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Transformation of *hpt* Gene to Indica Rice (*Oryza sativa* L.) by *Agrobacterium* Vector and by Electroporation of Intact Callus

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ABSTRACT. *Rice (Oryza sativa L.) is by far the world's most important crop species. However stress, pests and diseases have become the major constraints for the enhancement of crop production of rice. The most significant advancement in crop improvement, complementing the conventional tools of plant breeding has been the development and utilization of gene transformation techniques.*

Transformation of rice seeds of the indica variety Bg 450 attempted with the Agrobacterium tumefaciens strain LBA4404 which contains a binary vector pABK01. Calli derived from scutella co-cultivated with the above strain were selected on the medium supplemented with hygromycin and transformed calli were screened for its gus activity. Binary vector pABK01 encoding intron-gus reporter gene did not express its gene activity in Agrobacterium tumefaciens cells.

Callus induction was considerably inhibited by the presence of Cefotaxime in callus induction medium.

As an alternative to the use of Agrobacterium as a gene vector, intact cells of rice were transformed by introducing a bacterial hpt gene conferring resistance to hygromycin B, by electroporation.

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INTRODUCTION

Rice (*Oryza sativa* L.) is the world's single most important food crop and a primary food for more than a third of the world's population. Production and consumption are concentrated in Asia where more than 90% of all rice is produced and consumed.

However stress, diseases and pests specially insects have become the major constraints in the enhancement of production of rice. A variety effectively resistance to above stresses, but maintaining a good yield and quality is a distant goal.

Genetic transformation can significantly strengthen the rice breeding programs and helps to produce new varieties with higher yield potential and greater yield stability. The availability of reliable genetic transformation techniques will facilitate the elucidation of molecular principles of gene regulation and paves the way for transferring agronomically important genes into rice crop improvement.

Although a variety of successful transformation systems have been developed, *Agrobacterium*-mediated transformation systems has been the most useful vector system for transfer of foreign genes to dicotyledonous plants. Since monocotyledonous, particularly cereals have been considered outside the host range for *Agrobacterium tumefaciens*, there has been renewed interest in using the *Agrobacterium* system for gene transformation in cereals (Chan *et al.*, 1993; Wilmink and Dons, 1993; Binns, 1990).

However, genetically transformed indica rice is not yet available, as this crop is recalcitrant in its tissue-culture response. Thus, one aspect of this study is the use of *Agrobacterium* binary vector system for indica rice transformation.

As an alternative to the use of *Agrobacterium* as a gene vector system, electroporation of intact plant tissues adds a new dimension to the genetic transformation. Therefore, in addition to standard electroporation-mediated protoplast transformation methods, attempts were made to transform intact cells of rice by electroporation.

MATERIAL AND METHODS

Transformation with *Agrobacterium* binary vector

Bacterial strain

The *Agrobacterium tumefaciens* strain LBA4404 which contains the binary vector pABK01 was obtained from the Microbiology laboratory of the Department of Agricultural Biology. This binary vector containing a *hpt* gene encoding hygromycin resistance, *gus* gene under the control of the CaMV 35S promoter and, *npt* II gene which confers kanamycin resistance under the control of nos promoter was used for the transformation experiments (Figure 1).

The *Agrobacterium tumefaciens* LBA4404 (pABK01) was maintained on YEB medium (Luis and Simpson, 1988) supplemented with 50 mg/l Hygromycin and 50 mg/l kanamycin.

Inoculation of rice seeds

Mature rice seeds of variety Bg 450 were surface sterilized by 2.5% (w/v) mercuric chloride for 10 minutes followed by rinsing five times with sterile distilled water. Sterilized seeds were allowed to germinate at 25°C. Three to four days of postgermination, sterilized seeds were dehusked and emerging primary and secondary roots and sheath were removed and, used for the following experiments.

Inoculum was composed of colonies of *Agrobacterium* strain containing a binary vector pABK01 suspended in sterile distilled water with protocatechuic acid (40 µg/ml). Scutellum was wounded with a needle containing the inoculum. Inoculated seeds were transferred onto N6 medium. After a 48 hour co-cultivation at 27°C, seeds were transferred onto the same medium supplemented with 400 µg/ml cefotaxime to kill the bacterium. After two weeks seeds were subcultured onto the same medium containing 500 µg/ml cefotaxime.

