

Production of Transgenic Japonica Rice (*Oryza sativa*) Cultivar, Taichung 65, by the *Agrobacterium* – Mediated Method

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Abstract

Transgenic rice plants of Taiwanese japonica rice cultivar, Taichung 65, were obtained by co-cultivating scutellum calli with an *Agrobacterium tumefaciens* strain, EHA101, that carried a binary vector harboring the luciferase (*Luc*) gene driven by the CaMV35S promoter. The transformation efficiency of Taichung 65 was similar to that obtained by the methods routinely used for a transformable cultivar, Notohikari. There was no correlation between the length of the culture period (21 to 30 days) and the transient transformation efficiency in Taichung 65 and in Notohikari. In the T₀- and T₁-generations, the transgenes were integrated and stably expressed, indicating that the transgene was inherited to the next generation. The copy number of integrated transgene varied from one to three in the T₁-transformants, which was confirmed by Southern blot analysis. Moreover, approximately 60% of the T₁-transformants of Taichung 65 showed the LUC-positive phenotype. These results suggest that, in addition to Notohikari, Taichung 65 is a practical, transformable cultivar.

Keywords: *Agrobacterium* – mediated transformation, EHA101, Notohikari, pIG121Hm, Taichung 65, Transgenic rice.

One suitable tool for plant molecular breeding and genetics is gene transformation by the *Agrobacterium*-mediated gene transfer system (Hiei *et al.*, 1997). In contrast to the direct gene delivery systems such as polyethylene glycol (PEG)-mediated protoplast transformation (Peng *et al.*, 1992), the electroporation method (Yamamoto *et al.*, 1994) and the particle bombardment method (Wakita *et al.*, 1998), with the *Agrobacterium*-mediated method it was easy to produce independent transformants in a single series of experiments without complicated manipulations in tissue culture, and only one or a few copies of transgenes were integrated into the host genome. Although the *Agrobacterium*-mediated method has been used to produce transgenic rice of some cultivars (Hiei *et al.*, 1994), the frequency of transformation varies with the plant genotype and with the bacterial strains and vectors, since rice was not originally infected by *Agrobacterium* in the outfield conditions (Hiei *et al.*, 1997). Therefore, it is premature to conclude that all cultivars of rice are transformable.

Taichung 65 is one of the most vital cultivars in

the genetic study and breeding of rice. A number of genetic stocks with single and multiple phenotype markers that are very useful to screen mutants have been developed in the Taichung 65 background. Those mutants have been maintained at Kyushu University for the last decade (Yoshimura *et al.*, 1997). For example, the reduced embryo mutant (Hong *et al.*, 1996), giant embryo mutant (Hong *et al.*, 1996), shootless mutant (Sato *et al.*, 1999), dwarf mutant (Fujisawa *et al.*, 1999) and zebra mutant (Kusumi *et al.*, 2000) of Taichung 65 have been developed. Because all of these mutants share the genetic background with cultivar Taichung 65, establishment of transformation technique for this particular cultivar is essential for the genetic manipulation of these mutants and for acceleration of basic and applied studies on breeding programs of rice. In this study, we developed a method for consistent production of stable transformants of Taichung 65 at a frequency of 4.6% using scutellum-derived calli inoculated with *Agrobacterium tumefaciens*.

Mature seeds of Taichung 65 and Notohikari were

dehusked, and sterilized with 10% sodium hypochlorite solution for 1 h. Then, they were rinsed thoroughly with sterile water, and cultured on the callus induction medium, N6D2 (Table 1), at 28°C in continuous darkness for 21 to 30 days. On the 7th day of culture, scutellum tissues began to dedifferentiate on the N6D2 medium. Then, on the 20th day

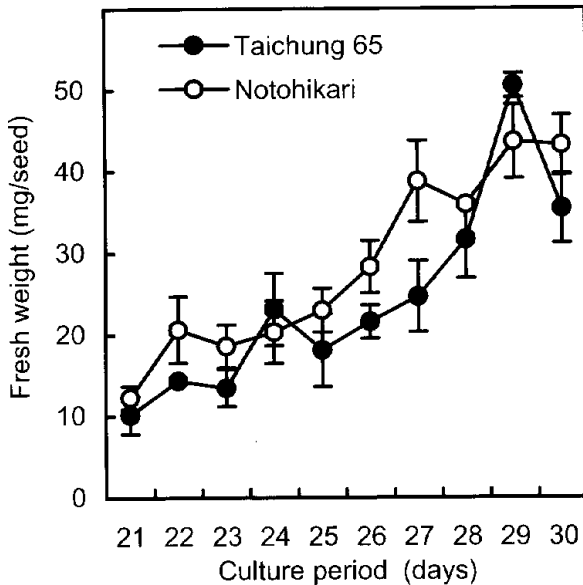


Table 1 Culture media used for callus induction (N6D2), selection (N6D2A,N6D2S), and plant regeneration (LSR, LS).

