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Efficient Transformation of Korean Rice Cultivars (*Oryza sativa* L.) Mediated by *Agrobacterium tumefaciens*

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The purpose of this study was to improve transformation efficiency for three Korean rice cultivars, Ilpum, Dasan, and Namyang. Using two different media with or without light, efficiencies of callus induction, regeneration, and transformation of the Korean cultivars were compared to Japanese cultivar, Nipponbare, as a control. Immature cv. Nipponbare seeds produced 35.5% and 16.1% regeneration efficiency on CIM and N6D media, respectively. Among the Korean cultivars, only cv. Ilpum induced on CIM in the dark was actively regenerated with efficiency of 8.2%. With LBA4404 (pTOK233), no difference for the efficiency of transformation was found between mature and immature seeds of cv. Ilpum. This result reveals that mature seeds can be substituted for this study with no difference. The anther-derived calli of cv. Namyang inoculated with either LBA4404 (pTOK233) or EHA101 (pSMABuba) showed regeneration efficiencies of 14.5% and 20.9%, respectively, even though efficiency of transformation did not differ with these two vectors. We suggest that the anther-derived calli are better-materials for transformation experiment due to their genotype-independent regeneration. In the assay of GUS, all of the calli that survived on the second selection medium were strongly stained. PCR-Southern blot analyses confirmed that T-DNA was stably transformed into all tissues selected. Cvs. Nipponbare and Namyang transformed by LBA4404 (pTOK233) showed positive color in the NPTII ELISA.

Keywords: *Agrobacterium tumefaciens*, ELISA, PCR-Southern blot analysis, rice (*Oryza sativa* L.), transformation

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops in the world, especially in Asia. Plant breeders have targeted it for improvement in productivity and quality for many years. Conventional breeding methods have contributed greatly to this goal. However, recent developments in biotechnology have shown great potential as efficient working tools for crop improvement when combined with traditional plant breeding skills. This application has overcome the barrier between different species by gene transfer and introgression of foreign genes. After the feasibility of transgenic plant was proven, the field of direct DNA transfer into plants was spotlighted and attempted in broad plant species (Gasser and Fraley, 1989). Not only are they useful for introducing foreign genes, the genetic engineering methods are also useful for understanding specific gene function and expression.

Agrobacterium-mediated transformation has been known as the most efficient and reliable system over other transformation methods. However, the use of *Agrobacterium tumefaciens* has been limited only to dicotyledonous species due to its specific infection capability. But trials to obtain transgenic plants by the *Agrobacterium*-mediated transformation have continuously been applied to enlarge the limit of its host range in monocot plants. With serious efforts to improve efficiency, upgraded methods for rice transformation have been developed. Hiei *et al.* (1994) proved the validity of different parts of rice plant as explants, and Toki (1997) showed the rapid regeneration of transformed calli derived from immature rice embryos where the whole procedure took only 8-9 weeks.

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However, it seems that *Agrobacterium*-mediated transformation in rice is still genotype-dependent and not successful in most cultivars. To overcome these barriers, use of improved tissue culture system and efficient vectors with favorable bacterial strains should be considered.

In this study, three Korean cultivars of japonica type rice, Ilpum (high-quality rice), Dasan (super-yield rice), and Namyang were investigated for their transformation capabilities compared to Japanese cultivar Nipponbare, which is known to be the genotype favorable for regeneration. Especially the use of anther-derived calli was to verify the possibility of obtaining directly homozygous transgenic plants. For this purpose, two different culture media were compared for callus induction, and two different vector systems were used to establish *Agrobacterium*-mediated transformation in these Korean cultivars.

MATERIALS AND METHODS

Plant Materials

Immature seeds of Japanese rice cv. Nipponbare were used as a control. Immature seeds, which were collected in 2 weeks after anthesis, and mature seeds of cvs. Ilpum and Dasan, and the anther-derived calli of cv. Namyang were used in this experiment.

