

Production of transgenic Indica rice (*Oryza sativa* L.) Cv. MR 81 via particle bombardment system

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Abstract: Embryogenic callus of rice (*Oryza sativa* L. cv. MR 81 and Taipei 309) were bombarded with the recombinant pRQ6 construct carrying the hygromycin phosphotransferase (*hph*) and β -glucuronidase (*gusA*) genes using particle bombardment system. Transformed embryogenic callus that are capable of growing on selection media containing 50mg/L hygromycin B developed GUS expression based on blue colouration in the presence of 5-bromo-4-chloro-3-glucuronic acid. These embryogenic callus consisted of both transformed (blue) and non-transformed (yellow or white cells). The integration of *hph* gene into rice genome was confirmed by Southern blot hybridization. Regenerated plantlets were transferred to soil in pots, and grown to maturity under glasshouse conditions. Rice (R1) seedlings derived from R0 transgenic plants germinated normally in a medium containing 50mg/L hygromycin B and also expressed GUS activity as revealed by histochemical assay. The Southern blot analysis further confirmed the insertion of the *hph* gene in the rice genome of the R1 plants of MR 81 and Taipei 309. A simple and economic procedure for screening large number of putative resistant rice plants was described.

Key words: Rice embryogenic callus, Particle bombardment, *gusA* gene, *hph* gene.

إنتاج الأرز الهندي المعدل وراثيا من خلال نظام القصف بالجسيمات

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الملخص: تم قصف الكالس الجنيني لنبات الأرز *Oryza sativa* L. (صنف 81 و تايبيه 309) وإعادة ضمها من خلال pRQ6 والمحتوي على جينات *hph* و hygromycin phosphotransferase (*hph*) و β -glucuronidase (*gusA*) باستخدام نظام القصف بالجسيمات. الكالس الجنيني المعدل القادر على النمو على وسط يحتوي على 50mg/L hygromycin B طور GUS بناء على اللون الأزرق بوجود حامض 5-bromo-4-chloro-3-glucuronic acid. هذا الكالس الجنيني يحتوي على خلايا متحولة (ذات اللون الأزرق) وخلايا غير متحولة (ذات اللون الأصفر أو الأبيض) وتم تأكيد اندماج جينات *hph* لجينوم الأرز بواسطة تهجين لطخة سذرن (Southern blot hybridization). وتم نقل بادرات الأرز وزراعتها في أصص تحتوي على التربة حتى مرحلة النضج تحت ظروف البيت الزجاجي. صنف الأرز R1 الناتج من النباتات المعدلة وراثيا R0 نمت بشكل طبيعي في الوسط المحتوي على 50mg/L hygromycin B كما يظهر نشاط GUS وذلك من خلال الفحص النسيجي. كما اكد تحليل لطخة سذرن على عمليات إدخال جين *hph* في جينوم الأرز R1 من نباتات 81 و تايبيه 309. في هذه الدراسة تم وصف طريقة بسيطة واقتصادية لفحص وفرز عدد كبير من نباتات الأرز المتحملة.

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Introduction

Direct foreign gene transfer into intact tissues by particle bombardment is regarded as the most efficient and consistent genotype-independent approach for gene transfer across japonica and indica rice lines (Christou et al., 1991; Valdez et al., 1998; Srivastava and Ow, 2001; Visarada and Sarma, 2004; Grewal et al., 2006). The bombardment technique has been also used to achieve stable transformation of soybean (McCabe et al., 1999), cotton (Finer and McMullen, 1990), barley (Bregitzer and Tonks, 2003), sugar cane (Bower and Birch, 1992), oats (Somers et al., 1992) and wheat (Weeks et al., 1993; Becker et al., 1994).

Our attempts at rice transformation have been concentrated using this method as the success has been widely reported for various crops and is a routine procedure in many laboratories (Srivastava and Ow, 2001; Visarada and Sarma, 2004; Grewal et al., 2006). The choice of target tissue is one of the major factors for successful transfer of the gene of interest into rice. This target tissue must eventually be able to produce fertile plants from the *in vitro* culture procedures with some researchers reported that the callus cultures derived from mature seeds or immature seeds have the potential to regenerate high number of fertile rice plants (Sivamani et al., 1996; Chen et al., 1998).

In this study, we were focused on the production of transgenic indica rice plants cv. MR 81 via particle bombardment system and the transmission of both *hph* and *gusA* to its progenies into the first generation (R1). Concurrently, transgenic japonica rice plants cv. Taipei 309 was also obtained as a universal control since the particle bombardment technique have been reported successful to produce fertile transgenic japonica rice plants (Chen et al., 1998). Thus, improvement in the rice production and productivity is achievable when genotypes of specific barriers using genetic engineering systems are overcome.

Materials and Method

Plant materials and tissue culture

Mature seeds of rice cv. MR 81 and Taipei 309 were obtained from the MARDI Rice Research Centre in Kepala Batas, Penang. Indica rice, MR 81 was chosen for use in the particle bombardment transformation experiment for it is one of the varieties released by MARDI. The parents of MR 81 are the two varieties, IR 36 and Pankhari. Taipei 309 serves as the control model as a comparison. Rice plants MR 81 and Taipei 309 were grown in the glasshouse at MARDI, Serdang, Malaysia.