Medium	Composition
Callus induction medium (N6D2)	N6 ^{*1} basal salts containing 2.9 g l ⁻¹ L-proline, 0.3 g l ⁻¹ casamino acid, 0.1 g l ⁻¹ myo-inositol, 2 mg 2,4-dichlorophenoxy acetic acid, 30 g l ⁻¹ sucrose, 3.2 g l ⁻¹ gelrite (pH 5.8)
Co-cultivation medium I (N6D2A)	N6D2 medium, 1 mg l ⁻¹ acetosyringone (pH 5.8)
Selection medium II (N6D2S)	N6D2 medium, 500 mg l ⁻¹ carbenicillin, 50 mg l ⁻¹ hygromycin (pH 5.8)
Regeneration medium (LSR)	LS ^b basal salts, 2 mg l ⁻¹ naphthalene acetic acid, 1 mg l ⁻¹ kinetin, 30 g l ⁻¹ sucrose, 30 g l ⁻¹ sorbitol, 1 g l ⁻¹ 2-morpholinoethane sulfonic acid monohydrate, 2 g l ⁻¹ casein acid hydrolysate, 500 mg l ⁻¹ carbenicillin, 50 mg l ⁻¹ hygromycin, 3.2 g l ⁻¹ gelrite (pH 5.8)
Rooting medium (LS)	LS ^{*2} basal salts, 500 mg l ⁻¹ carbenicillin, 50 mg l ⁻¹ hygromycin, 3.2 g l ⁻¹ gelrite (pH 5.8)

^{*1} N6, from Chu *et al.* (1975); ^{*2} LS, from Linsmaier and Skoog (1965).

Table 2 Comparison of the callus induction rate and the transformation rate between two cultivars of japonica rice. Scutellum calli were inoculated with *A. Tumefaciens* harboring pBILucHm.

Cultivar	Number of Seeds	Callus induction rate ^{*1} (%)	Percentage of hygromycin-resistant ^{*2}	
			Calli (%)	Plants (%)
Taichung 65	371	97.9	6.2	4.6
Notohikari	261	98.3	2.7	2.7

^{*1} Callus induction rate was measured after culturing for 30 days.

^{*2} Number of regenerated calli or plants/Number of seeds × 100.

of culture, secondary calli gradually began to grow from the scutellum tissues. Actively growing pieces of calli were used in the transformation experiments. The fresh weights of scutellum calli obtained from seeds that had been cultured for different lengths of time were measured and compared, before they were co-cultivated with *A. tumefaciens*. The frequency of callus induction (Table 2) and the fresh weight of induced calli (Fig. 1) from scutellum of Taichung 65 were the same as those from Notohikari.

pIG121Hm is a binary vector that contains a reporter gene, the β -glucuronidase gene (intron-*GusA*) and two selectable marker genes, the kana-

Fig. 1 Relationship between the duration of cultivation of scutellum calli and their fresh weight. Mature seeds that had been dehusked and sterilized, were cultured on N6D2 medium. The fresh weight of calli introduced from one seed of Notohikari or Taichung 65, is shown. Bars indicate standard error. Each point represents the average of 5 samples. One sample consisted of approximately 50 seeds.

A pIG121Hm



B pBILucHm

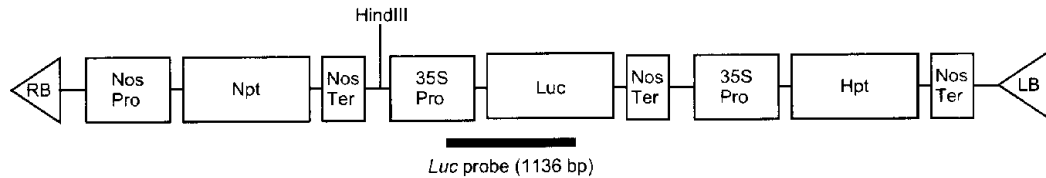


Fig. 2 Physical map of the T-DNA region of the introduced genes.