Callus Induction and Transformation

Dehulled seeds were soaked in 70% ethanol for 1 min and washed once with distilled water. The pre-treated seeds were sterilized in 2.5% sodium hypochlorite for 20 min with vigorous shaking (about 220 rpm), and rinsed thoroughly with sterilized water. Ten seeds were placed on an N6D (Hiei *et al.*, 1994) or CIM (Chu basal salt, glycine 2 mg/L, nicotinic acid 0.5 mg/L, pyridoxine·HCl 0.5 mg/L, thiamine·HCl 0.1 mg/L, casein hydrolysate 1 g/L, 2,4-D 4 mg/L, sucrose 30 g/L, gelrite 3 g/L, pH 5.8) plate, and incubated at 25°C under a dark or light condition for 4 weeks.

Agrobacterium strains, LBA4404 and EHA101, were used in this study and each vector, pTOK233 or pSMABuba, was introduced into the *Agrobacterium* by freeze-thaw or electroporation (Sambrook *et al.*, 1989). The vectors, pTOK233 and pSMABuba, were kindly provided by Drs. T. Komari and S. Toki, respectively (Fig. 1). Calli induced on N6D and CIM media were transferred to fresh media and precultured for 3 days at 28°C under a light or dark condition. *A. tumefaciens* cultures which were grown for 3 days on a solid AB (Hiei *et al.*, 1994)

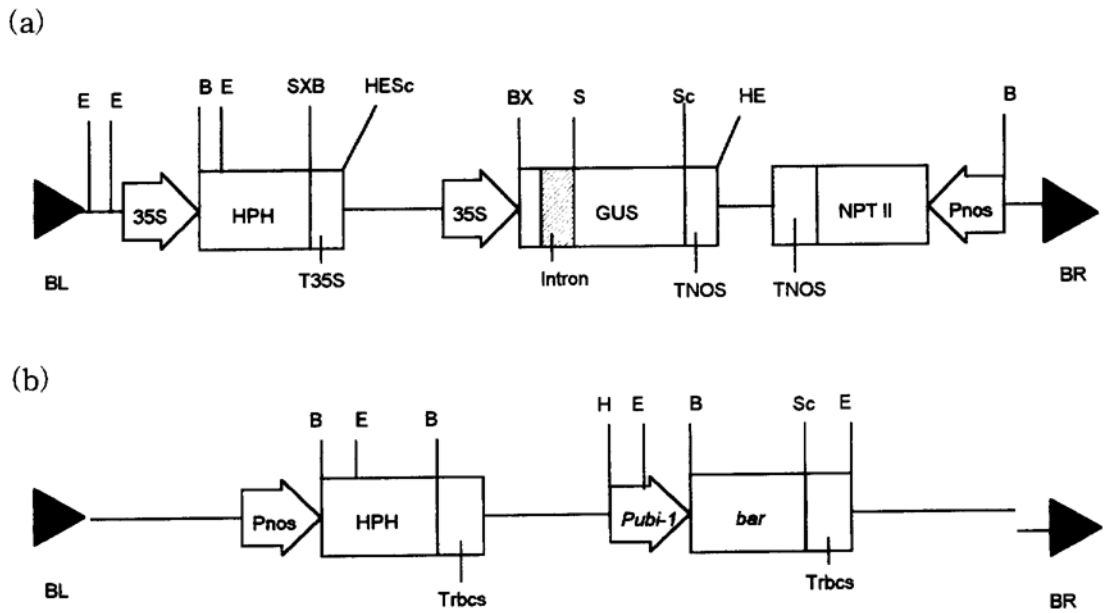

Fig. 1. T-DNA regions of (a) pTOK233 (Hiei *et al.*, 1994) and (b) pSMABuba (Toki, 1997).
BR, right border; BL, left border; NPTII, neomycin phosphotransferase; GUS, β-glucuronidase; HPH, hygromycin phosphotransferase; 35S, CaMV 35S promoter; TNOS, terminater of nopaline synthase; T35S, terminater of 35S RNA; Pnos, nopaline synthase promoter; Trbcs, terminater of *RbcS*; *Pubi-1*, promoter from maize *Ubq1*; bar, bialaphos-resistant gene; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I; Sc, *Sac*I; X, *Xba*I.