In order to evaluate the effect of cefotaxime on callus induction, uninoculated seeds were cultured on N6 medium supplemented with cefotaxime (500 µg/ml), and N6 medium without any Cefotaxime.

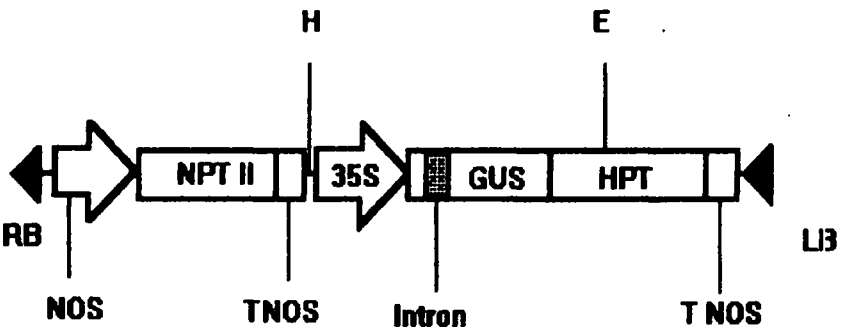

Figure 1. T-DNA region of pABK01.

RB - right border LB - left border E - *Eco*R1 H - *Hind*III
NPTII - neomycin phosphotransferase GUS - ß-glucuronidase
HPT - hygromycin phosphotransferase; 35S - 35 Spromotor
TNOS - 3'signal of nopaline synthase NOS - nopaline synthase

Co-cultivation of calli derived from scutella with *Agrobacterium*

Calli developed from excised scutella were multiplied on N6 medium containing 2.5 mg/l 2,4-D. *Agrobacterium* cells were collected with a loop and suspended at a density of 3-5 x 10^9 cells/ml in the AAM medium (Hiei *et al.*, 1994). Protocatechuic acid was used in place of acetosyringone. Rice calli were immersed in the bacterial suspension for 3 minutes and transferred without rinsing onto 2N6-AS medium (Hiei *et al.*, 1994) and incubated at 25°C in the dark for 3 days.

After co-cultivation materials were rinsed thoroughly with 250 µg/ml cefotaxime in sterilized distilled water and placed on 2N6-CH medium (first selective medium) which contained 50 µg/ml kanamycin and 50 µg/ml hygromycin B (Hiei *et al.*, 1994) and cultured for 3 weeks. Then proliferating calli on the first selective medium were excised and transferred onto N6-7-CH medium (second selective medium) which contained a higher concentration of

hygromycin B (100 µg/ml) (Hiei *et al.*, 1994). After 10 days transformed calli on second selective medium were plated on regeneration medium containing 50 µg/ml hygromycin B and 250 µg/ml cefotaxime (Hiei *et al.*, 1994) and incubated at 25°C.

Effect of protocatechiuc acid on transformation

Rice tissues were immersed in protocatechuic acid free AAM medium containing bacterium and co-cultivated on protocatechuic acid free 2N6-AS medium. Three days after co-cultivation calli were transferred onto first selective medium.

Assay of *gus* activity

Some of the transformed calli were incubated on a second selective medium containing histochemical substrate (salmon-beta-D-glc-A) in order to screen for the *gus* gene activity of transformed tissues.

Confirmation of the presence of intron-*gus* reporter gene in binary vector pABK01

Agrobacterium tumefaciens LBA4404 (pABK01) and *Agrobacterium tumefaciens* C58 C1 Rif^r pGV 2260:P260 were plated on YEB medium containing histochemical substrate to evaluate the activity of intron-*gus* reporter gene of binary vector pABK01.

Gene transfer into intact cells of rice by electroporation

Plant materials

Rice calli derived from scutella were obtained as described above. Less than 1mm size small calli were used for the electroporation experiment.

Suspension cultures were initiated by transferring small calli each of which is 2-5 mm in diameter in to a 100 ml flask containing 20 ml of N6 suspension medium. Suspension cultures were shaken regularly on gyratory shaker (80 rpm) under light (ca. 3000 lx) at 25°C for 4-6 weeks. Suspension

cultures were subcultured every 7 days. Intact cells from rice suspension culture on the 4th day were used for the electroporation.

