 ± 0.3**	11.2 ± 0.6*
150	5.2 ± 0.3*	10.4 ± 0.9**	5.5 ± 0.3**	8.4 ±0.5*	4.3 ± 0.4**	6.3 ± 0.1**
200	3.6 ± 0.3*	7.5 ± 0.4*	2.2 ± 0.2**	5.2 ±0.1**	1.1 ± 0.1**	3.3 ± 0.2**
250	0	0	0	0	0	0
300	0	0	0	0	0	0

Values represent means ± standard deviation; *$P < 0.5$; **$P < 0.05$ (significance per columns)

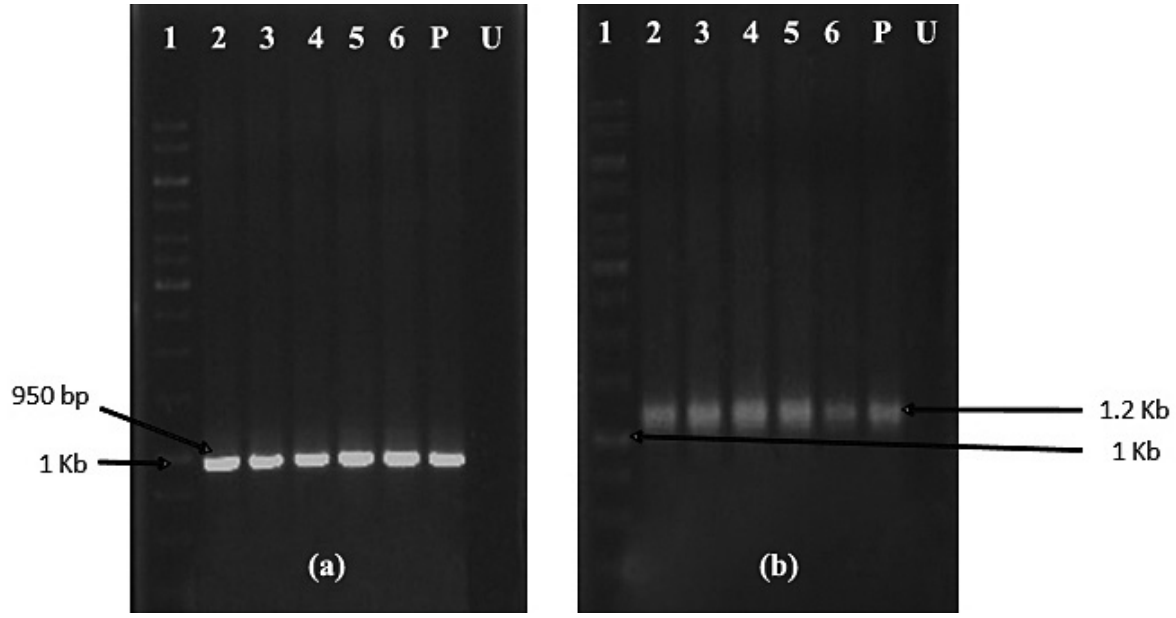

Figure 3. PCR analysis of randomly selected putatively transformed IR 64 plants for the integration of (a) *hpt* (950 bp) and (b) *gus* (1.2 kb) genes; lane M – 1 kb marker; lane 1 to 5 – transformants; lane P – pCAMBIA 1301 (positive control); lane U – untransformed plantlet (negative control)

confirmed by the amplification of expected *gus* (1.2 kb) and *hpt* (0.95 kb) gene fragments while the presence of *gusA* and *nptII* genes is evident in all the seven plants (Figures 3a, b). As expected, there was no amplification from the DNA of the untransformed control plants.

Table 4. Frequency of callus survival, regeneration and transformation

Experiment No.	No. of explants	Callus survival (%)		Regeneration (%)	Stable transformants (%)
		selection 1	selection 2		
1	30	90.0	70.3	42.3	23.3
2	30	86.6	61.5	50.0	20.0
3	30	83.3	68.0	46.6	26.6

DISCUSSION

In crop plants, development of an effective *Agrobacterium*-mediated transformation system with high efficiency of plant regeneration in a relatively short period depends on the transformation parameters that permit efficient T-DNA delivery, and true selection of transformed cells. Many factors including the vector, the explants used for transformation, co-cultivation parameters etc. contribute to an optimal performance in any transformation system. The vector pCAMBIA 1301 used in the present study has been proved ideal for rice transformation as reported by other groups (Hiei *et al*. 1994; Ilag *et al*. 2000; Chern *et al*. 2001; Saharan *et al*. 2004; Kumar *et al*. 2005). Mature seed derived calli were widely considered as suitable target tissue for *Agrobacterium*-mediated and biolistic transformation (Kumar *et al*. 2005). Previously, one-month-old calli were used for transformation (Hiei *et al*. 1994; Rashid *et al*. 1996), but breaking of calli before one month resulted in browning and phenolics production which ultimately resulted in low frequency callus proliferation and somatic embryogenesis (Kumar *et al*. 2005). In the present study, it was observed that co-cultivation with 1 OD culture suspended in 5 ml of 3% liquid MS medium supplemented with 100μM AS for 1 min possibly reduced the damage to explants during *Agrobacterium* infection. Our results are in line with the previous findings stating that the addition of AS (100μM) during co-culture was found inevitable for successful transformation (Kumar *et al*. 2005, 2009; Ashok *et al*. 2007) as it induces vir genes, extends the host range to *Agrobacterium* strains, and was found necessary for rice transformation as reported in other rice cultivars (Godwin *et al*. 1992; Hiei *et al*. 1994; Tyagi *et al*. 2007). The explants co-cultivated for 2 min with OD = 1 culture were proved to be optimum as an increase in the co-cultivation time will lead to overgrowth of *Agrobacterium* and death of the explants (Rao *et al*. 2009) as longer exposure in the infection medium may cause hypertonic conditions which may be lethal to the explants and hence results in a low frequency of transformation. GUS expression was not detected in any of the control (non-transformed) explants. Transient GUS expression was used as a parameter to optimize *Agrobacterium*-mediated transformation as it was rapid and easy to perform. Ashok *et al*. (2007) reported about the successful *Agrobacterium*-mediated transformation of cereals by means of transient expression analysis.