medium were used to inoculate to the actively growing precultured embryogenic calli. To be inoculated, the calli were immersed in a bacterial suspension for 1 min and kept on 2N6-AS (Hiei *et al.*, 1994) media for 3 days at 28°C in dark conditions. After co-cultivation, the calli were rinsed five times with distilled water containing 250 mg/L cefotaxime and 500 mg/L carbenicillin, and placed on the first-selection media (N6D+hygromycin 50 mg/L+carbenicillin 250 mg/L+cefotaxime 200 mg/L) for 2 weeks at 28°C under continuous illumination. The calli that had pro liferated on the first-selection media, were transferred to the second-selection media (N6D+hygromycin 50 mg/L+carbenicillin 200 mg/L+cefotaxime 100 mg/L). After 2 weeks on this medium the calli were transferred to the first-regeneration media (MS salt and vitamins, sucrose 30 g/L, sorbitol 30 g/L, casamino acids 2 g/L, kinetine 2 mg/L, NAA 0.02 mg/L, gelrite 4 g/L, pH 5.8, hygromycin 50 mg/L+ carbenicillin 100 mg/L) for 2 weeks, then cultured on the second-regeneration media (all the compositions in the first-regeneration media except carbenicillin) for another 2 weeks.

DNA Isolation from Callus

DNA was extracted as described by Michaels *et al.* (1994). Calli 100 mg were homogenized in a 250 μL extraction buffer (0.25 M NaCl, 50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% SDS, 0.25% β-mercaptoethanol). The sample was extracted with phenol/chloroform (Sambrook *et al.*, 1989). The supernatant was transferred into a fresh tube to which was added 0.35 volume of absolute ethanol, and then incubated for 20 min on ice. After centrifugation at 7,000 g for 5 min, supernatant was transferred to a new tube. One volume of isopropanol was added into this tube, and then centrifuged for 10 min at 9,000 g. The DNA pellet was resuspended in 50 μL of TE buffer (pH 8.0).

GUS Expression Assay

Expression of GUS was assayed by the modified method of protocol described by Jefferson *et al.* (1987). Four weeks after the innoculation, the randomly selected hygromycin resistant (Hyg^+) calli were preconditioned a in 0.1 M phosphate buffer (pH 6.8) containing 1% Triton X-100 and placed at 37°C for 1 h. To make 1 mL of GUS assay solution, 3 mg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) was dissolved in 150 μL of dimethyl formamide and mixed with 850 μL of Solution B (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1 M phosphate buffer, pH 7.0, 0.1% Triton X-100). The calli were placed in the GUS assay solution at 37°C for 4 h and were evaluated by visual examination.