Plasmid

Intact cells of rice suspension cultures and intact calli were transformed using a plasmid pROB5 (Roland *et al.*, 1991) which contained a bacterial *hpt* gene conferring resistance to hygromycin B under the control of the 35S promoter of cauliflower mosaic virus (Figure 2).

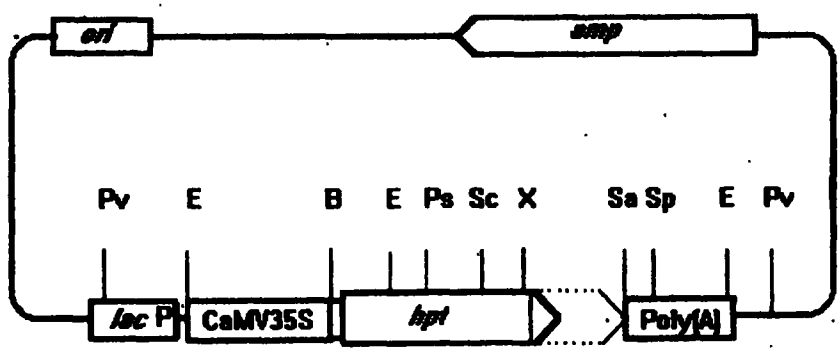

Figure 2. Restriction map of pROB5.

B - *Bam*H1 E - *Eco*R1 Ps - *Pst*1 Sp - *sph*1
Pv - *Pvu*II Sa - *Sal*1 Sc - *Sac*II X - *Xma*III
amp - Ampicillin resistance lac P - acterial *lac* promoter
Ori - Origin of replication CaMV 35 S - CaMV 35 S promoter
hpt - Hygromycin Phosphotransferase
Poly (A) - CaMV 35S Polyadenylation signals

Electroporation of intact rice cells

Small cell aggregates (calli) and single cells were incubated for 3 hours in electroporation buffer (EPR) with 0.2 mM spermidine (Dekeyser *et.al.*, 1990).

After incubation, buffer was removed and they were washed twice with EPR buffer. Calli and cells were incubated in 0.2 ml of EPR containing 0.2 mM spermidine with approximately 20 µg of DNA for one hour on ice. Eleven micro litres of 3 M NaCl was added and tubes were placed on ice for 10 minutes. Calli/cells-DNA mixture was transferred into a 0.1 cm plastic cuvette pre-cooled in ice and electroporated by a capacitor-discharge system (Invitrogen Electroporater II-version 3.0). One pulse with an electrical field strength of 150 V was discharged from a 1000 µF capacitor for 100 ms. After 15 minutes on ice, calli/cells were rinsed with N6 medium and, electroporated calli and suspension cells were cultured in N6 suspension medium. After 10 days, calli/cells were transferred into fresh N6 suspension medium supplemented with 30 µg/ml hygromycin B and incubated for another 10 days. Hygromycin resistant calli were selected and placed on N6 solid medium containing 30 µg/ml hygromycin B. Electroporated suspension cells were cultured by the mixed nurse culture method. After 10 days, transformed calli were incubated on hygromycin free N6 medium for 1 week before transferring onto N6 regeneration medium.

RESULTS AND DISCUSSION

Transformation of rice seeds with *Agrobacterium tumefaciens*

All inoculated seeds turned brown and eventually died within seven days without producing callus. Callus induction could not be observed even in uninoculated seeds in the control which were cultured on the N6 medium supplemented with cefotaxime. However, 100% callus induction was shown only from those seeds which were cultured on cefotaxime free N6 medium (Figure 3b). These results suggest that cefotaxime inhibits callus induction.

Effect of protocatechuic acid

Results of the experiment conducted to evaluate the effect of protocatechuic acid on infection are shown in Table 1. Results were similar to that of the uninoculated calli in the control. Calli which showed 93.3% proliferation in the first selective medium in the presence of kanamycin also turned brown and died when it was transferred to second selective medium containing hygromycin only. Phenolic compounds such as acetosyringone and α-hydroxy acetosyringone exuded by the wounded cells which activate the *vir* genes were responsible for the initiation of infection by *Agrobacterium tumefaciens* to the wounded host cells (Stachel *et al.*, 1985). These signal molecules appear to be very important in allowing *Agrobacterium tumefaciens* to

Table 1. Proliferation of inoculated calli on first selective medium (2N6-CH) after co-cultivated on protocatechiuc acid free 2N6-AS medium.

