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Transgenic plant production mediated by *Agrobacterium* in *Indica* rice

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Summary. A reproducible system has been developed for the production of transgenic plants in *indica* rice using *Agrobacterium*-mediated gene transfer. Three-week-old scutella calli served as an excellent starting material. These were infected with an *Agrobacterium tumefaciens* strain EHA101 carrying a plasmid pIG121Hm containing genes for β-glucuronidase (GUS) and hygromycin resistnace (HygR). Hygromycin (50 mg/l) was used as a selectable agent. Inclusion of acetosyringone (50μM) in the *Agrobacterium* suspension and co-culture media proved to be indispensable for successful transformation. Transformation efficiency of Basmati 370 was 22% which was as high as reported in *japonica* rice and dicots. A large number of morphologically normal, fertile transgenic plants were obtained. Integration of foreign genes into the genome of transgenic plants was confirmed by Southern blot analysis. GUS and HygR genes were inherited and expressed in R1 progeny. Mendelian segregation was observed in some R1 progeny.

Key words: Acetosyringone - *Agrobacterium* - *Indica* rice - *Oryza sativa* L. - Transgenic plants

Abbreviations. GUS= ß-glucuronidase, HygR= hygromycin-resistance, AS= acetosyringone

Introduction

Transformation of dicotyledonous plants by *Agrobacterium*-mediated gene transfer is well established and has produced stable transgenic plants expressing a number of foreign genes. This has not been the case for monocotyledonous plants in general. In the past, the monocots and particularly the graminaceous crop species have been considered to be outside the *Agrobacterium* host range (Bevan1984; Decleene 1985), and gene transfer methods developed for monocots had been restricted to direct gene transfer into protoplasts or particle discharge methods of direct DNA transfer into intact cells of embryogenic callus or suspension cells. However, transformation methods based on the use of *Agrobacterium tumefaciens* are still preferred in many instances, as *Agrobacterium*-mediated transformation does not require protoplasts, and, in general, results in higher transformation efficiency and a more predictable pattern of foreign DNA integration rather than any other transformation techniques (Czernilofsky et al. 1986; Chan et al. 1993; Hiei et al. 1994).

In recent years, some examples on the transformation of monocots using *Agrobacterium* were presented, for example, in *Asparagus officinalis* (Bytebier et al. 1987), maize (Gould et al. 1991; Citovsky et al. 1994). In rice, the successful transformation with subsequent integration into DNA has been restricted to *japonica* rice (Raineri et al. 1990; Chan et al. 1992, 1993; Hiei et al. 1994). Recently, Hiei et al. (1994) obtained transformation frequency of *japonica* rice as high as that of dicots and demonstrated the Mendelian transmission of the introduced DNA to the progeny. The genetic transformation of *indica* rice employing *Agrobacterium*, however, has been restricted to a few reports without an established transformation procedure and only transgenic calli with a low frequency have been obtained (Chan et al. 1992; Li et al. 1992).

In this study, we employed Basmati cultivars, which comprise an important group of *indica* rice, and described the production of transgenic plants by *Agrobacterium* with a frequency as high as that of dicotyledonous plants. Further, expression of GUS and HygR genes in R0 and R1 plants is also described.

Materials and methods

Rice cultivars and culture media. *Indica* rice cultivars (*Oryza sativa* L.) Basmati 370, Basmati 385 and Basmati 6129 were obtained from National Agricultural Research Center Islamabad, Pakistan. Media used for tissue culture and transformation are listed in Table 1. Nine-cm-diameter petri plates were used in this experiment and all the plates except for co-cultivation were sealed with medical gas-permeable tape (Micropore Surgical Tape; 3M).

Calli derived from scutellum. Mature seeds were dehusked, washed by tap water, first sterilized with 70% ethanol for one minute and then with 2.50 % sodium hypochlorite for 20 minutes. The seeds were further washed 3 times with sterilized deionized water. These were cultured on CI medium (Table 1) for Basmati 370 and Basmati 385 for callus induction. For Basmati 6129, 2 mg/l 2,4-D in CI medium was replaced by 3 mg/l 2,4-D. The cultures were incubated in the dark at 25°C for two weeks and then shifted to 16 hours photo period light for one week. After three weeks, the proliferated calli derived from the scutella were separated with scalpel. The compact calli (1-2 mm in diameter) were selected for transformation.

