ORIGINAL ARTICLE

Transformation of indica rice (*Oryza sativa* L.) cv. RD6 mediated by *Agrobacterium tumefaciens*

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Abstract

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High percentage of callus induction at 97% was obtained when seeds of rice (*Oryza sativa* L.) cv. RD6 were cultured on modified N6 medium supplemented with 3% (w/v) sucrose, 22.5 µM 2,4-D and 0.8% agar under light condition. The suitable regeneration medium was N6 medium supplemented with 3% (w/v) sucrose, 2.5 µM IAA, 18 µM BA and 0.8% agar. A test had been performed to determine the effect of antibiotics on the regeneration of rice cv. RD6. It was found that kanamycin concentration up to 150 mg l^{-1} and hygromycin concentration at 10 mg l^{-1} were effective for selection of transformants. Cefotaxime and carbenicillin concentration up to 250 mg l^{-1} had the highest phytotoxicity to plant regeneration. *Agrobacterium*-mediated gene transfer protocols for rice cv. RD6 were performed using *A. tumefaciens* strain LBA4404, which harbored the plasmid pBI121 containing genes for β-glucuronidase (GUS) and kanamycin resistance (*nptII*), and strain EHA105, which harbored plasmid pCAMBIA1301 containing genes for β-glucuronidase (GUS) and hygromycin resistance (*hptII*). GUS activities were found in rice calli after co-cultivation. A

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number of morphologically normal fertile transgenic rice plants were obtained. Stable integration, expression and inheritance of transgenes were demonstrated by molecular and genetic analysis of transgenic plants in T_0 and T_1 generation. Mendelian segregation was observed in T_1 progeny.

Key words : *Agrobacterium*-mediated transformation, rice transformation, transgenic plants

บทคัดย่อ

ทศพร พิพัฒน์ภานุกูล สุมนทิพย์ บุนนาค ปิยะดา ธีระกุลพิสุทธิ์ และ มานิตย์ โฆษิตตระกูล
การส่งถ่ายยีนในข้าวพันธุ์ กข6 โดย *Agrobacterium tumefaciens*
ว. สงขลานครินทร์ วทท. 2547 26(1) : 1-13

อาหารสูตร N6 ดัดแปลง ที่เติมน้ำตาลซูโครส 3% (น้ำหนัก/ปริมาตร), 2,4-D 22.5 ไมโคร- โมลาร์ และผงวุ้น 0.8% (น้ำหนัก/ปริมาตร) ภายใต้สภาวะที่มีแสงมีประสิทธิภาพสูงสุดในการชักนำเมล็ดข้าวพันธุ์ กข6 ให้เจริญเป็นแคลลัส และอาหารสูตร N6 ดัดแปลง ที่เติมน้ำตาลซูโครส 3% (น้ำหนัก/ปริมาตร), IAA 2.5 ไมโครโมลาร์, BA 18 ไมโครโมลาร์ และผงวุ้น 0.8% (น้ำหนัก/ปริมาตร) เป็นสูตรอาหารที่เหมาะสมต่อการเพาะเลี้ยงแคลลัสข้าวพันธุ์ กข6 ให้เจริญเป็นต้น การศึกษาอิทธิพลของสารปฏิชีวนะต่อการเจริญเป็นต้นของแคลลัสข้าวพบว่ากานามัยซินที่ความเข้มข้น 150 มก./ล. และไฮโกรมัยซินที่ความเข้มข้น 10 มก./ล. เหมาะสมที่จะใช้ในการคัดเลือกต้นข้าวแปลงพันธุ์ กข6 ที่ทนทานต่อกานามัยซินและไฮโกรมัยซินตามลำดับ สำหรับความเข้มข้นของซีโฟแทคซีมและคาบินิซินลินที่มีผลยับยั้งการเจริญเป็นต้นของแคลลัสได้สูงที่สุด คือ 250 มก./ล. การศึกษาการส่งถ่ายยีนสู่ข้าวโดย *Agrobacterium tumefaciens* สายพันธุ์ LBA4404(pBI121) ที่มียีน *nptII* เป็นยีนคัดเลือก และมียีน GUS เป็นยีนรายงานผล และสายพันธุ์ EHA105(pCAMBIA1301) ที่มียีน *hptII* เป็นยีนคัดเลือก และมียีน GUS เป็นยีนรายงานผล ผลการศึกษาสามารถส่งถ่ายยีนสู่ข้าวพันธุ์ กข6 ได้ โดยพบการแสดงออกของยีน GUS ในแคลลัสและต้นข้าวแปลงพันธุ์ และการถ่ายทอดลักษณะทนทานต่อไฮโกรมัยซินจากต้นข้าวแปลงพันธุ์รุ่น T_0 ไปสู่รุ่น T_1

ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002

Rice (*Oryza sativa* L.) is one of the most important cereal crops, providing a staple diet for almost half of the world's population. However, rice yield and quality are affected by pests and diseases, as well as by environmental stress. Plant genetic engineering provides an opportunity to incorporate novel resistance traits into rice. The method for plant transformation, either through direct DNA delivery by particle bombardment or *Agrobacterium*-mediated transformation are well established for dicotyledonous species. In recent years, the successful transformation of rice by *Agrobacterium* had been reported, although monocotyledonous plants were considered to be outside the host range of *A. tumefaciens* (Gould *et al.*, 1991; Raineri *et al.*, 1990). Transformed rice callus was first reported by Chan *et al.* (1992) but no transgenic plants were obtained. Chan *et al.* (1993) reported the generation of first transgenic japonica rice plants by inoculating immature embryos with *Agrobacterium*. Park *et al.* (1996) described the transfer of gene coding for glufosinate-ammonium (PPT) resistance into japonica rice by using *Agrobacterium*. They confirmed the inheritance of the transferred genes to the progeny by Southern blot analysis. Insertion of the reporter gene glucuronidase (GUS) gene and the selectable marker, hygromycin phosphotransferase (hpt*II*) gene, into several cultivars of indica rice using *Agrobacterium*-mediated transformation was also subsequently demonstrated (Rashid *et al.*, 1996). Cheng *et al.* (1998) used *Agrobacterium*, which

harbored the *cryIA(b)* and *cryIA(c)* genes to introduce stem borer resistance into a japonica rice. Recently, Datta *et al.* (2000) introduced the chimeric chitinase gene in rice cultivars by using *Agrobacterium*. The transgenic plants exhibited resistance to sheath blight pathogens. Stable integration, inheritance and expression of the chitinase gene were demonstrated by Southern blot and Western blot analysis of transformants.

This research was set up to verify a transformation system using *Agrobacterium*-mediated transformation into indica rice. The rice cultivar used for the transformation was RD6, which is a glutinous rice widely grown in the northeast of Thailand. It is a Thai commercial rice cultivar represented in the Thai rice export market. This cultivar is susceptible to insect pests and diseases. The successful production of transgenic plants of this cultivar will open the way for introducing agronomically useful traits into any commercially grown cultivars, providing the best opportunity for maximizing yields.

Materials and Methods

Callus induction and regeneration

Dehulled mature seeds of rice cv. RD6 were initially washed with mild detergent and then soaked in 1% (w/v) calcium hypochlorite for 15 min. After being rinsed 4 times with sterile distilled water, seeds were cultured on Nitsch & Nitsch (1969) (N6) medium containing 4.5, 9.0, 13.5, 18.0 and 22.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 300 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose and 0.8% agar, pH 5.7. The explants were divided into two groups; the first was cultured under 16 hr photoperoid (light intensity 20 µmole $m^{-2}s^{-1}$) and the second was maintained under darkness at 25±2ºC for 4 weeks.

To evaluate the effects of IAA and BA on shoot regeneration of the calli, three-week-old calli were used as explants. Calli were cut into small pieces (5 mm in diameter) and cultured on N6 medium containing 2.5 and 5 µM IAA in combination with 4.5, 9, 13.5 and 18 µM BA. The cultures were then maintained under 16 hr photoperoid (light intensity 20 µmole $m^{-2}s^{-1}$) at 25±2ºC.

Effect of antibiotics on plant regeneration

Surface-sterilized seeds of rice cv. RD6 were induced to form calli on N6 medium containing 9 µM 2,4-D, 300 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose and 0.8% agar, at pH 5.7. Primary calli obtained after 3 weeks of culture were then transferred to the regeneration medium (N6 medium containing 18 µM BA, 2.5 µM IAA, 30 g l^{-1} sucrose and 0.8% (w/v) agar, pH 5.7) supplemented with antibiotics.

To determine the effects of the antibiotics on callus regeneration, carbenicillin and cefotaxime were added to the regeneration medium at concentrations of 0, 50, 100, 150, 200 and 250 mg l^{-1}. The effective concentrations of kanamycin and hygromycin were determined. The concentrations tested were 0, 50, 100, 150, 200 and 250 mg l^{-1} for kanamycin and 0, 5, 10, 15, 20 and 25 mg l^{-1} for hygromycin. All kinds of antibiotics were added to the regeneration medium after autoclaving. Calli were then cultured at 25±2ºC under 16 hr photoperoid (light intensity 20 µmole $m^{-2}s^{-1}$). The regeneration percentage and number of shoots per regenerating callus were evaluated after 4 weeks of culture. The experiment was repeated 5 times, each treatment with 5 explants.