Medium	Number of calli inoculated	% of callli proliferated
2N6/CH + C + kn	30	93.3
2N6/CH + C + hy	30	0
2N6/CH + C + kn + hy	30	0
2N6/CH + C - kn - ky	30	96.6

C - cefotaxime kn - kanamycin hy - hygromycin

recognize suitable hosts, and they activate the *vir* loci on the Ti plasmid (Binns, 1990). Bolton *et al.* (1986) utilized seven phenolic compounds including protocatechuic acid to induce *vir* activity. Monocotyledons particularly grasses may not produce these compounds or if they do, they may not be at sufficient levels to serve as signal molecules. So many investigators believe that inoculation of monocotyledons with *Agrobacterium tumefaciens* treated with inducing compounds will significantly increase the number of transformation events in monocotyledons from *Agrobacterium tumefaciens* treatment (Schafer *et al.*, 1987). Since our results also demonstrated that protocatechuic acid contributed to successful transformation and protocatechuic acid was routinely used in all our experiments.

Co-cultivation of scutella-derived calli

Cell proliferation was observed in rice tissues that had been co-cultivated with *Agrobacterium tumefaciens* LBA4404 (pABK01), cultured on selective medium containing hygromycin and kanamycin. Most of the colonies of cells that recovered from the first round of selection were proliferated on the second selective medium that contained a higher concentration of hygromycin B, while uninoculated calli in the control turned brown and eventually died within ten days (Figure 3c). Therefore, proliferated calli on second selective medium were selected as a transformed calli based on the expression of hygromycin resistant gene. Early reports showed that high natural resistance to kanamycin is found in