PCR-Southern Analyses and NPTII ELISA

To identify stable gene integration, transformant tissues were subjected to PCR-Southern blot analyses which required a small quantity of callus tissues. Two sets of primers, 5'-ACAGCGTCTCCGACCTGATGCA-3' (sense) and 5'-AGTCAATGACCGCTGTTATGCG-3' (antisense), and 5'-TGCACCATCGTCAACCACTA-3' (sense) and 5'-ACAGCGACCACGCTCTTGAA-3' (antisense) were utilized to amplify the internal part of the *hph* and *bar* genes, respectively. The optimum reaction mix for a 30 μL PCR was comprised of 50 ng of genomic DNA, dNTP each at 200 μM final concentration, 20 pmol of each oligonucleotide primer, 1 × PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM of $MgCl_2$, 50 mM of KCl, and 0.1% of Triton X-100), and 1 unit of *Ex*Taq DNA polymerase (Takara, Japan). The amplification reaction was performed in a DNA thermal cycler (Takara, Japan) with the following thermal profile: 35 cycles of 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C. The initial denaturation was for 4 min at 95°C and the last extension was for 10 min at 72°C. Amplified fragments were electrophoresed in 0.8% agarose gel and transferred to a positively charged nylon membrane (Hybond-N+) as described by Chomczynski (1992). The probe manipulation and Southern analysis were performed according to manufacturer's manual (ECL™, Amersham, USA). For the detection of the neomycin phosphotransferase II (NPTII) protein in crude cellular extract, an enzyme-linked immunosorbant assay (ELISA) was carried out following manufacturer's manual (NPTII ELISA kit, 5 Prime → 3 Prime Inc., USA).

RESULTS AND DISCUSSION

Callus Induction on Different Media and Conditions

Selection of yellow and compact rice calli, especially 1-2 mm in diameter, was essential for successful regeneration after transformation. The calli derived from control Nipponbare showed optimum size and proliferation for transformation. Four weeks after plating on callus induction media, N6D and CIM,

the calli derived from cv. Nipponbare broke into small and compact calli. However, no difference in either treatments, media composition, or light conditions, was observed for callus induction. In cv. Ilpum, slower callus induction and lower percentage of callus breakage (around 30-40%, data not shown) than cv. Nipponbare was found. The pattern of callus induction in cv. Dasan was greatly different from the others in that the calli grew bigger and never broke into small pieces. These calli interrupted washing the removal of *A. tumefaciens* in the washing step. Embryogenic calli derived from the anther culture of cv. Namyang were also used in this study. The calli were bigger in size than optimum (3-5 mm in diameter) but the color and the compactness indicated that they were suitable for transformation. It is noteworthy that there was no great difference in callus induction among media. Toki (1997) emphasized that the modification of medium components by addition of casamino acids and proline resulted in shorter regeneration time. However, we observed that the addition did not affect callus induction efficiency in these cultivars.

Production of Hygromycin-resistant Calli

Table 1 shows the productivity of hygromycin-resistant calli on the second selection media four weeks after inoculation with the LBA4404 harboring cointegrate vector, pTOK233. The highest frequency (95.3%) was observed from the mature seeds of cv. Ilpum on an N6D medium in a light condition. But overall productivity was high in most conditions except cv. Dasan on a CIM medium. The poor performance of cv. Dasan in resistant calli induction came to a total failure of regeneration in later stage, irrespective of seed maturity, medium composition, or light condition. To obtain embryogenic calli from Dasan, special efforts, such as using different nitrogen sources or anther-cultured calli, will have to be attempted in the future.

Maturity of seeds, different light, or media condition did not affect production of resistant calli. Considering the view that most researchers have favored immature seeds as transformation materials, this result reveals that mature seeds can be used in this purpose with no difference. This will eliminate the need for the burdensome effort to obtain immature seeds. An encouraging result is that the most popular Korean cv. Ilpum has competitive frequency in resistant calli formation compared to that of control Nipponbare.

The anther-derived calli from cv. Namyang showed high hygromycin-resistance (95.8%) and excellent conditions for transformation and regeneration. Therefore, once induced into calli, these materials seem to be genotype-independent for regeneration. Homozygous plants can be readily obtained by a natural or artificial doubling procedure.