Agrobacterium-mediated transformation

A. tumefaciens strain LBA4404 (pBI121) and EHA105 (pCAMBIA1301) were used for the establishment of the transformation. The plasmid pBI121 contained a chimeric genes for β-glucuronidase (GUS) gene and kanamycin-resistance (*nptII*), each driven by CaMV35S promotor. The plasmid pCAMBIA1301 carried GUS gene and hygromycin-resistance (*hptII*) gene, each expressed under the CaMV35S promotor. Sterilized mature seeds and three-week-old seed-derived calli of rice cv. RD6 were used as the explants for transformation in this experiment.

A. tumefaciens strain LBA4404 (pBI121) was cultured in LB liquid medium supplemented with 100 mg l^{-1} kanamycin and 50 mg l^{-1} streptomycin while *A. tumefaciens* strain EHA105

(pCAMBIA1301) was grown in LB medium supplemented with 50 mg l^{-1} kanamycin and 50 mg l^{-1} hygromycin. Bacterial cultures were grown on a reciprocal shaker at 28ºC for 48 hr until OD_{600} = 1.0. The explants were soaked in *Agrobacterium* suspension for 30 min. They were then co-cultivated on the callus induction medium for 3 days. After co-cultivation, the seeds and seed-derived calli were washed thoroughly in sterile distilled water containing 250 mg l^{-1} cefotaxime. Explants co-cultivated with *A. tumefaciens* LBA4404 (pBI 121) were transferred to the same medium supplemented with 200 mg l^{-1} cefotaxime and 150 mg l^{-1} kanamycin while explants co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA1301) were transferred to the same medium supplemented with 200 mg l^{-1} cefotaxime and 10 mg l^{-1} hygromycin. The surviving calli were then transferred to the regeneration medium supplemented with the same selective agents. After 45 days of culture, regenerating plants with well-developing roots were potted and grown in a greenhouse.

Assay for β-glucuronidase (GUS) activity

The histochemical assay for GUS gene expression was performed according to the method of Jefferson (1987), using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as a substrate. An incubation temperature of 37ºC was used.

PCR analysis

Total genomic DNA was extracted from the leaves of transformed plants and non-transformed control plants by the CTAB method. Primer pairs specific for CaMV35S gene as follows: forward primer 5'-GCTCCTACAAATGCCATCA-3', reverse primer 5'-GATAGTGGGATTGTGCGTCA-3' were used for PCR analysis. The expected product size was 189 basepairs. Reaction conditions were as follows: denaturation at 94ºC for 3 min followed by 40 amplification cycles (94ºC/20 sec, 50ºC/40 sec, 72ºC/60 sec) and final extension step at 72ºC for 3 min. The PCR products were visualized by running the completed reaction on a 1.5% agarose gel containing ethidium bromide.

Inheritance and Mendelian segregation

To determine the lethal dose of hygromycin during the seed-germination stage, sterilized seeds of nontransformed rice cv. RD6 were cultured on a hormone-free N6 medium containing 30 g l^{-1} sucrose, 0.8% (w/v) agar and different concentrations of hygromycin (0, 5, 10, 15, 20 and 25 mg l^{-1}) at pH 5.7. Cultures were maintained at 25±2ºC under 16 hr photoperoid (light intensity 20 µmole $m^{-2}s^{-1}$). After 14 days of culture the survival rates of nontransformed rice seeds were determined. The lowest concentration that caused the death of all seedlings was chosen for the selection of transformants.

Seeds of T_1 generation of hygromycin resistant rice were collected. In order to test their hygromycin resistance under *in vitro* condition, the seeds were surface-sterilized and placed on a hormone-free N6 medium supplemented with 30 mg l^{-1} sucrose, 0.8% (w/v) agar and a lethal dose of hygromycin (20 mg l^{-1} hygromycin), at pH 5.7. After 14 days of culture at 25±2ºC under 16 hr light, numbers of germinating seedlings were collected.

Results

Callus induction and regeneration of rice

Swelling of scutellum region of rice embryos were observed 2-3 days after culture on N6 medium containing 2,4-D. This was followed by development of primary calli from the scutellum. Scutellum-derived callus was yellow in color under light condition while the color of the calli cultured in darkness was creamy white. Differences in induction percentage were recorded (Table 1). The highest callus induction percentage at 97 was obtained in the medium containing 22.5 µM 2,4-D. It was found that callus induction under light condition was more effective than that under darkness.