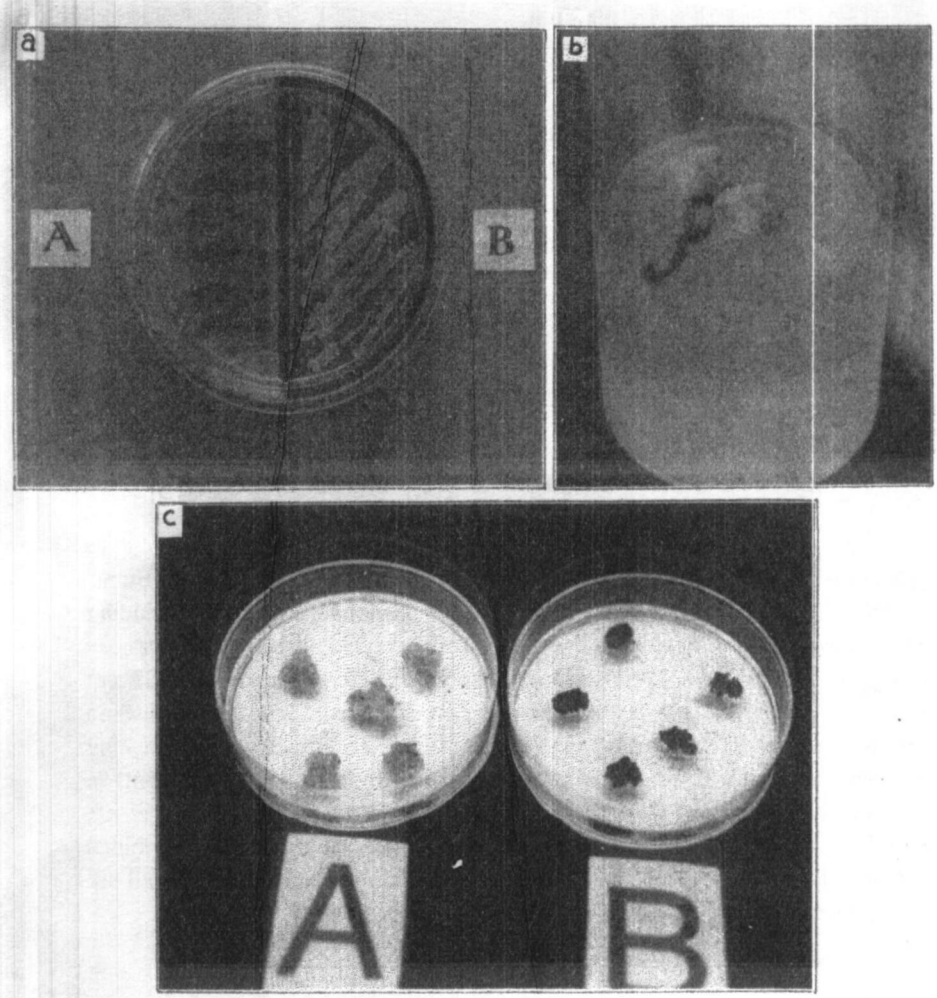

Figure 3. **(a) A: Expression of non-intron - *gus* gene activity of C58 C1 Rif^{r} PGV2260:P260, and B: intron-*gus* gene activity of LBA4404 (pABK01); (b) Scutellum-derived rice calli on N6 medium; (c) A: hygromycin resistant calli on 2nd selective medium, B: uninoculated calli on 2nd selective medium.**

most important species of grasses (Raineri *et al.*, 1990). Therefore this has limited use as a selectable marker for the transformation experiments. Since both transformed and uninoculated calli, expressed resistance to kanamycin, use of kanamycin as a selectable marker was discontinued. Results (Table 2) revealed that the rice calli derived from scutella were carrying *hpt* gene through *Agrobacterium* mediated transformation.

Intron-*gus* reporter gene

The *Agrobacterium tumefaciens* C58 C1 Rif' pGV2260:P260 which encode non-intron-*gus* reporter gene expressed the colour change with salmon-beta-glc-A. Colonies appeared in salmon-pink colour while colonies of *Agrobacterium tumefaciens* LBA4404 (pABK01) exhibited a creamy white colour (Figure 3a). Results confirmed that intron-*gus* gene was present only in the binary vector pABK01. Therefore, intron-*gus* gene (Ohta *et al.*, 1990) can be used as a convenient marker gene for transformation studies since this gene was strongly expressed in plant cells but not in *Agrobacterium tumefaciens* cells attached to the plant tissues.

GUS activity assay

The non-selectable gene encoding β-glucuronidase (*gus*) was also transferred with *hpt* and *npt*II genes. Of the selected calli screened for *gus* activity, transformed calli failed to exhibit co-expression of this non-selectable marker. This suggested that the *gus* gene was either not present or else was present but not-expressed in transformed calli. Generation of kanamycin resistant transgenic plants which do not possess the β-glucuronidase gene is possible because of rearrangements, deletions and amplifications leading irregular T-DNA insertions have been widely reported (De Blaere *et al.*, 1985). However position effect may also explain this phenomenon whereby integration in different chromosomal regions might influence the level of expression of the introduced gene (Raineri *et al.*, 1990).

Electroporation

Electroporation is a very efficient technique to introduce DNA into many types of protoplasts and previous reports have indicated that intact plant cells can be permeabilized by electroporation. After 10 days of incubation in N6 suspension medium supplemented with hygromycin B, calli electroporated with

Table 2. **Efficiency of transformation of rice calli by *Agrobacterium tumefaciens* LBA4404 (pABK01).**

Experiment	Number of scutellum derived calli			
	Inoculated (A)	Hm^R calli on 1st selective medium	Hm^R calli on 2nd selective medium (B)	Frequency % (B/A) x 100
1	40	28	25	62.5
2	40	35	21	52.5
3	40	32	26	65.0

Hm^R - Hygromycin resistance

DNA showed low levels of *hpt* resistance while calli electroporated without DNA (control) never showed any *hpt* resistance (Figure 4a). They turned brown and eventually died upon transferring onto N6 solid medium containing hygromycin B. After 10 days electroporated calli which were proliferating in N6 suspension medium containing hygromycin, were transferred onto a N6 solid medium containing 30 µg/ml hygromycin B. These selected calli showed further proliferation after they were transferred on to N6 solid medium supplemented with 30 µg/ml hygromycin (Figure 4b). Electroporated suspension cells failed to form micro calli by mixed nurse culture method.