(A) pIG121Hm and (B) pBILucHm. RB, Right border; LB, Left border; Nos Pro, nopaline synthase promoter; 35S Pro, cauliflower mosaic virus 35S promoter; Npt, kanamycin resistance gene (NPTII); Nos Ter, nopaline synthase terminator; Intron-GUS, β -glucuronidase gene with intron; Hpt, hygromycin resistance gene (HPT); Luc, luciferase gene of firefly. *Hind*III endonuclease site is indicated above the box. Southern blot analysis of the *Hind*III-digested DNAs was performed using the 1136-bp PCR product indicated below the box as a hybridization probe.

mycin-resistance gene (*Npt*) and the hygromycin-resistance gene (*Hpt*) in the T-DNA region (Ohta *et al.*, 1990) (Fig. 2A). With this vector, GUS is expressed in plant cells but not in *A. tumefaciens* cells. Ti-plasmid pBILucHm was constructed by replacing the intron-*Gus* with the firefly luciferase (*Luc*) cDNA fragment that was obtained as an *Nco*I/*Sac*I fragment from pNtPrbcLm-LUC as described previously (Inada and Iba, 1998) (Fig. 2B). For blunt-end ligation, the *Xba*I site of the pIG121Hm vector and the *Nco*I site of *Luc* fragments were treated with the klenow fragment of DNA polymerase I.

The *A. tumefaciens* strain, EHA101, harboring pIG121Hm was grown at 28°C on LB agar medium supplemented with 50 mg l⁻¹ hygromycin and 50 mg l⁻¹ kanamycin for 3 days. The grown bacteria were collected with a small spoon, and suspended at a density of 2 × 10⁹ CFU ml⁻¹ in N6D2A liquid medium (Table 1). The calli of each cultivar that had been cultured for various lengths of time, were immersed in a bacterial suspension for several minutes and then transferred onto dry sterile filter paper to remove the excess liquid. Then, the calli were co-cultivated on N6D2A medium solidified by 0.32% gelrite at 28°C in the dark for 3 days. After co-cultivation, the level of transient GUS expression in the calli was examined and compared with that in the non-transformed calli (Fig. 3). The GUS assay was performed as described previously (Jefferson *et al.*, 1987). The level of GUS activity

was calibrated against 4-methyl-umbelliferone and expressed as milligrams of total soluble protein (Bradford, 1976). Although a small peak of GUS activity was observed in the 29-day-old calli of Taichung 65 and the 25-day-old calli of Notohikari, GUS activity was detected in all of the transformed calli of Taichung 65 and Notohikari. There was no correlation between the duration of culture of the calli and the level of transient expression of GUS in Taichung 65 or in Notohikari.

After co-cultivation, the calli that had been infected with the EHA101 harboring pBILucHm, were rinsed thoroughly with 500 mg l⁻¹ carbenicillin in sterile water, placed on the selection medium N6D2S (Table 1), and cultured at 28°C in the dark for 2 weeks. The calli were transferred onto fresh N6D2S medium, and subcultured every 2 weeks. After the subculture, the actively growing calli were transferred onto the regeneration medium, LSR (Table 1), and cultured at 28°C in a daily cycle of 15 hours of light and 9 hours of darkness. Then, the regenerated shoots were transferred onto a hormone-free rooting medium (LS) (Table 1) supplemented with 50 mg l⁻¹ hygromycin and 500 mg l⁻¹ carbenicillin (Shimada *et al.*, 1995). The transformation efficiency of scutellum calli from Taichung 65 was compared with that from Notohikari. In Taichung 65 and Notohikari, a few independent hygromycin-resistant calli (6.2% and 2.7%, respectively), and hygromycin-resistant plants (4.6% and 2.7%, respectively) were obtained

(Table 2). As a result, the stable transformation efficiency of hygromycin-resistant transformants in the Taichung 65 was the same as that in Notohikari. In addition, all of the regenerated plants grew into fully fertile mature plants. The product of polymerase chain reaction (PCR) for *Luc* from the plantlets that integrated the *Luc* gene showed a positive band (data not shown). To confirm the