Various concentrations of IAA and BA were used for regeneration of shoots. The highest regeneration percentage at 61 and 6 shoots per callus were obtained when calli were cultured on N6

Table 1. Effect of different concentrations of 2,4-D on percent callus induction of rice cv. RD6 cultured on N6 medium under light and darkness condition

2,4-D concentration (µM)	Callus induction (%)	
	Darkness condition	Light condition
4.5	53.13c	81.25c
9.0	80.0b	89.28b
13.5	90.0a	80.56c
18.0	82.5b	93.75a
22.5	87.5a	97.22a
F-test	*	*
CV (%)	22.00	17.095

*** significant difference at ρ<0.01. Means within a column not sharing a common letter differ significantly by DMRT**

medium supplemented with 2.5 µM IAA and 18 µM BA (Figure 1). The results from all tested combinations revealed that shoots developed without forming roots.

Effect of antibiotics on rice regeneration

Antibiotics strongly reduced regeneration capacities of rice calli. In the presence of 50 and 100 mg l^{-1} carbenicillin and cefotaxime, a slightly inhibitory effect was seen. The highest dose of both carbenicillin and cefotaxime (250 mg l^{-1}) completely inhibited regeneration (Figure 2). The presence of kanamycin and hygromycin decreased the number of shoots and percent regeneration in all treatments. Hygromycin clearly affected regeneration capacities as compared to kanamycin. Complete inhibition of plant regeneration from rice calli occurred at 150 mg l^{-1} kanamycin, while

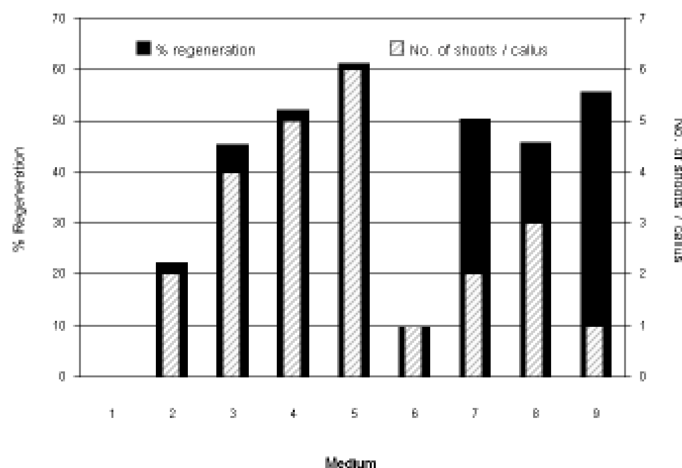


Figure 1. Effect of various concentrations of IAA and BA on plant regeneration from the calli of rice cv. RD6. medium 1: growth hormone free, media 2 to 5: contained 2.5 µM IAA in combination with 4.5, 9, 13.5 and 18 µM BA, media 6 to 9: contained 5 µM IAA in combination with 4.5, 9, 13.5 and 18 µM BA. The plotted values are averages of 5 replicates.

no shoot regeneration was observed at 10-50 mg l^{-1} hygromycin (Figure 3).

***Agrobacterium*-mediated transformation of rice**

Co-cultivated rice seeds and seed-derived calli that had been transformed with *A. tumefaciens* strain LBA4404 (pBI121) and EHA105 (pCAMBIA1301) were cultured on the induction medium containing selecting agents, kanamycin and hygromycin. After 5 days, GUS activities in rice calli were detected (Figure 4). Proliferation of scutellum calli on the selective medium was observed. After resistant calli were transferred to a regeneration medium that contained the same selective agents, plants were regenerated (Figure 5(a), (b) and (c)). Surviving plants showed stable expression of GUS activities as shown in Figure 5 (d), (f), (g), (h), (i), (j) and (k). Regenerated plants with well-developed roots were then selected and grown in a greenhouse. In three months all primary transformed plants grew normally and produced normal seeds after self pollination (Figure 6).