Callus induction and regeneration

Mature seeds were harvested, dehusked and sterilized with 70% ethanol for 5 min, followed by 20% sodium hypochloride and washed three times with sterile distilled water. Five seeds were placed onto each Petri dish with callus induction (CI) medium, described below (Table 1), and the plates incubated in the dark at 24°C. The different medium for Indica rice MR 81 used in these experiments was based on those described by Murashige and Skoog (1962). All the medium namely callus induction (CI), pre-regeneration medium (PM), callus regeneration (CR) and osmoticum medium (OM) contained the following basic ingredients: MS (Murashige and Skoog, 1962) basal salts, 30g/L sucrose, 1.0g/L thiamine-HCL, 1.0g/L casein hydrolysate, 0.5g/L proline, gelrite, pH 5.8. The CO medium contained an additional of 0.4M total carbohydrates, 30g/L sorbitol and mannitol. Optimisation of phytohormone levels, auxin (2,4-dichlophenoxyacetic acid (2,4-D), and cytokinin (6-benzylaminopurine) BAP, in CI using medium was carried out previously (Hamidah, unpublished data) to determine the medium formulation for callus induction. To test the callus regeneration ability after selection on hygromycin B containing medium, CI medium was supplied with 20, 30, 40, and 50 mg/L

hygromycin B, and callus regeneration frequency (CRF%) was recorded. The callus induction medium and the procedure

to transform and regenerate Taipei 309 seeds are as described by Li et al. (1993).

Table 1. Summary of the comparison of the protocol from callus induction to transformed plants expressing GUS and hygromycin resistance in Taipei 309 (Japonica) and MR81 (Indica).

	Taipei 309 (seeds)	MR 81 (seeds)
1. Callus induction medium	N6 macro and B5 micro salts, B5 vitamins, 2 mg/L 2,4-D (NB medium)	MS macro and micro salts, B5 vitamins, 2 mg/L 2,4-D (CI medium)
2. Callus Age	4 weeks	4 weeks
3. Pre-bombardment Treatment	N6 macro and B5 micro salts, B5 vitamins, 2 mg/L 2,4-D, 30 mg/L sorbitol and 30 mg/L mannitol (NBO)	MB + 30 mg/L sorbitol + 30 mg/L mannitol (CO medium)
4. Duration	4 hr prior to bombardment	4 hr prior to bombardment
5. Post-bombardment medium	NB	CI medium
6. Duration	7 days post-bombardment	7 days post-bombardment
7. Subculture medium I	NB + 50 mg/L hygromycin	CI + 50 mg/L hygromycin
8. Subculture interval	15 days (twice)	15 days (twice)
9. Pre-regeneration medium	NB (without 2,4-D) + 5 mg/L ABA + 2 mg/L BAP + 1 mg/L NAA + 50 mg/L hygromycin	CI (without 2,4-D) + 5 mg/L ABA + 2 mg/L Kinetin + 1 mg/L NAA + 50 mg/L hygromycin
10. Subculture Interval	15days	15 days
11. Regeneration Media	NB (without 2,4-D) + 3 mg/L BAP + 0.5 mg/L NAA + 50 mg/L hygromycin	MB (without 2,4-D) + 3 mg/L Kinetin + 0.5 mg/L NAA + 50 mg/L hygromycin
12. Subculture Interval	15 days	15 days
13. Plant Growth Media I	MS	MS
14. Subculture Interval	15days	15days

Plasmid construct

Plasmid pRQ6 (Figure 1), the vector used for the rice transformation experiments, given by Dr. Rhonda Qu of the International Laboratory for Tropical Agriculture Biotechnology at the Scripps Research Institute (ILTAB/TSRI). This plasmid contains β -glucuronidase (GUS)

gene under the control of cauliflower mosaic virus (CaMV) 35S promoter and the hygromycin phosphotransferase (*hph*) gene, encoding the enzyme hygromycin phosphotransferase which enhances resistance to hygromycin B under the control of CaMV 35S promoter.

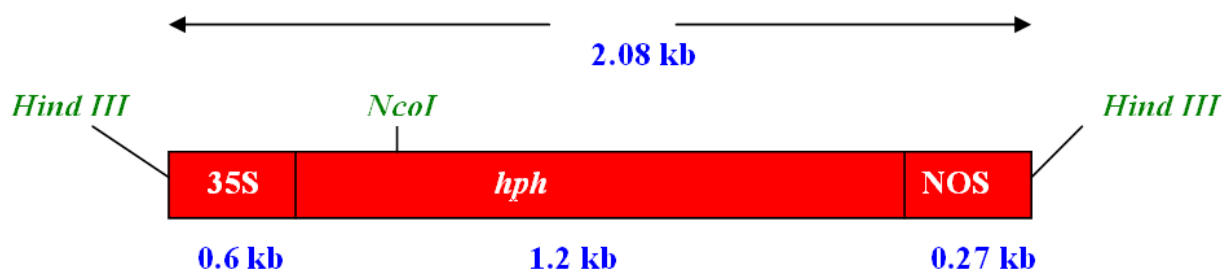


Figure 1. Restriction Enzyme Map of the *hph* and the *gusA* genes construct of pRQ6. Numerical values indicate approximate kilobase lengths.

Preparation of DNA and particle delivery

All DNA introductions into rice calli were done using a Biolistic PDS-1000/He Particle Delivery System (BioRad, USA). DNA preparation for bombardment was carried out by adding in order, 25µl of gold suspensions, 2.5µg DNA, 10µl spermidine, 50µl CaCl₂ and left at room temperature for 10 min. A 7.5µl pf the particle DNA mixture was distributed on the microcarrier (Sivamani et al., 1996). Approximately, 50 pieces of four week old embryogenic callus were placed evenly on CO medium. The DNA delivery was used according to the manufacturer's instructions. All target tissues were placed at a distance of 5 cm from the stopping plate in the gene gun chamber at helium pressure of 1100 psi and were bombarded with single shots.