Table 1. Efficiency of hygromycin-resistant transformants inoculated with LBA4404 (pTOK233) survived on the second selection medium

Cultivar	Light condition	Medium	
		N6D	CIM
Nipponbare	L	299/349 (85.7)	370/408 (90.7)
	D	427/468 (91.2)	518/589 (87.9)
Ilpum M	L	283/297 (95.3)	255/272 (93.8)
	D	325/368 (88.3)	159/172 (92.4)
Ilpum IM	L	264/327 (80.7)	164/175 (93.7)
	D	508/543 (93.6)	260/287 (90.6)
Dasan M	L	107/119 (89.9)	27/35 (77.1)
	D	133/157 (84.7)	116/165 (70.3)
Dasan IM	L	159/186 (85.5)	12/19 (63.2)
	D	197/228 (86.4)	69/78 (88.5)

Ten seeds per cultivar were placed on an N6D or CIM plate and induction of callus was carried at 25°C under dark (D) or light (L) condition for 4 weeks. Each value represents the number of survived calli per total number of calli tested and percentages in the parenthesis. M, mature seed: IM, immature seed

Regeneration of Transgenic Calli

Table 2 shows the number of plantlets regenerated from hygromycin-resistant calli. Within 2-3 weeks, parts of the callus took on green color. The green calli were transferred immediately to the first regeneration medium. Two or three weeks after this stage, shoots as well as roots became visible (Fig. 2).

Table 2. Frequency of transgenic plants regenerated from the resistant calli inoculated with LBA4404 (pTOK233)

Cultivar	Light condition	N6D	CIM
Nipponbare	L	34/299 (11.4)	117/370 (31.6)
	D	89/427 (20.8)	204/518 (39.4)
Ilpum M	D	0/283 (0)	13/159 (8.2)

The survived calli on the second selection medium were transfered on the regeneration medium and incubated at 25°C under light condition. L and D means the light conditions at the callus induction stage. Each value represents the number of survived calli per total number of calli tested and percentages in the parenthesis. M, mature seed.

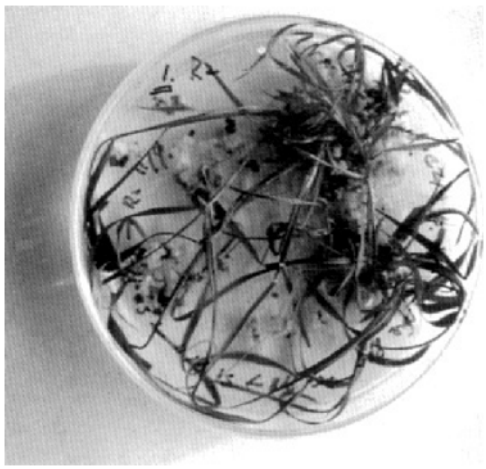

Fig. 2. Regenerated cv. Nipponbare induced on the CIM medium at 25°C light condition.

Cultivar Nipponbare produced an average of 36.1% and 16.9% regeneration efficiency on CIM and N6D media, respectively. This result was not fully concordant with Toki's result, but the growth of calli placed on N6D medium were much faster. Cultivar Ilpum calli induced on a CIM medium in the dark regenerated into plants with a lower percentage of survival (8.2%) but cv. Ilpum calli did not regenerate in the other conditions. The addition of casamino acids and proline did not enhance the frequency of regeneration for the Korean rice cultivars tested.

When the anther-derived calli were used for transformation, a much higher efficiency of plant regeneration was observed compared to the embryo-derived calli of cv. Ilpum (Table 3). Although the efficiency of regeneration from the calli inoculated with EHA101 harboring pSMABuba was higher (20.9%), the state of regeneration appeared rather vigorous in LBA4404 harboring pTOK233 inoculation. LBA 4404 has been known as a common strain, not like so-called superstrain EHA101; therefore the result indicates that the superior state of transformants must have resulted from the excellent transformation system of cointegrate vector, pTOK233 (Hoekema *et al.*, 1983; Hiei *et al.*, 1994). Otherwise, the better growth on selection media might have resulted from the strong promoter driving hygromycin resistance gene in pTOK233 vector. The hygromycin resistant gene in binary vector, pSMABuba, was driven by the promoter from nopaline synthases, known as a relatively weak promoter in transgene expression. Many research scientists have been looking for a strong promoter for better transgene expression, and the 35S promoter of cauliflower mosaic virus was one with a satisfying level of gene expression in dicot species. However, gene expression in monocots is still variable, showing great plant-species dependency. So far, the 5' region of the rice actin 1 gene has been known as the strongest promoter in overall monocots, and the alternative choice for promoters was 35S promoter fused to introns from other known genes, such as the maize *Adh1* gene (McElroy *et al.*, 1991). The results in this study suggested that a vector/promoter combination would be an important key to improve transformation efficiency in rice transformation, even though we did not try exact comparison of the vector efficiencies.