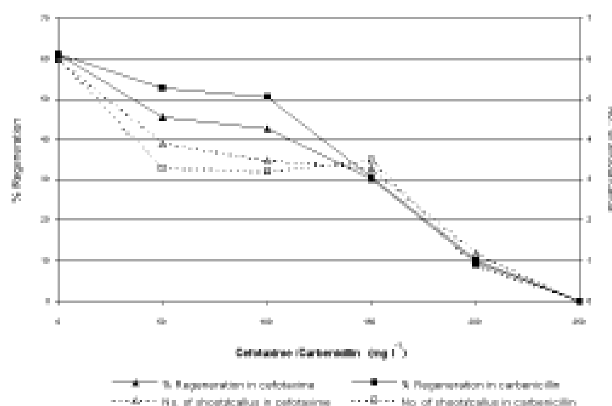
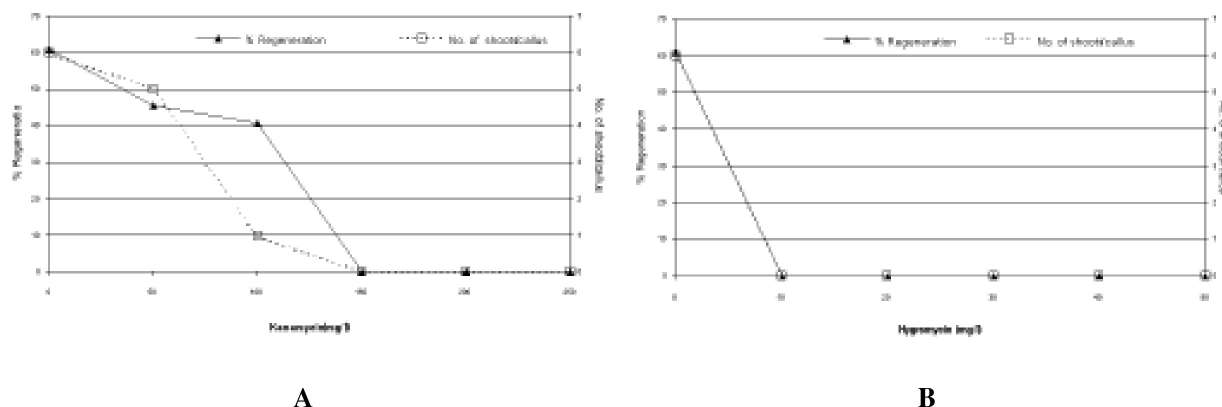


Figure2. Effect of different concentrations of cefotaxime and carbenicillin on plant regeneration from the calli of rice cv. RD6. The plotted values are averages of 5 replicates.



A **B**

Figure 3. Effect of different concentrations of kanamycin (a) and hygromycin (b) on plant regeneration of the calli of rice cv. RD6. The plotted values are averages of 5 replicates.

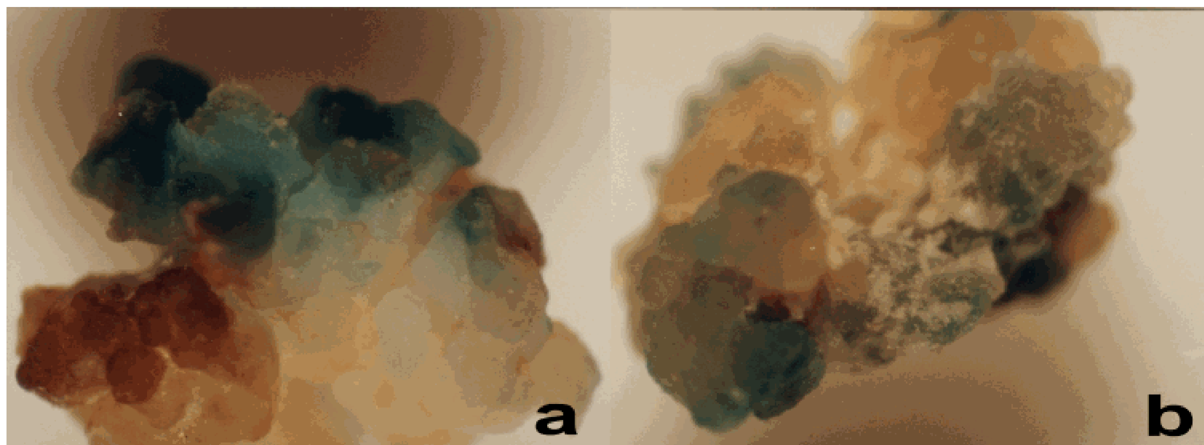


Figure 4. Visual detection of histochemical staining for GUS activity in the calli of rice cv. RD6 following *Agrobacterium*-mediated transformation.
(a) five days after co-cultivation with LBA4404 (pBl121)
(b) five days after co-cultivation with EHA105 (pCAMBIA1301)