Selection of transformants and recovery of hygromycin phosphotransferase (hpt) resistant rice plants

Following bombardment, the callus cultures of Taipei 309 and MR 81 were subsequently then transferred to modified N6 and CI mediums, respectively. Hygromycin as a selection agent was chosen to select for callus that was stably transformed with *hph* gene. While callus growth still occurred at 30mg/L hygromycin B, callus development was effectively eliminated at 50mg/L. To minimize the possibility of escapes, a concentration of 50mg/L was finally chosen for routine selection of

transformants application (unpublished data). This concentration was also found to be efficient in preventing the growth of established non-transformed callus (data not shown). The time of the initial application of the selection agent (0 day or 7 days) did not influence the selection stringency during the callus regeneration phase. Therefore, day 7 was chosen by which time the expression of hygromycin should have been well established and first callus growth are imminent. After seven days post-bombardment, the callus were transferred with medium supplemented with 50mg/L hygromycin B. Surviving white callus was further subcultured twice on a media of N6 or MS medium, with 50 mg/L hygromycin B for another 14 days. After 4 weeks, shoots were transferred to MS hormone free medium with 50mg/L hygromycin B to allow further development. Regenerated plantlets were transferred to soil in pots, and grown to maturity under glasshouse conditions. Two independent R1 plants which were progeny of plants M4 (cv MR 81) and R1 (cv Taipei 309) were examined. For each line, 40 seeds were randomly chosen from R0 plants and germinated on plant growth medium with 50mg/L hygromycin B for three weeks.

Histochemical GUS assay

Transient gene expression experiments with rice callus for both varieties were done 2 days after bombardment in a GUS substrate mixture (Jefferson, 1987). The percentages of

callus expressing GUS were recorded after being incubated in staining solution containing 1mM of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Research Organics, Cleveland, Ohio, USA) in 50mM phosphate buffer, pH 7.0 at 37°C overnight. The same procedure was used to assay GUS expression in leaf pieces, whole plantlet and inflorescence of putatively transformed R0 and R1 plants. For the leaves, chlorophyll was extracted after staining by incubation in 70% ethanol. Two independent R1 lines which were the progeny of M4 (cv. MR 81) and T1 (cv. Taipei 309) were examined. For each line, five seeds were randomly chosen from one panicle of R0 plants. A total of 40 seeds were inoculated on plant growth media containing 50mg/L hygromycin B. Leaves and roots were excised from each plant and examined for the presence of GUS activity (blue sectors).

PCR assay of the putative transgenic rice plants

Polymerase chain reaction (PCR) assay was used to detect *hph* gene in the R0 and R1 plants. Total DNA from putatively regenerated R0 and R1 rice plants of MR 81 and Taipei 309 were analysed by PCR using the primers that were specific to the coding region of the *hph* gene. The complete PCR mixture contained about 100ng genomic DNA, 5µl of 10x Taq-polymerase buffer (Boehringer), 0.5µl of dNTP mixture (10mM concentration each), 0.5µl Taq-polymerase (2.5 units), 0.5µl of both primers (10µM each) in a final volume of 50µl. The PCR cycles (Perkin Elmer) were at 94°C for 3 min for one cycle, 95°C for 30 sec, 55°C for 1 min for 30 cycles and 72°C for 7 min for one cycle. An aliquot of 10µl of each reaction was used for electrophoresis on a 1% TAE agarose gel.

DNA Isolation and Southern blot analysis

Genomic DNA was extracted from leaves of rice plants based on Dellaporta et al. (1983) with modifications. About 5µg of genomic DNA from each sample, undigested or digested with restriction endonucleases (*Hind III* and *NcoI*) was used for electrophoresis in 0.8% agarose gel. Southern blot transfer to Hybond-N⁺ nylon membrane was according to manufacturer's instructions (Amersham, Arlington Heights, USA). Protocols for fixation, prehybridization, hybridization and washing of the membrane was in accordance to manufacturer's instructions. The ³²P-probe for Hybridization was prepared using a PCR product of the *hph* gene with the Prime it II random priming kit (Strangene, CA, USA) according to the procedure supplied by the manufacturer.

Results

Histochemical analysis of the GUS gene driven by CaMV 35S promoter

Forty-eight hours after bombardment, the embryogenic callus cultures produced detectable levels of GUS activity after bombardment of particles coated with pRQ6 plasmid containing the β -glucuronidase gene as determined by the appearance of the blue colourations following the addition of enzyme assay substrate, while being cultured in 50 mg/L hygromycin B. All transgenic plant samples expressed the GUS gene in the roots, shoot tissues and inflorescence (Figure 2A-E). GUS activity was also observed in the hygromycin-resistant seedlings of R1. GUS expression in different tissues of R1 plants are shown in Figure 2A-E. About 25% of the Taipei 309 exhibited GUS activity while 20% of the MR 81 exhibited GUS activity. Hygromycin resistant somatic embryos induced from the transformed callus cultures were found to be uniformly GUS positive by histochemical assay (Figure 2B). This was an indicative of the presence

and expression of *gusA* as a reporter gene as well as the selectable marker gene. Untransformed control callus remain yellowish white in the presence of the X-Gluc. All transgenic plant samples of R0

and R1 generation expressed the GUS gene in roots, shoots tissues and inflorescence. Multiple shoots and roots emerged from resistant callus on the regeneration medium with selection within 2-3 weeks.