Bacterial strain and plasmid. The infection of the rice calli was carried out using *Agrobacterium tumefaciens* strain EHA101 containing pIG121Hm (Hiei et al. 1994). pIG121Hm is a binary vector that contains genes for hygromycin resistance and GUS in the T-DNA region, as well as for kanamycin resistance (Fig. 1a). The gene for GUS has an intron in the 5' end of the coding sequence and is connected to the 35S promoter of cauliflower mosaic virus (Ohta et al. 1990). This intron-GUS reporter gene expresses GUS activity in plant cells but not in the cells of *Agrobacterium tumefaciens*.

Transformation. Although some modifications were tested, the general protocol was as follows. The *Agrobacterium* strain EHA101 (pIG121Hm) was grown overnight at 28°C in YEP medium (An et al. 1988) containing 50 mg/l kanamycin and 50 mg/l hygromycin. The culture was centrifuged at 3,000 rpm for 10 minutes and the pellet was resuspended in equal volume of AA-AS medium (Table 1). The calli were soaked in bacterial suspension for 3 minutes, blotted dry with sterile filter paper to remove excess bacteria. Then the calli were transferred on a piece of filter paper placed on a co-culture medium. A co-culture medium was prepared by spreading one ml of a liquid medium (AA-AS medium) on a bottom medium (CI-AS medium; Table 1).

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Table 1. Media used for tissue culture and transformation of *indica* rice cultivars Basmati 370 and Basmati 385. For Basmati 6129, 2 mg/l 2,4-D in CI medium was replaced by 3 mg/l 2,4-D.

Medium	Composition
CI	N6 salts and vitamins (Chu et al. 1975), 30 g/l sucrose, 2 mg/l 2,4-D, 4 g/l gel rite, pH 5.8.
CI-AS	CI with 50 μM acetosyringone.
AA-AS	AA medium (Toriyama and Hinata 1985), 20 g/l sucrose, 1mg/l 2,4-D, 50 μM acetosyringone, pH 5.8.
CI-HmCb	CI with 50 mg/l hygromycin and 500 mg/l carbenicillin.
RE1-Hm	MS salts and vitamins (Murashige and Skoog, 1962), 30 g/l sorbitol, 30 g/l sucrose, 2g/l casamino acids, 2 mg/l benzylaminopurine, 1 mg/l naphthaleneacetic acid, 50 mg/l hygromycin, 4 g/l gel rite, pH 5.8.
RE2-Hm	MS salts and vitamins (Murashige and Skoog, 1962), 30 g/l sorbitol, 30 g/l sucrose, 2g/l casamino acids, 50 mg/l hygromycin, 4 g/l gel rite, pH 5.8.
P	MS salts and vitamins (Murashige and Skoog, 1962), 30 g/l sucrose, 4 g/l gel rite, pH 5.8.
P-Hm	P with 50 mg/l hygromycin.

The plates were sealed with parafilm. Co-cultivation was carried out in the dark at 28°C for 2-3 days. The calli labeled as control were not infected with *Agrobacterium*. After co-cultivation, the infected calli were washed with N6 medium (Chu et al. 1975) containing 500 mg/l carbenicillin to kill the *Agrobacterium* and then transferred to CI-HmCb medium (Table 1).

Selection and regeneration of transformants. After selection for 3 weeks, calli were transferred to RE1-Hm medium (Table 1) for shoot regeneration and root development. The regenerated shoots were further transferred to RE2-Hm medium (Table 1) for full plant formation with extensive root system. After rooting, the transgenic plants were transferred to a glasshouse and grown to maturity.

DNA isolation and Southern hybridization. Total genomic DNA was extracted from young leaf tissues (1.5-2.0 g) of transgenic rice plants and untransformed control plants according to the CTAB method (Murray and Thompson 1980). Genomic DNA was digested with *Bam*HI and fractionated on 0.8% agarose gel. The bands of DNA were transferred to a nylon membrane (Nytran; Schleicher and Schunell) by standard Southern's method (Southern 1975). The GUS DNA used as a probe was prepared from *Bam*HI-*Eco*RI restricted fragment of pBI221(Clontech) and labeled with digoxigenin. Labeling, hybridization and washing were performed according to the instruction manual of the DIG labeling and Luminescent Detection Kits (Boehringer Mannheim).

Assay of GUS activity. Expression of GUS in rice cells was assayed essentially as described by Jefferson (Jefferson 1987). The segments of rice tissues were incubated in X-gluc solution containing 50 mM phosphate buffer, 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 0.5% Triton X-100 and 20% methanol. The reaction mixture was placed under a mild vacuum for a few minutes and then incubated overnight at 37°C. For chlorophyll removal, the materials were soaked in 70% ethanol and the blue staining was scored visually.