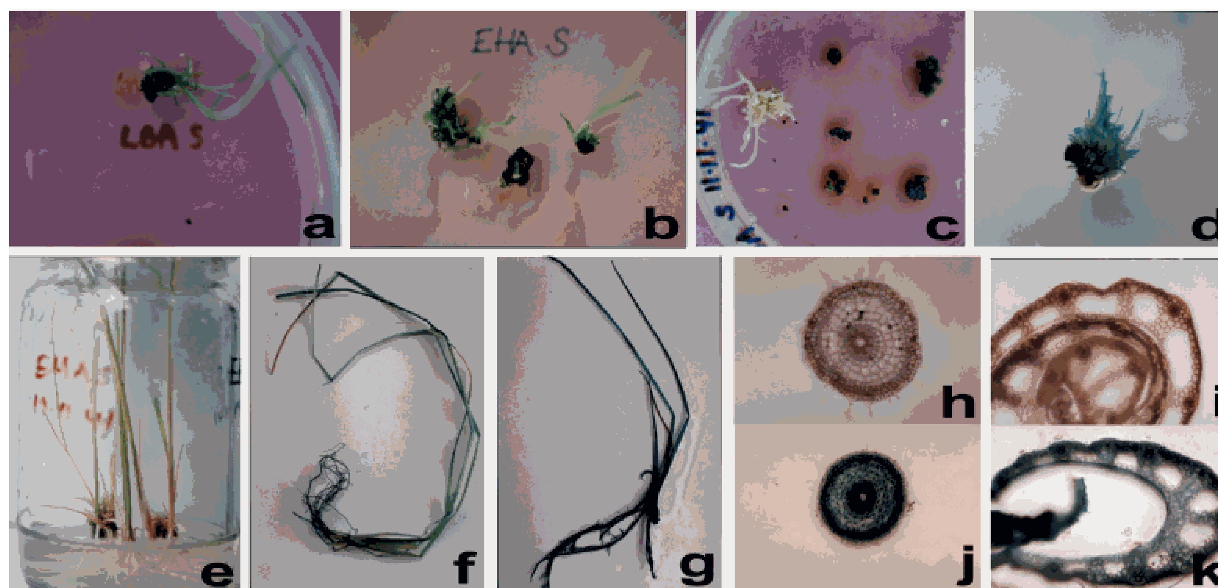


Figure 5. (a), (b) Transgenic plants derived from calli co-cultivated with *A. tumefaciens* strain LBA 4404(pBI121) and EHA105(pCAMBIA1301), (c) Albino transgenic plants regenerated from calli co-cultivated with *A. tumefaciens* strain LBA4404(pBI121), (d) Expression of GUS gene in albino kanamycin resistant plants, (e) Plant regeneration at four weeks after selected calli was transferred to regeneration medium,(f), (g) Expression of GUS gene in transformants, (h), (i) Root and leave sheath cross section of untransformed plant, (j), (k) Root and leave sheath cross section of transgenic plants, showing GUS activity compared with control.

Figure 6. Hygromycin-resistant plants at maturation.

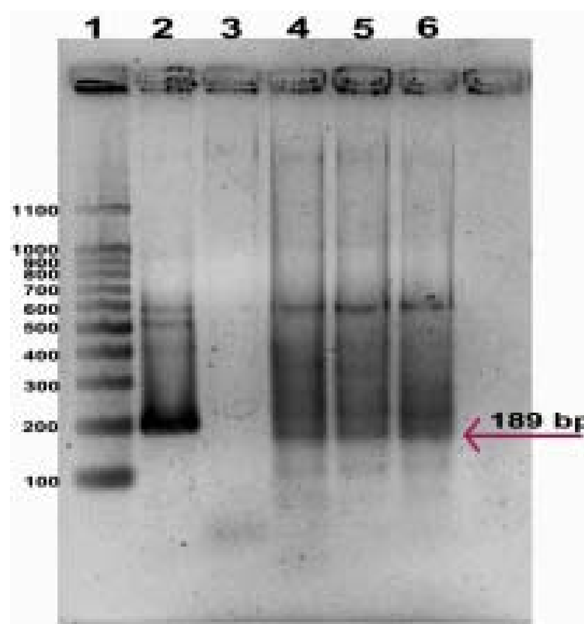


Figure 7. PCR analysis of transformed T_1 plants of rice cv. RD6.
lane 1: 100 bp ladder, lane 2: pCAMBIA1301, lane 3: nontransformed control rice plant, lane 4, 5 and 6: hygromycin resistant plants, showing the expected 189 bp fragment.

Efficiency of transformation in rice cv. RD6 by both strains of *A. tumefaciens* is shown in Table 2.

To determine the integration of T-DNA fragments in hygromycin-resistant plants, polymerase chain reaction (PCR) analysis was carried out. Leaves of transgenic plants were analyzed using PCR with primer designed to amplify the CaMV35S promotor. Figure 7 shows an analysis of the PCR amplification band of genomic DNA of hygromycin-resistant rice plants. The expected 189 basepairs fragment of CaMV35S promotor was presented.

Inheritance and Mendelian segregation

An appropriate concentration of hygromycin for selecting hygromycin-resistant rice seeds was verified. The survival rates of non-transformed rice seeds on the medium containing hygromycin at 0, 5, 10 and 15 mg l^{-1} were 92.3, 62.96, 27.5 and 10% respectively. No seed survived on the medium containing 20 and 25 mg l^{-1} hygromycin. Therefore, hygromycin at 20 mg l^{-1} was used as a selective agent (Figure 8(a)).