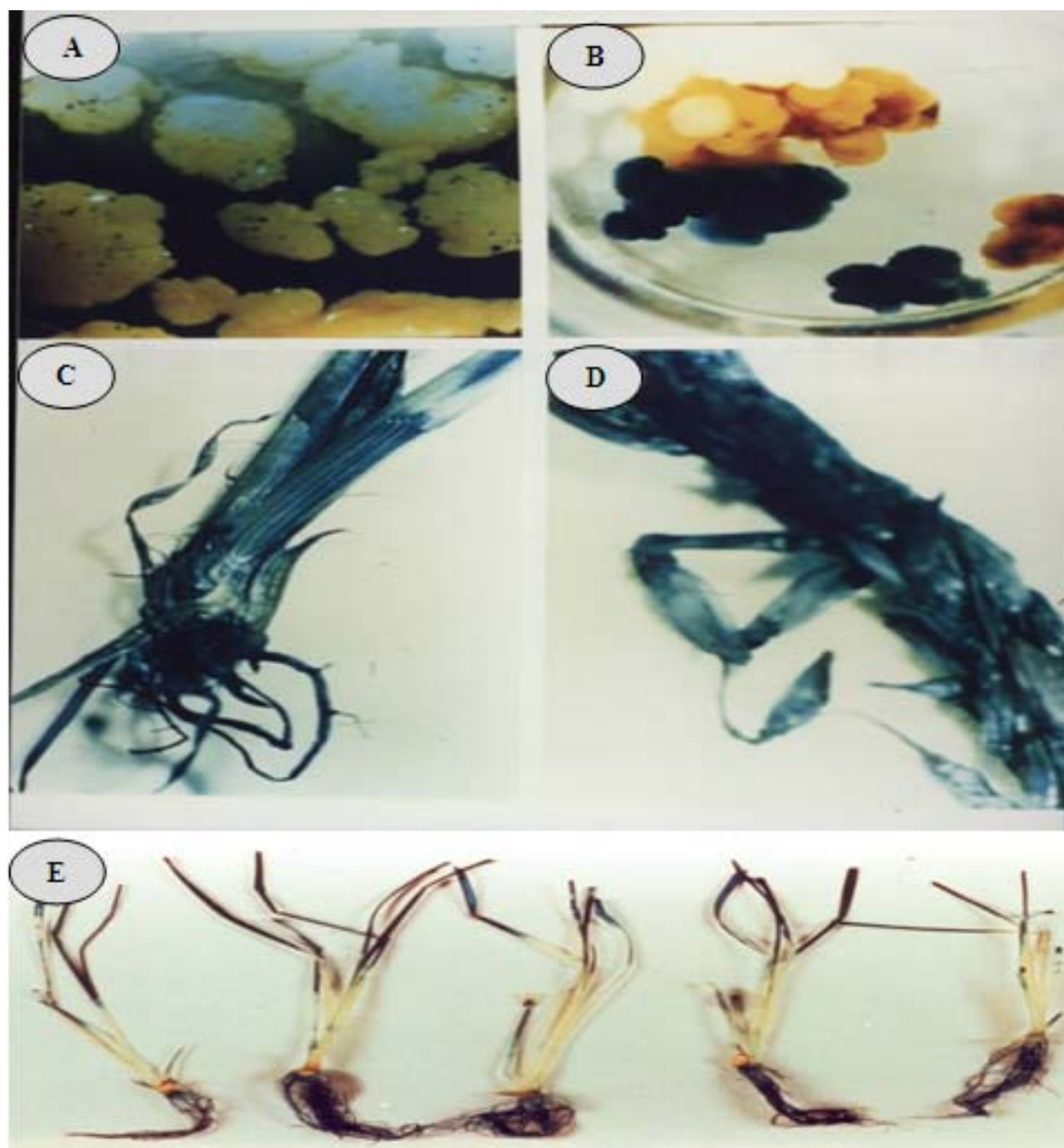


Figure 2. (A) Transient GUS expression in rice callus culture; (B) Hygromycin-resistant callus culture staining for GUS activity (blue) after 1 month of continuous culture onto hygromycin selection medium. The untransformed control callus does not express the GUS activity (white); (C, D): Hygromycin-resistant plants regenerated from transformed callus cultures, expressed GUS in the roots, leaves, stem and inflorescence; (E) GUS positive rice seedlings of MR 8.

DNA transfer and selection of transgenic rice plants

Callus bombarded with plasmid pQR6 grew well when placed on modified MS medium for the first 7 days. After being subjected to HPH selection for the following 3 weeks, most calli stopped growing, while a few putative transformants continued to grow (Figure 3B). In control treatment, the callus that was bombarded without plasmid DNA (i.e., no DNA) did not continue to grow under HPH selection. Since 50mg/L of hygromycin B were effective in selecting the transformants (data not shown), we have used this concentration in the subsequent experiments. We have also observed that a selection pressure of 50mg/L hygromycin B was strong enough to inhibit further development of the non-resistant somatic embryos at early stages of development and the same concentration could be used continuously at different phases of rice plant regeneration (Figure 3) and growth.

After being transferred to regeneration medium, HPH-resistant callus

formed morphological structures resembling coleoptiles or shoots within 3 weeks. These structures developed into plantlets (Figure 3) when transferred to MS medium lacking plant growth regulators. After 5 weeks in the MS medium, these rice plantlets were readily transplanted into soil and grown in the greenhouse. Forty-three HPH-resistant callus from six separate experiments gave rise to at least 1 plantlets with some callus could produced more than 10 plantlets (Table 2). In five separate experiments, the measurement of morphological characters showed that the R0 transgenic plants of MR 81 (Table 3) and Taipei 309 had much lower average height and a higher number of tillers per plant. All transgenic R0 rice plants of Taipei 309 and MR 81 were set seeds upon self-pollination. The R0 plants of MR 81 and Taipei 309 and that of their progeny having the HPH gene germinated normally in the growth medium with 50mg/L hygromycin B. All the 5 independent regenerated calli were positive for GUS and resistant on 50mg/L hygromycin B.