Segregation analysis for HygR and GUS genes in R1 progeny. Selfed seeds of R1 generation were sown on P medium (Table 1) and, 3 days later, the root segment was used for GUS assay. The seedlings were transferred to P-Hm medium (Table 1). Drug resistance was scored 7 days after the transfer.

Results

Factors affecting transformation efficiency

We exploited three factors, inclusion of acetosyringone, type of callus and co-cultivation period, for efficient transformation in Basmati cultivars of *indica* rice. Transient GUS expression was examined for fifteen infected calli per treatment soon after co-cultivation. At first, we tested the effect of acetosyringone on transformation efficiency using 3-week-old scutellum calli. Expression of GUS was detected in all of the calli examined by inclusion of acetosyringone during co-cultivation (Table 2). These calli proliferated on selective medium and GUS expression was also observed two weeks after co-cultivation (Table 2). No

Table 2. Effect of acetosyringone on transformation efficiency evaluated by number of GUS-expressing (GUS+) calli in Basmati 370. *Agrobacterium* cells were suspended in AA-AS medium and infected rice calli was co-cultured on the filter paper overlayed on a liquid medium (AA-AS medium) which was spread on a solid bottom medium (CI-AS medium). + and - denote the presence and absence of acetosyringone, respectively. Number of GUS+ calli/ total calli was checked immmediately (A) and two weeks (B) after co-cultivation. Five calli per treatment were selected and experiment was repeated twice.

Liquid medium	Bottom medium	Number of GUS+ calli/ total calli	
		(A)	(B)
+	+	13/15	15/15
+	-	0/15	0/15
-	+	0/15	0/15
-	-	0/15	0/15

transformation efficiency was recorded when acetosyringone was omitted either from the liquid and the bottom medium or from both (Table 2). The calli turned brown and died during selection. The result suggested that inclusion of acetosyringone during co-culture is vital for Basmati rice transformation.

When 3-week-old scutellum callus and calli after 3 months of culture were co-cultivated in the above described conditions, the 3-week-old calli displayed GUS expression immediately after co-cultivation, whereas the latter did not show any blue staining at all or yield any transformants.

Co-cultivation period was varied 1, 2 ,3 and 5 days. Infected calli for one day did not show any GUS activity. GUS activity was observed from all of the calli co-cultivated for 2-3 days. Although the calli infected for 5 days showed some GUS activity, the tissues were adversely affected during prolonged co-cultivation period. It is clear from the result that the co-cultivation time with *Agrobacterium* needed to be 2-3 days to obtain efficient expression of GUS in Basmati rice. It yielded the maximum transformation efficiency and further transgenic plant production.

Transgenic plant formation

The scutellum calli (Fig. 2a) from Basmati 370, Basmati 385 and Basmati 6129 were co-cultivated with *Agrobacterium tumefaciens* strain EHA101 (pIG121Hm). They were further cultured on the CI-HmCb medium, which contained 50 mg/l hygromycin for selecting transformed cells. All the three varieties responded but good transient expression was detected in Basmati 370 and Basmati 385 and to a less extent in Basmati 6129 (Table 3). Maximum (86%) number of hygromycin resistant calli were recovered from Basmati 370 (Table 3; Fig. 2b). After selection, the hygromycin resistant calli from all varieties were transferred to the RE1-Hm where shoot initiation and subsequently multiple shoot formation occured (Fig. 2c). The transformed plants were readily regenerated on the RE2-Hm medium containing hygromycin without any hormone. The frequency of regeneration varied from 0% -36% of selected calli. Basmati 370 showed a transformation frequency of 22% (Table 3). More than hundred hygromycin resistant and GUS expressing plants were produced and transferred to a glasshouse.

Table 3. Transformation efficiency (T.F.) by *Agrobacterium* in *indica* rice cultivars Basmati 370, Basmati 385 and Basmati 6129.

Rice	Number of scutellum-derived calli					
Cultivars	Co-cultivated calli (A)	Produced HygR calli	Plantlet forming calli	Produced HygR plants	Produced HygR and GUS+ plants (B)	T.F. % (B/A)
370	118	102	40	37	26	22
385	84	41	5	4	4	4.8
6129	53	22	0	0	0	0

Plants from 11 independent hygromycin resistant cells of Basmati 370 and two from Basmati 385 were evaluated for phenotypic characterization and fertility (Fig. 2d and e). Albinos appeared in 11% of the plants and they failed to survive in soil. All the other plants exhibited normal phenotype with a usual life cycle. Twenty one transgenic plants grew to maturity, flowered and set seeds. They had the same seed fertility as compared to control.