T_1 hygromycin-resistant seeds of rice cv. RD6 (Hm2, Hm3, Hm4, Hm5, Hm6, Hm7, Hm8, Hm9) were recovered on the medium containing a lethal dose of hygromycin (20 mg l^{-1}) compared with seeds of the control. No control rice seeds escaped from this selection medium, whereas some T_1 progeny seeds regenerated plants in 4-5 days (Figure 8(b) and (c)). The genetic segregation to hygromycin resistance was observed in T_1 progeny plants. Mendelian segregation was observed in Hm4, Hm5 and Hm7 progeny, while the others had the different patterns of segregation as shown in Table 3.

Discussion

Plant regulators have an important role in callus cultures and the effect of growth regulators on plant regeneration in rice has been investigated (Rueb *et al*., 1994; Zhang *et al*., 1996; Marassi *et al*., 1996; Oinam and Kothari,1993). 2,4-D is preferred in the medium for induction and proliferation of rice callus while a growth regulator free medium or a medium containing a combination of auxin and cytokinin is used for plant regeneration (Yang *et al*., 1999).

Concentrations of selective agents are needed to avoid development of undesirable numbers of the escapes. In this experiment, two selectable marker genes were used in the production of transgenic rice: *nptII* (encoding resistance to kanamycin) and hpt*II* (encoding resistance to hygromycin). Kanamycin and hygromycin are aminoglycoside antibiotics which cause harmful death to plant cells by inhibiting transcription and translation. Chan *et al*. (1992) reported a transformation of *nptII* gene into indica rice cv. Taichung Native 1 by *A. tumefaciens* and kanamycin at 20 mg l^{-1} was used in selecting media. Cheng *et al*. (1998) reported the presence of 50 mg l^{-1} hygromycin throughout the callusing as well as regeneration period to avoid development of the escapes.

In order to eliminate *A. tumefaciens* after co-cultivation, the use of antibiotics in the culture medium is required. Unfortunately, the concentra-

Table 2. Efficiency of transformation by *Agrobacterium tumefaciens* strain LBA4404(pBI121) and EHA105 (pCAMBIA1301)

Strains	Plant material	No. of inoculated explants	No. of plants in T_0 generation		No. of tested plants		No. of GUS+ plants	
			Normal	Albino	Normal	Albino	Normal	Albino
LBA4404	seeds	450	40	30	22	30	22	30
(pBI121)	calli	300	-	5	-	5	-	5
EHA105	seeds	450	12	-	5	-	5	-
(pCAMBIA1301)	calli	300	-	-	-	-	-	-

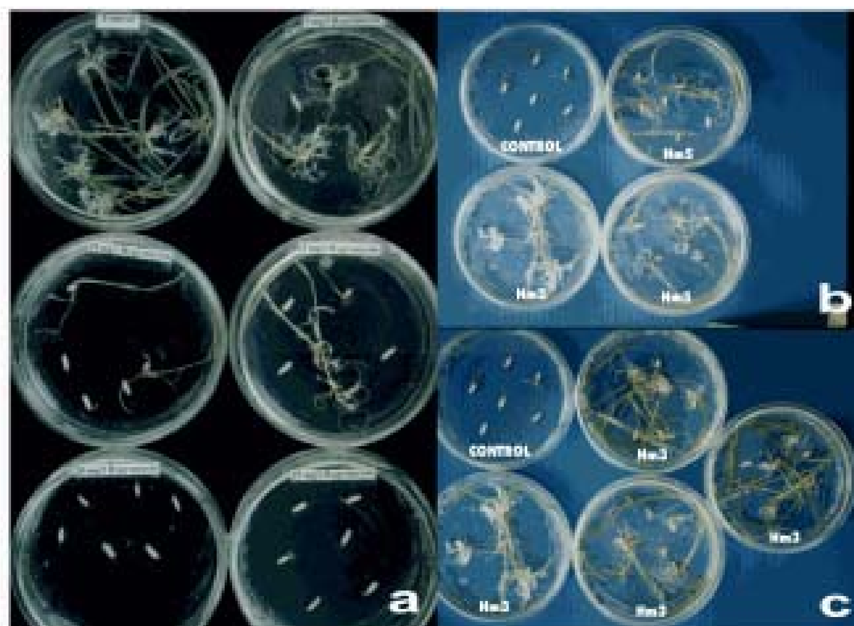


Figure 8. (a) Seed germination test on media containing six different concentrations of hygromycin (0, 5, 10, 15, 20 and 25 mg l^{-1}), (b) and (c) Test of the progenies for resistance to hygromycin on media containing 20 mg l^{-1} hygromycin. Control seeds produced no seedlings on the selection medium, while progenies from Hm3 and Hm5 produced vigorous roots and green shoots.