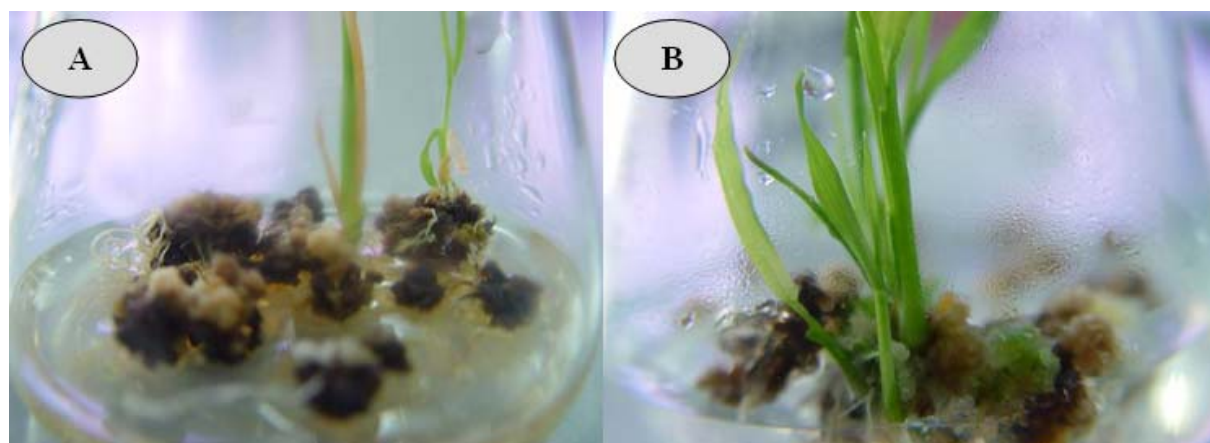


Figure 3. (A, B) Plant Regeneration of Taipei 309 and MR81 callus under selection medium containing 50mg/L hygromycin B, respectively.

Table 2. Regeneration of transgenic plantlets from HPH-resistant callus of MR 81.

Experiment number	No of HPH-resistant callus selected for regeneration	Regenerated rice plantlets
A	6	18
B	13	33
C	4	12
D	11	31
E	7	34
f	2	13

^a Each HPH-resistant callus produced at least one plant. Some calluses were capable of generating of 10 plantlets.

Table 3. Plant height, tiller number, flat leaf length and length of second leaf of non-transformed (control) MR 81 and transformed MR 81 (7 weeks after transplanting).

Trough number	Plant number*	Plant height (cm)	Tiller number	Flat leaf length (cm)	Second leaf length (cm)
1	5312	98	24	50	55
Control	Non-transformant	117	17	56	63
	5312	90	17	35	40
	5291	85	17	29	36
Seed	Non-transformant	123	17	60	70
	5312	130	30	47	61
2	5291	103	33	47	55
Seed	Non-transformant	120	16	56	62
	5264	98	12	43	53
	5264	106	13	53	64
Control	Non-transformant	115	14	51	69
	5291	106	34	44	54
3	5410	104	22	41	54
Seed	Non-transformant	127	17	63	67
	5312	100	14	37	54
	5291	62	20	29	33
Control	Non-transformant	116	21	52	65
	5291	100	31	46	53
Control	Non-transformant	120	19	57	67
	5291	121	28	63	65
4	5351	121	28	63	65
	5441	115	42	47	58
	5351	111	39	48	63
Seed	Non-transformant	123	33	57	69
5	5410	108	33	48	51
Seed	Non-transformant	127	25	56	67
	5410	110	31	55	66
	5410	109	33	51	54
Control	Non-transformant	109	22	55	66
	5431	96	18	44	56

* Non-transformed MR81 are seed derived plants or control tissue culture plants regenerated by bombarding (without plasmid) or selection.

PCR analysis

Amplification of the 900 base pair (bp) fragment could be identified from DNA of 5 putatively transformed (R0)

Taipei 309 and 12 of MR 81 plants indicating the presence of the *hph* gene in the R0 plants (Figure 4) and R1 plants.

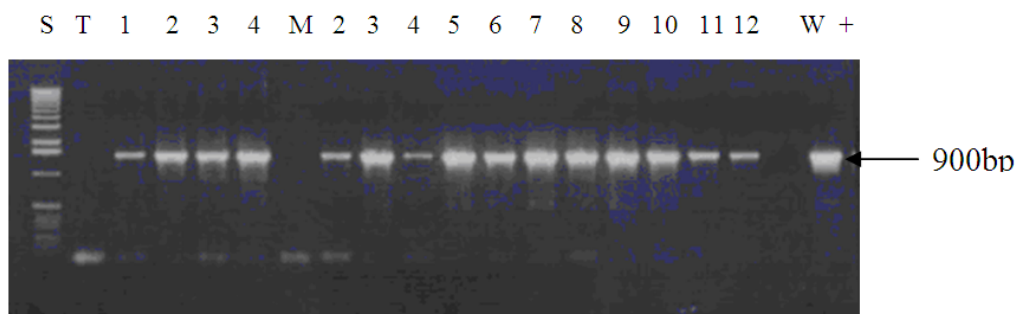


Figure 4. Agarose gel electrophoresis of PCR-amplified DNA from putative transgenic Ro rice plants. The results revealed the presence of the 900bp fragment of the *hph* gene in the rice genome in transformed plants of Taipei 309 (T onwards; Lanes 1-4), MR81 (M onwards; Lanes 2-12) and W⁺ (positive control from the plasmid). The *hph* gene is not detected in the non-transformed plants (Lanes T and M).