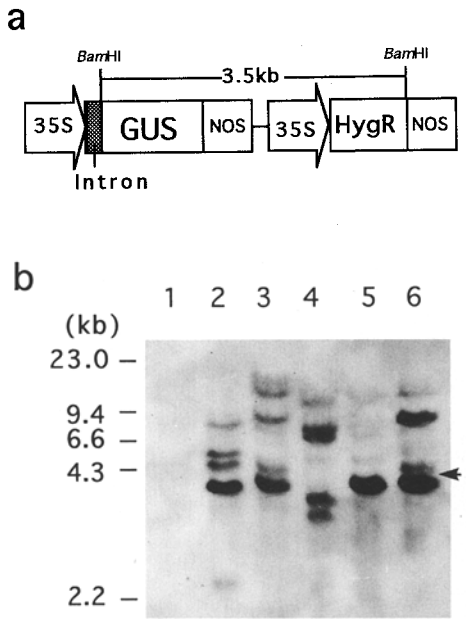

Fig. 1. Transformation vector and Southern blot analysis. (**a**) Schematic diagram of a part of the T-DNA region of transformation vector pIG121Hm. 35S, 35S promoter of cauliflower mosaic virus; intron, the first intron of catalase gene of caster bean, GUS, gene for β-glucuronidase; HPT; gene for hygromycin phosphotransferase; NOS; terminator of nopaline synthatase. (**b**) Southern blot analysis of five transgenic plants of Basmati 370 (lanes 2-6) and an untransformed plant (lane 1). DNA was digested with *Bam*HI and allowed to hybridize to GUS probe. An arrow indicates the expected band of 3.5 kb. Molecular markers are indicated on the left.

Many of the hygromycin resistant plants exhibited blue staining for GUS. However 30% hygromycin resistant plants exhibited no GUS activity in Basmati 370. In Basmati 385, all of hygromycin resistant plants have shown GUS activity. The segment of root resulted in more deep blue staining in comparison to leaves (data not shown).

Integration of foreign DNA in the genome of transgenic plants

Southern hybridization was carried out on the DNA from five GUS expressing plants derived from independent hygromycin resistant calli of Basmati 370. The GUS DNA was detected as a fragment of the expected size of 3.5 kb (Fig. 1a and 1b lanes 2, 3, 5 and 6). One plant did not show the expected band of 3.5 kb (Fig. 1b, lanes 4). Some bands of unexpected sizes were also observed in all the transgenic plants. These unexpected DNA fragments were probably produced by rearrangements of DNA upon transformation. Since the GUS DNA probe only hybridized to DNA from transgenic plants but not to the DNA from the untransformed control plant (Fig. 1b, lane 1), the result indicated that the GUS DNA was integrated into rice genome.

Inheritance of HygR and GUS genes in R1 progeny

We chose five plants derived form independent calli, N332, NE320, N322, N382 and N38 from Basmati 370 and one plant, N81, from Basmati 385. The selfed progeny of these plants were evaluated for hygromycin resistance and GUS expression (Table 4). Most of the seedlings were either fully positive or fully

Table 4. Expression of HygR and GUS genes in selfed progeny (R1 seedlings) of transgenic *indica* rice cultivars, Basmati 370 (a) and Basmati 385 (b).

Selected plants	Total R1 seedlings tested	HygR		HygS		Segregation of Hyg		Segregation of GUS	
		GUS+	GUS-	GUS+	GUS-	Expected ratio	P value	Expected ratio	P value
N38a	36	6	3	0	27	NT	NT	NT	NT
N320a	89	6	2	0	81	NT	NT	NT	NT
N322a	77	7	0	2	68	NT	NT	NT	NT
N332a	77	52	5	3	17	3:1	0.84	3:1	0.47
N382a	71	68	0	0	3	15:1	0.48	3:1	0.48
N81b	56	36	2	1	17	3:1	0.22	3:1	0.12

NT= not known

Fig. 2. Production of transgenic plants in *indica* rice cv. Basmati 370 and test of R1 progeny for GUS expression and hygromycin resistance.
(**a**) Three-week-old scutellum derived calli used for co-cultivation with *Agrobacterium*. (**b**) Hygromycin-resistant colonies of cells proliferated on hygromycin-containing medium 3 weeks after selection. (**c**) Plant regeneration 4 weeks after transfer. (**d**) Seeds at maturity stage. (**e**) A transgenic plant at flowering stage. (**f**) Test for GUS expression of root segments in R1 progeny. Blue staining indicates GUS expression. Segregation of GUS positive and negative plants were observed. (**g**) Hygromycin resistance in R1 progeny of transgenic plants (right) and untransformed plants (left). Seedlings were plated on a hygromycin-containing medium. Transgenic plants showed normal growth, where as untransformed plants were died 7 days later.