Table 3. Mendelian segregation in T_1 hygromycin resistant plants

Transformants (T_1 line)	Total assayed	Expected ratio	χ^2-value	Prob.
Hm2	40	1:1	0.167	0.683
Hm3	40	1:1	1.084	0.297
Hm4	40	3:1	0.025	0.755
Hm5	40	3:1	0.026	0.873
Hm6	40	1:1	0.043	0.835
Hm7	40	3:1	0.71	0.399
Hm8	45	1:1	0.04	0.841
Hm9	50	1:1	0.00	1.0

tions required in many cases inhibit regeneration of the plant tissues (Barette and Cassells, 1994). The capable of carbenicillin and cefotaxime in controlling the growth of *Agrobacterium* on the regeneration of the calli of rice cv. RD6 showed that there was strong inhibition of the regeneration potential. The reduced regeneration capacity is in agreement with the results obtained by Nauerby *et al.* (1997), who reported that 500 mg l^{-1} cefotaxime and 1000 mg l^{-1} carbenicillin played an inhibitory effect on regeneration of *Nicotiana tabacum*. However, a positive effect of concentration up to 1000 mg l^{-1} was seen in some plants (Robert *et al.*, 1989; Eapen and George, 1990). Barrett and

Cassells (1997) found no influence of 500 mg l^{-1} cefotaxime on regeneration of *Pelargonium domesticum* cv. Grand Slam.

In this study, a number of transformed indica rice cv. RD6 were produced using *Agrobacterium* transformation system. The results confirmed that seeds and calli of rice cv. RD6 can be used as the target tissue for *Agrobacterium*-mediated transformation. We also confirmed the result of Cheng *et al.* (1998) that it is not necessary to use the supervirulent strain for rice transformation. Datta *et al.* (2000) reported rice transformation in three indica rice cultivars without using the supervirulent strains of *A. tumefaciens*. However, supervirulent strains of *A. tumefaciens* were reported to be very useful in some rice callus transformation (Hiei *et al.*, 1994; Hauptmann *et al.*, 1988).

Our results showed that CaMV35S promotor was useful for rice transformation. Hiei *et al.* (1994) reported high level of gene expression with 35S promotor. This is in contrast to Park *et al.* (1996), who reported that the rice *actin1* might be a more effective promotor for driving high expression of gene than CaMV35S promotor. Hauptmann *et al.* (1998) demonstrated that the CaMV35S promotor expression was less effective in cereal cells than in dicot cells.

We reported the efficiency in rice transformation without using any *vir* inducer. This is in agreement with Raineri *et al.* (1990), who reported a successful transformation in rice without using a *vir* gene inducing compound. However, no transgenic plant was obtained. Vijayachandra *et al.* (1995) reported that scutellum tissue of rice seeds which was cultured on the medium containing 2, 4-D promoted the activation of *virE* expression. They suggested that rice scutellum and scutellum-derived calli were excellent explants in *Agrobacterium*-mediated transformation. However, the pretreatment of *A. tumefaciens* with *vir* inducing compound, particularly acetosyringone and potato suspension, before co-cultivation was nescessary for promoting the efficiency of transformation (Park *et al.*, 1996; Godwin *et al.*, 1991; James *et al.*, 1993)

T-DNA was shown to be stably maintained in transformed rice calli. PCR analysis was consistent with genomic integration of these genes. Inheritance studies of the T_1 progeny of hygromycin-resistant rice cv. RD6 showed that the *hptII* gene was transferred to the T_1 progeny, demonstrating stable incorporation of T-DNA into the rice nuclear genome. The 3:1 segregation ratio suggested that the *hptII* gene was integrated at a single locus. However, different patterns of segregation were observed, and these led to the assumption that there might be some multiple T-DNA integration events.

The low efficiency in plant regeneration of rice calli co-cultivated with both strains of *Agrobacterium* was achieved in this study. This might be related to the prolonged period of tissue culture. The calli were almost four weeks old before co-cultivation and were maintained for more than four weeks on the selection medium to ensure no escape of nontransformants. Wu *et al.* (1998) reported the loss of morphogenetic capacity in the established transformed callus and suggested that reduction of the culture period enhanced plant regeneration.

In summary, this report described the use of *A. tumefaciens* strain LBA4404(pBI121) and EHA105(pCAMBIA1301) to transfer screenable and selectable marker genes into rice cv. RD6 and showed molecular evidence of primary transgenic plants and T_1 progeny which demonstrated stable integration and inheritance of transgenes. We confirm that rice seeds are the suitable target tissues for *Agrobacterium*-mediated transformation. We also propose that this method can be used to transform novel genes into other rice cultivars and other cereal species, particularly genes conferring disease or pest resistance.

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