Southern blot analysis of R0 rice plants

Southern blot analysis of DNA isolated from leaf blades of plants MR 81 and Taipei 309 regenerated from HPH-resistant callus confirmed the presence of the HPH gene (Figure 5). As expected, neither undigested nor the digested genomic DNA from nontransformed plants hybridized with the x-[³²P]-labeled *hph* gene probe. The nontransformed plant did not show any hybridization band. Integration of the HPH gene was illustrated by the formation of a smear in only the high molecular weight region (Figure 5) when undigested genomic DNA was hybridized with the *hph* probe. Six of the 12 plants illustrated in Figure 5 showed a hybridization signal in the 900kb region corresponding to the intact *hph* gene coding sequence.

All the 5 plants of Taipei 309 in the Southern blot analysis showed similar pattern indicating that they were siblings (data not shown). The intact plasmid cassette is present as seen in the lane loaded with R1 genomic DNA digested with *Hind III*. Out of the 12 lines of MR 81 tested, 4 distinct lines were identified

by Southern blot analysis (Figure 5a and 5b). Lines M4 and M9 have more copies of the *hph* gene than lines M3 and M12 based on the intensity of the bands. Lines M3 and M12 have at least one insertion based on the visible bands. Lines M3 and M10 are siblings since they show a similar Southern pattern analysis.

Phenotype of R0 plants and inheritance of the HPH gene

The majority (>95%) of hygromycin-resistant plants survived transfer to soil and flowered. As shown in Figure 6 most (>85%) developed normally and set seeds. Sometimes R0 plants showed characteristics typical for tissue cultured-induced stress such as reduced stature. Nevertheless, the seeds of R0 plants are similar to seed-derived plants. Plants regenerated from HPH-resistant callus were designated as R0 plants. Seed of self-pollinated R0 plants gave rise to R1 plants.

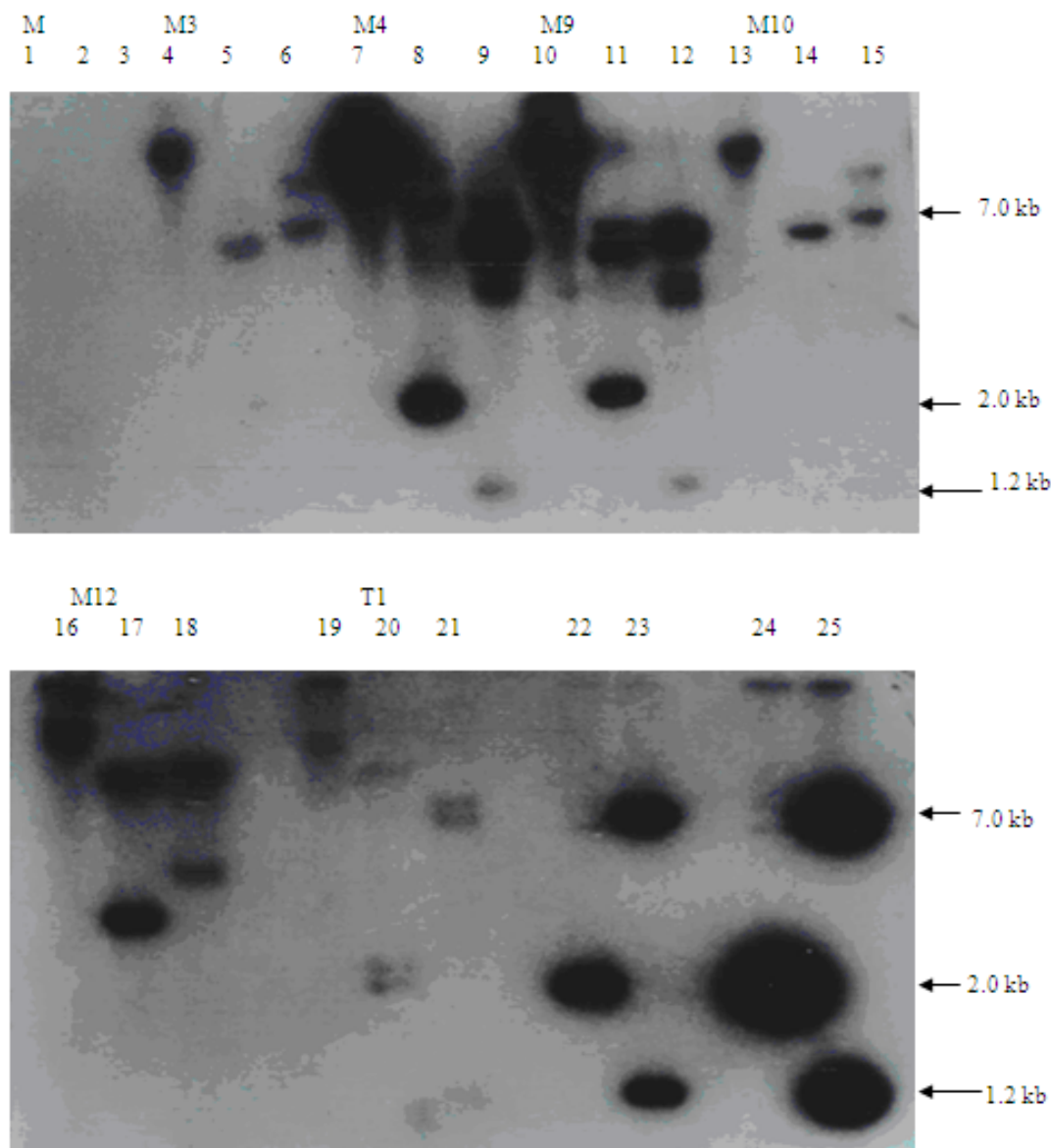


Figure 5. The Southern blot contains the genomic DNA samples of untransformed plants (MR 81) as control and 4 independent Ro transgenic lines (M 3, M 4, M 9 and M 12) of MR 81 and T 1 of Taipei 309. 5µg of genomic DNA was used in all the lanes. Lanes 22 and 23: about 50pg of pRQ6, the plasmid DNA used in the transformation digested with *Hind III* and *NcoI*, respectively. Lanes 24 and 25: 100pg of pRQ6 digested with *Hind III* and *NcoI*, respectively. Lanes 1, 4, 7, 10, 13, 16 and 19: undigested genomic DNA of MR 81 and Taipei 309, respectively. Lanes 5, 8, 11, 14, 17 and 20: digested with *Hind III*. Lanes 6, 9, 12, 15, 18 and 21: digested with *NcoI*. The numbers on the side of the panel indicate the DNA size in kb, based on the calibration with the 1 kb ladder (Gibco-BRL).