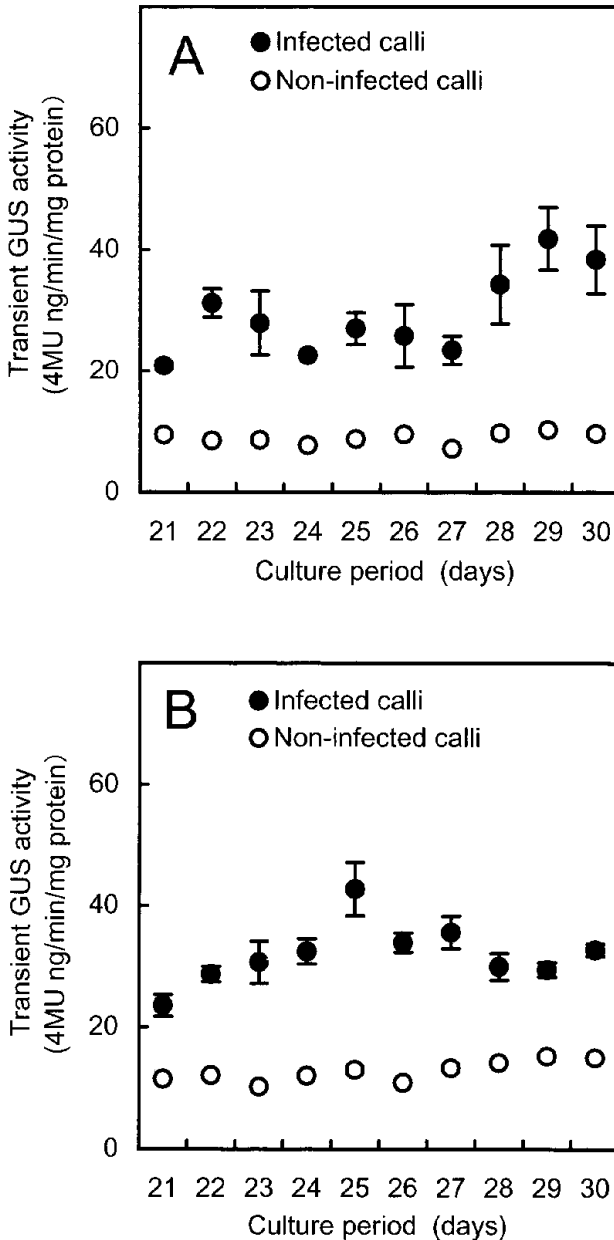


Fig. 3 Relationship between the duration of cultivation of scutellum calli and the level of transient GUS activity. Mature seeds that had been dehusked and sterilized, were cultured on N6D2 medium. The level of GUS activity in extracts of transformed and non-transformed rice calli of (A) Taichung 65 or (B) Notohikari is shown; bars indicate standard error. Each point represents the average of 5 to 8 samples. One sample consisted of approximately 100 mg of the induced scutellum calli.

expression of *Luc*, the hygromycin-resistant plants were assayed. Transgenic plants containing the *Luc* gene were evenly sprayed with 1 mM D-luciferin dissolved in water, and kept in the dark for 5 min before imaging. The luminescence of LUC activity from the transformants was captured using a photon-counting VIM camera equipped with an ARGUS-20 image processor and the U4470 software (Hamamatsu Phototronics, Hamamatsu, Japan). Expression of *Luc* was observed in all of the hygromycin-resistant plants, whereas the non-transformed control plants displayed no luminescence (data not shown).

Some of the selfed T_1 -transformants were analyzed by Southern blot analysis to detect the integrated genes in their genomic DNA and to estimate the transgene copy number (Fig. 4). Genomic DNAs were isolated from eight hygromycin-resistant T_1 -plants of Notohikari and three hygromycin-resistant T_1 -plants of Taichung 65 by the CTAB method (Murray and Thompson, 1980). Eight micrograms of genomic DNA was digested with *Hind*III, which has a unique site within the T-DNA region (Fig. 2), and fractionated by electrophoresis on a 0.8% agarose gel. The fractionated DNA segments were transferred to positively charged nylon membranes (Pall Biodyne™) Transfer Membrane), and hybridized with the *Luc* probe. The 1136-bp PCR product of the *Luc* gene was used as the probe. Primers BIN5-1 (5'-GTGTGGAATTGTGAGCGGA-3') and LUC3-1 (5'-CATAGCTTCTGCCAACCGA-3') were designed and synthesized according to the sequence of