negative in GUS expression (Fig. 2f). Resistant and sensitive seedlings were clearly distinguishable on the P-Hm medium that contained hygromycin (Fig. 2g). The R1 progeny of plants N332 and N81 displayed segregation pattern of 3:1 and N382 showed 15:1 segregation pattern for both hygromycin resistance and GUS expression (significant at the 5% level based on X^2 tests). The plants N320, N322 and N381 showed much lower segregation pattern than expected for both hygromycin resistance and GUS expression. The unexpected appearence of hygromycin resistance and GUS negative plants, or hygromycin sensitive and GUS positive plants was observed in most of the R1 progeny.

Discussion

There are various factors which affect the transformation efficiency of Basmati cultivars, but optimization of the suitable conditions for co-cultivation and selection of a suitable starting material are shown to be vital. During co-cultivation, presence of acetosyringone has played a vital role for transformation efficiency (Table 2). Previously Chan et al. (1993) indicated co-incubation of potato suspension cells (PSC) with *Agrobacterium* significantly improved the transformation efficiency of rice. PSC was rich in acetosyringone and sinapic acid, which were generally believed to enhance transformation in various plant species (Czernilofsky et al. 1986). Our method did not require such addition. Hiei et al. (1994) reported that three factors, inclusion of acetosyringone and glucose and pH of the medium (pH 5.2), during co-cultivation were important for enhancing transformation in *japonica* rice. In our study, only acetosyringone at 50 μM was shown to be required.

The selection of suitable explant source was one of the most important factors. Scutellum-derived calli were shown to be served as an excellent starting material in our transformation experimrnt, as reported for *japonica* rice by Hiei et al. (1994). Hiei et al. (1994) also described that prior to infection, pre-culture of calli in a fresh medium for 4 days was an important step for *japonica* rice transformation. Pre-treatment of tissues, for example, by wounding or enzymatic digestion of cell walls were also found to be essential in other studies (Chan et al. 1993; Mooney et al. 1991; Raineri et al. 1990). Such pre-treatments were not needed in transforming *indica* rice in our study.

We used a binary vector, pIG121Hm, which was derived from one of the most common binary vector, pBI121 (Ohta et al. 1990). Previous workers reported that a super-binary vector, in which a DNA fragment from the virulence region was introduced into a binary vector, was more effective for transforming rice and they thought that difficult cultivars can more easily be transformed by using the super-binary vector (Hiei et al. 1994). Our data indicate that Basmati cultivars were transformed by using an ordinary vector.

High transformation efficiency of Basmati 370 (22%) was observed (Table 3) which was as high as reported in *japonica* cultivars (Hiei et al. 1994). In Basmati 385, low regeneration from selected calli was due to adverse effects of *Agrobacterium* infection. The transgenic plants exhibited normal growth in terms of phenotype and yield of seeds. It may be due to regeneration from short term culture, as reported by Hiei et al. (1994).

Histochemical localization of GUS expression driven by cauliflower mosaic virus 35S promoter has been extensively studied (Terada and Shimamoto 1990; Battraw and Hall 1990). Similar pattern of expression was observed in this study. However, more strong expression of GUS was observed. This would be attributed to the presence of the intron in 5' end of GUS genes of pIG121Hm, because the intron-containing plasmid, the intron-GUS gene, was reported to increase the level of GUS activity 80 to 90-fold as compared with the intronless plasmid (Tanaka et al. 1990). The intron-GUS gene used in this study was a convenient marker gene for rice transformation.

Inheritance of foreign genes to the further progeny has been reported in *japonica* rice (Shimamoto et al. 1989; Chan et al. 1993; Hiei et al. 1994). As for *indica* rice, this is the first report to demonstrate the stabe transmission of inheritance in R1 progeny (Fig. 2f and g) . Three out of the six R0 plants examined exhibited a Mendelian segregation (Table 4). It is not clear why the segregation of transgenes in some R0 plants was deviated from Mendelian law. The lack of GUS activity and hygromycin- resistance may indicate that the foreign DNA was either absent or present but non-functional.

In summary, we have developed a simple method for production of transgenic plants in *indica* rice cultivars, Basmati 370 and Basmati 385. The protocol was modified in a more simple manner than that reported for *japonica* rice. The foreign genes transferred were inherited to the offspring of the transgenic plants. We are now transferring some useful genes in Basmati cultivars by using this approach. It may be possible to transform other recalcitrant cultivars of rice and to other monocots by empolying the method descibed in this study.

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