The Southern blot analysis conducted on progenies of MR 81 (M 4) and Taipei 309 T1 in the R1 generation confirmed the presence of the *hph* gene in the plants (Figure 7) that were also GUS positive. From the transformation experiments using calli derived from mature seeds of MR 81

and Taipei 309, four transformed lines of MR 81 and one line of Taipei 309 were chosen randomly for further assays. In all of these lines both GUS activity and hygromycin resistance has been confirmed by histochemical assay, PCR assay and Southern blot analysis.

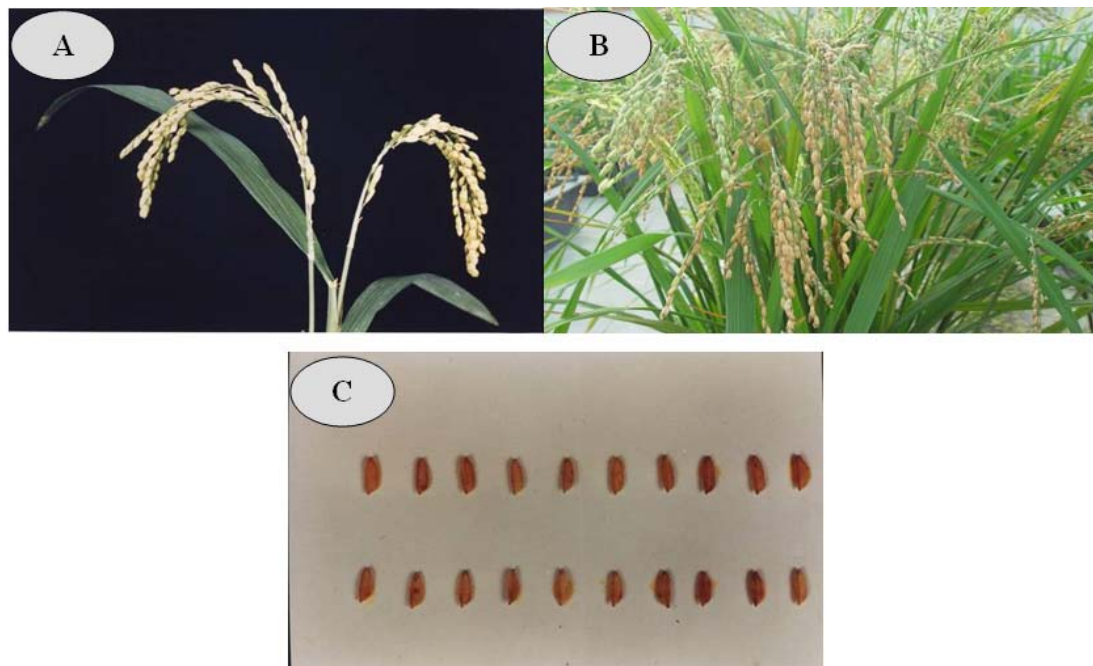


Figure 6. (A) Fertile transgenic R1 plants of Taipei 309; (B) Fertile transgenic R1 plants of MR 81; (C) Seeds collected from transformed R1 plants of MR 81 are similar to the seeds of non-transformed plants grown under glasshouse conditions.

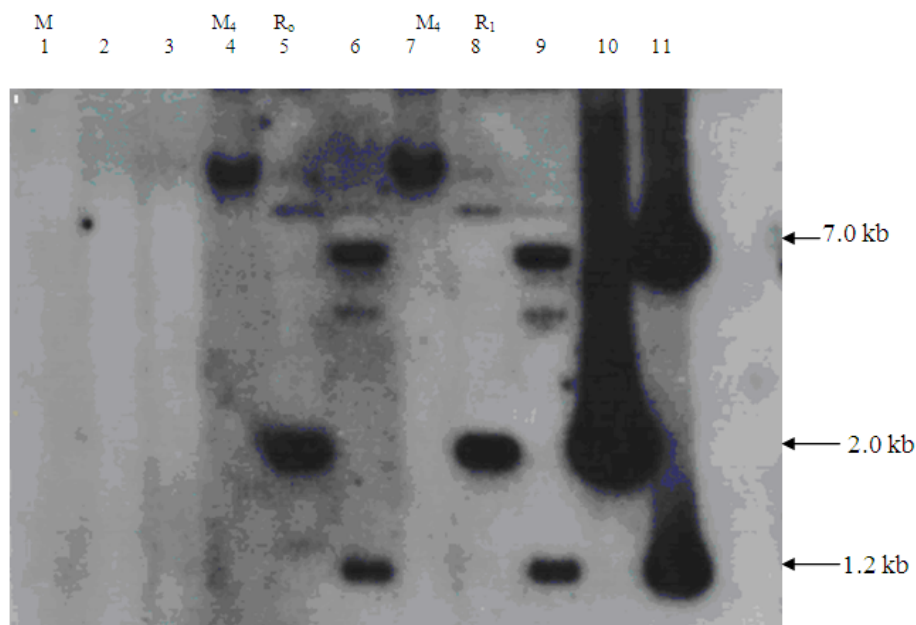


Figure 7. Southern blot analysis of R1 plants. The Southern blot contains the genomic DNA samples of untransformed plants of the MR81 (M) and Taipei 309 (T) as control, two independent R0 plants (M4 and T1). Lanes 10 and 11/11 and 12: about 50pg of pRQ6, digested with *Hind III* and *NcoI*, respectively. Lanes 1,4,7: undigested genomic DNA of M4/T1. Lanes 2, 5 and 8: digested with *Hind III*. Lanes 3, 6 and 9 digested with *NcoI*.