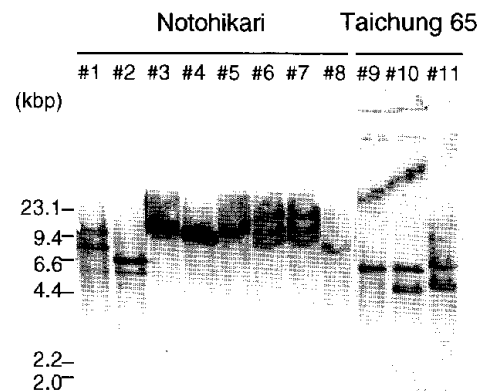


Fig. 4 Southern blot analysis of *Hind*III-digested DNAs from transformants, which were probed, with the 1136-bp PCR product of the *Luc* gene (Fig. 2B). The transformants were produced by EHA101 (pBILucHm). The T-DNA of pBILucHm has a single *Hind*III site. #1-8, T_1 transformant lines of Notohikari; #9-11, T_1 transformant lines of Taichung 65. Estimated length of DNA fragments are also given (in kbp).

Table 3 Estimation of the stability of the transgenes in the selfed T₁ plants produced by EHA101 (pBILucHm), and segregation patterns for expression of *Luc* in the progeny.

Cultivar	Transformant	Number of T ₁ Plants		Rate of LUC- positive plants	Ratio of LUC+/LUC-	χ^2
		LUC+	LUC-			
Taichung 65	#1	59	20	74.7%	3:1	0.00 ns* ¹
	#2	32	45	41.6%	1:1	1.10 ns
	#3	43	37	53.8%	1:1	0.23 ns
	#4	47	32	59.5%	1:1	1.42 ns
	#5	56	23	70.9%	3:1	0.71 ns
Notohikari	#1	30	15	66.7%	3:1	1.67 ns
	#2	40	9	81.6%	3:1	1.15 ns
	#3	38	15	71.7%	3:1	0.31 ns
	#4	34	16	68.0%	3:1	1.31 ns
	#5	31	19	62.0%	1:1	1.44 ns

*¹ ns, not significant.

the 35S promoter and the *Luc* gene. Southern blot analysis was carried out as described in the protocol of the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Life Science). We found that the transgene was integrated in all of the transformants examined. The copy number of transgene varied from one to three in the transformants. In a recent report (Wakita *et al.*, 1998), Southern blot analysis of transformants that had been produced by particle bombardment, revealed between 1 and more than 20 hybridizing bands of the transgenes. In general, particle bombardment more frequently results in the insertion of multiple copies and complex rearranged transgenes than the *Agrobacterium*-mediated method (Iyer *et al.*, 2000), and this was also demonstrated in the present study.

To confirm that the transgene is inherited to the next generation, the expression of *Luc* was studied in seedlings of selfed T₁-plants. The selfed T₁-progeny were evaluated for LUC expression. The patterns of segregation of the offspring from five independent transformants of Taichung 65 and Notohikari that have expressed a *Luc* gene in the T₀-generation, are shown in **Table 3**. T₁-seeds were dehusked and germinated on moist filter paper at 28 °C in continuous light for 5 days. The germination percentage of T₁ seeds was approximately 80%. During germination, there was a significant amount of LUC expression throughout the seedlings except in the albumen. As a result, transformants #1 and #5 of Taichung 65 and #1, #2, #3, and #4 of Notohikari showed a segregation ratio of 3:1 as expected from the mendelian inheritance of a single locus, which indicated inheritance of the transgene to the next generation. The other transformants

showed a segregation ratio less than 3:1. All of the LUC-positive seedlings of Taichung 65 #3 showed low LUC activity. We tested whether the *Luc* gene was integrated in the LUC-negative seedlings by PCR amplification. We found that the *Luc* gene was integrated in their genome. Some of the LUC-positive seedlings had been counted as negative due to weak expression of the gene. These genetic analyses suggest silencing by rearrangement of DNA upon transformation.

In this study, we produced stable transformants of Taichung 65 at a frequency of 4.6% using scutellum-derived calli inoculated with *A. tumefaciens*, EHA101. Taichung 65 is one of the genetic backgrounds of many genetic stocks with single and multiple phenotype markers for screening of various mutants; therefore, the *Agrobacterium*-mediated transformation method described here has wide application to molecular analyses with Taichung 65.

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