During transformation a frequent phenomenon is the necrotic response that the explants develop after co-cultivation (Ozawa 2009) and it is considered a limiting step to improve the transformation efficiency. To overcome the problem the addition of 10% coconut water and 1% PVP in the co-cultivation and callus induction media improved embryo recovery and callus formation. This was proved in the previous findings of *Agrobacterium*-mediated transformation of other cereal crops like sorghum. According to Matzk *et al*. (1996) it is essential to suppress and eliminate the extra *Agrobacterium* present in the plant tissue after transformation to reduce the detrimental effects on growth and regeneration as well as to prevent the release of genetically modified microorganisms. The influence of different antibiotics (carbenicillin, claforan, and timentin) on the elimination of *Agrobacterium* and differentiation of transgenic calli and transgenic plantlet regeneration through *Agrobacterium*-mediated transformation in loblolly pine was well proved (Tang *et al*. 2004). The influence of antibiotics on the growth of transgenic calli is similar to that of polyamines that improve the growth of transformed tissues (Bais *et al*. 1999). The positive effects of ticarcillin/potassium clavulanate (150 mg/l) on callus growth and shoot regeneration have been reported in the tomato cultivar Moneymaker (Ling *et al*. 1998); this is in correlation with our present study on the ability of timentin to enhance callus proliferation. The inhibitory effect of cefotaxime on shooting and rooting was explained by Nauerby *et al*. (1997). In contradiction to our investigation implying the negative effects of carbenicillin, the influence of carbenicillin on regeneration has been proved as it possesses auxin-like structural features and when broken down, displays effects similar to those of the weak auxin phenylacetic acid in the culture medium, thereby, increasing the regeneration potential of cultured explants (Nauerby *et al*. 1997; Ling *et al*. 1998; Tang *et al*. 2004). Cheng *et al*. (1998) reported that timentin can be considered to be an alternative antibiotic for those species in which the regeneration potential is negatively affected by carbenicillin and cefotaxime. Timentin was also as effective as carbenicillin and cefotaxime in suppressing *A. tumefaciens* at concentrations commonly used in transformation. Transient GUS expression was frequently used to assess stable

transformation efficiency either by evaluating the frequency of GUS explants or by evaluating the quality/quantity of GUS expression. The latter demonstrated a better correlation between transient and stable transformation (Švábová & Griga 2008). Similarly, the transient GUS expression was determined by GUS^+ explants and their intensity of blue colour. Based on this the embryogenic calli showed better results; besides this, the quality of GUS expression was also high marked by the highest score of 3 for strong GUS expression, therefore it was used for further selection and regeneration. The cross-sectional microscopic analysis of leaf bases showed intense blue staining in the vascular tissue (Figure 2a), whereas in the case of leaf blade sclerenchyma and vascular tissue it showed no staining (Figure 2b). In the coleoptile cross-section the epidermis as well as mesophyll exhibited intense staining but the metaxylem tracheary elements appeared to be without any blue staining (Figure 2c). Similar results were observed in rice in the cross-sections of the leaf, strong blue staining was seen in mesophyll cortex, epidermis, and vascular cells but the sclerenchyma fibres showed no staining (Verdaguer *et al*. 1996; Nomura *et al*. 2005). In the coleoptile cross-section the bundle sheath cells exhibited intense staining (Figure 2c).

Following the histochemical analysis of GUS expression, molecular characterization of the putative transgenic plantlet was carried out to assess the amplification of the expected *gus* and *hpt* gene fragments. The *hpt* primers gave an amplification of about 950 bp and the *gus* primers gave the expected amplification of 1.2 kb. All plants that grew in this selection medium were tested positive in molecular analysis. The tissue culture standardization and optimization of conditions for the delivery of *gus* gene in IR 64 might help in the transformation of other related genotypes for their genetic improvement.

CONCLUSION

In summary, we have concluded that agro-infiltration using different explants of indica rice IR 64 along with the use of the antibiotic timentin, PVP and coconut water was efficient during selection for producing the transgenic plants. This combination had significant utility in indica rice transformation towards the development of a high frequency of transgenic plantlets. During selection the presence of antibiotics other than timentin had a deleterious effect on callus proliferation and regeneration. The addition of PVP and coconut water prevents necrosis, thereby promoting the callus proliferation. Positive GUS assay and PCR amplification of *hpt* and *gus* genes finally confirmed stable transformation. The transformation efficiency obtained using this method was around 3.5 fold higher than that reported using scutellar derived calli (Kumar *et al*. 2005). Overall, the ease and high efficiency of transformation obtained lead us to conclude that the method established in this study is an effective protocol for obtaining the transformation of IR 64 (23.3%, Table 4). The information reported here may also facilitate *Agrobacterium*-mediated transformation of recalcitrant indica rice cultivars which in turn may result in their genetic improvement.

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