All plants were fertile, with more than 85% fertility in the panicles of all lines and are phenotypically normal. Both the *hph* and *gusA* genes were transmitted to the progeny as confirmed by histochemical assay, PCR assay and Southern blot analysis. Thus, the *hph* gene has proven to be a useful selectable and screenable marker for the transformation of MR 81 rice plants.

Discussion

Rice embryogenic callus is an effective starting material as explants, the choice of a biolistic process (also known as the particle gun bombardment) based system brought with it a number of both expected and fortuitous advantages. Brisibie et al. (2000) reported that wheat transformation using callus explant is profound importance in obtaining high percentage of tissues with transient and stable GUS activity. Proliferation of transformed cells and selection of transformed sectors is an important event since the transformed cells are at a disadvantage in terms of their retarded cell growth and multiplication in comparison to non-transformed ones (Visarada and Sarma, 2004). By utilising a somatic embryogenesis-based system of rice plants MR 81 and Taipei 309, we have reduced the probability production of transgenic chimera.

Competence was also assured by having an established totipotency while β -glucuronidase (*gusA*) gene immediately confirmed that these calluses were also competent for transformation. High frequencies of transient GUS expression, 25% to 30% range which are routinely obtained. The GUS reporter gene used to obtain data for the transient expression in these experiments, was found to be a reliable indicator for subsequent stable transgenic callus production. The GUS gene linked to the hygromycin (*hph*) gene was convenient to follow and confirm transformation events. Also, other authors

have demonstrated that the CaMv35S can confer GUS expression in seeds of transgenic rice plant providing a useful marker for detecting transmission of the transgene to the progeny of transgenic plants. The method of selecting transferred rice resistant to hygromycin B has been well-established (Li et al., 1993). The hygromycin caused yellowing, stunted growth and necrosis of the untransformed seedlings. Ortiz et al. (1996) working on wheat has also reported the efficiency of utilizing hygromycin resistance gene as a selectable marker for stable transformation.

In the present study, the *hph* gene was absent in control rice tissue but present in both transformed leaves of the primary regenerants of R0 plants of Taipei 309 and MR 81 (Figure 5). Data showed that these R0 plants not only harbour the *hph* gene but also express it. Hygromycin resistant plants exhibited normal growth and tillering. Conveniently, there are few escapes at the callus level apparently had an altered physiological state that was not conducive to subsequent rice regeneration. The results presented here clearly indicate that DNA uptake into callus mediated by the particle bombardment transformation system is a reliable and reproducible method for genetically engineering indica rice plants when combined with appropriate callus culture and HPH selection protocols. When the *hph* gene was used as a selectable marker with the use of hygromycin B as a selective agent was crucial for identifying the transformed cells and subsequently regenerating plants from the transformed callus. The transformation efficiency of our experiment depended on the consistent quality of the callus. In our laboratory, transformation efficiency is measured by the number of HPH-resistant callus per plate. An average frequency of seven HPH-resistant callus produced transgenic plants from per plate from 50 bombarded calli (Table 2). The optimum regeneration

procedure was found to be appropriate for both the genotype tested.

Particle bombardment transformation system of an organized and easily regenerable tissue such as embryogenic callus allowed us to generate transgenic rice plants. In rice, earlier reports (Sivamani et al., 1996; Chen et al., 1998; Srivastava and Ow, 2001; Visarada and Sarma, 2004; Grewal et al., 2006) have successfully used embryogenic callus as starting material for transformation particle bombardment. The method presented in this study requires a short tissue culture period under optimal conditions, and rooted transformed plants could be transferred to the greenhouse about four months after bombardment. In our transformation experiments, no phenotypic abnormalities or reduced fertility of the regenerated plants were observed using MR 81 and Taipei 309 callus. Fertile plants from transformation events has been reported for transgenic maize (Gordon-Kamm et al., 1990), wheat (Vasil et al., 1992), and transgenic oat plants (Somers et al., 1992) were obtained from particle bombardment of callus cultured or embryogenic suspensions. We observed various integration patterns of the introduced *hph* gene (Figure 6). The presence of multiple bands with molecular weights higher than expected is an indication of multiple insertion events and rearrangements of the integrated gene, both of which have been commonly observed in transformed plant materials (Gordon-Kamm et al., 1990; Srivastava and Ow, 2001). Transformation experiments using calli derived from mature seeds of MR 81 and Taipei 309 have yielded 4 transformed lines of MR 81 and I line of Taipei 309. In all of these transgenic lines, both GUS activity and hygromycin resistance has been confirmed by histochemical assay, PCR assay and Southern blot analysis. All the rice plants were fertile, with more than 85% fertility in the panicles of all lines and are phenotypically normal. Both the *hph*

and *gusA* genes were transmitted to the progenies as confirmed by histochemical assay, PCR assay and Southern blot analysis. Thus, the *hph* and *gusA* genes have proven to be useful selectable and screenable markers for the transformation of MR 81 rice plants. The next goal will be to use this transformation methodology to produce value-added transgenic plants by transferring single or many transgenes into commercially important rice varieties.

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