The protocol involve elimination of target cells released nucleases from the electroporation buffer prior to addition of the DNA and increasing the DNA/cells incubation time. Enriching incubation and electroporation solutions with 0.2 mM spermidine enhanced expression. Spermidine is known to reduce nuclease degradation of DNA and also has been shown to prevent lysis of oat protoplasts (Weissinger, 1992). Thus, spermidine treatment may increase transformation efficiency by protecting DNA and by increasing cell viability. Cells had to pre-incubate for 3 hours in EPR buffer before transferring to fresh medium containing plasmid DNA to remove nucleases excreted by the damaged cells. Co-cultivation of plasmid DNA with the plant material probably reflects the slow diffusion of DNA through the cell wall pores. Early reports revealed that one electrical pulse can introduce DNA into several layers of cells and, both super coiled and linearized plasmid DNA provided similar levels of transgene activity (Dekeyser *et al.*, 1990). Therefore, results presented demonstrate that the

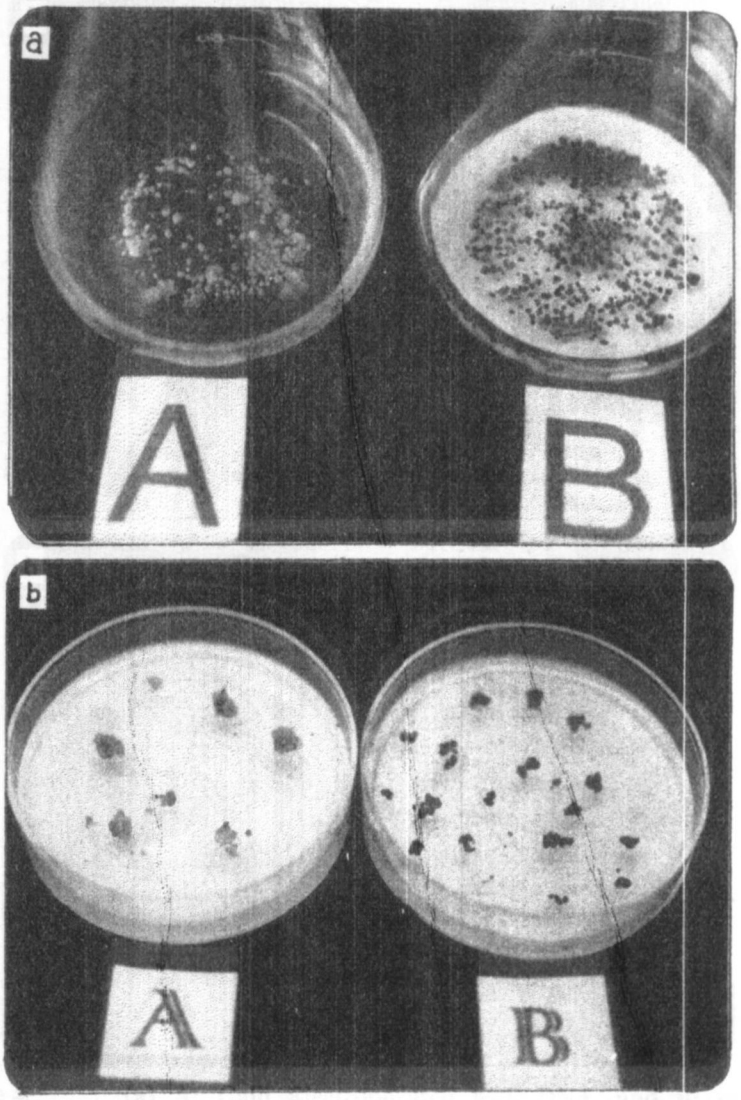

Figure 4. **(a) A: Calli Electroporated with DNA, and B: calli electroporated without DNA/control, in N6 suspension medium supplimented with Hygromycin B.**
(b) A: Poliferating calli on N6 solid medium containing Hygromycin B after transferring from N6 suspension medium containing Hygromycin B, and B: Control : electroporated calli without DNA on N6 solid medium containing Hygromycin B (B).

electroporation protocols for intact tissues could provide an important alternative to microprojectile bombardment for tests involving regulated gene expression in some tissues.

Thus far, we have been unable to generate rice plants from our transformed tissues perhaps due to unoptimum conditions employed for both transformation and selection procedures. However, we are hopeful that future experiments employing detection of transgene expression in transformed tissues and their progeny of stable transformants allow us to generate transgenic rice plant where protoplast culture has proven to be difficult.

CONCLUSIONS

The following conclusions can be made from this study.

1. Indica rice calli derived from scutella were susceptible to the *Agrobacterium*-mediated transformation.

2. Cefotaxime considerably inhibited callus induction.

3. Intact cells of rice can be transformed by introducing foreign genes by electroporation.

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