Table 3. Frequency of plant regeneration of anther-derived calli of cv. Namyang inoculated with two different vectors (pTOK233 and pSMABuba)

Vector system	
pTOK233	pSMABuba
34/234[a] (14.5)	33/158 (20.9)

[a]It represents the number of regenerated plants per the number of survived calli on the second selection medium.

Analysis of Transformed Rice Plants

GUS activity was assayed for the confirmation of transformation. Although no GUS activity could be positively detected in uninoculated embryo-derived calli tissues, the calli transformed with *A. tumefaciens* showed accumulated GUS staining. The high level of gus expression observed in Fig. 3 confirmed the strong enhancing effect of the intron from castor

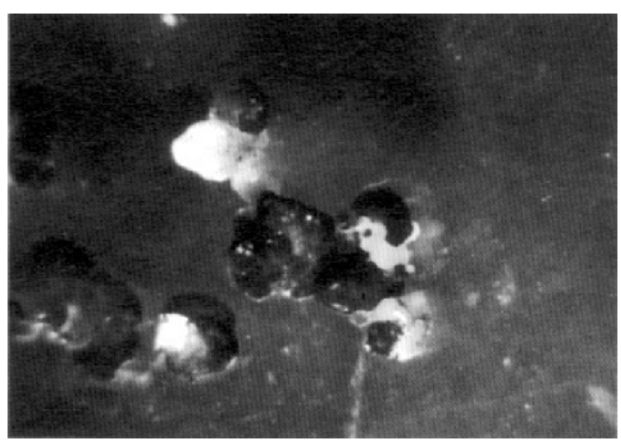

Fig. 3. Expression of GUS in four weeks after co-cultivation with LBA4404 harboring pTOK233. Calli were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc).

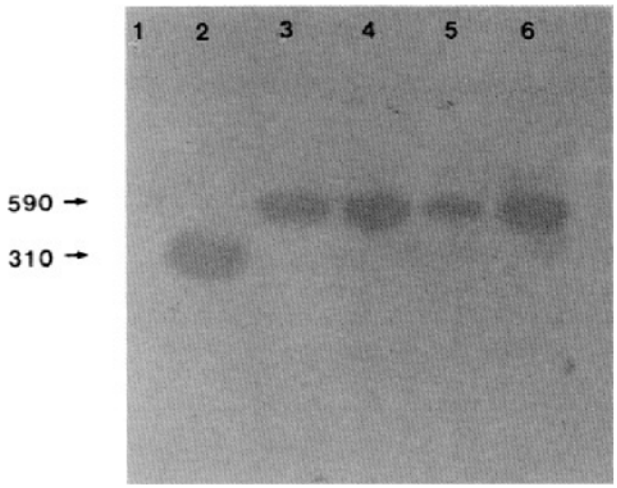

Fig. 4. PCR-Southern blot analysis of transformed calli on the second selection medium. Lane 1, Negative control (Nipponbare inoculated with *Agrobacterium* only); lane 2, Namyang inoculated with EHA101 harboring pSMABuba; lane 3, Namyang inoculated with LBA4404 harboring pTOK233; lane 4, Control inoculated with LBA4404 harboring pTOK233; lane 5, Dasan inoculated with LBA4404 harboring pTOK233; and lane 6, Ilpum inoculated with LBA4404 harboring pTOK233.

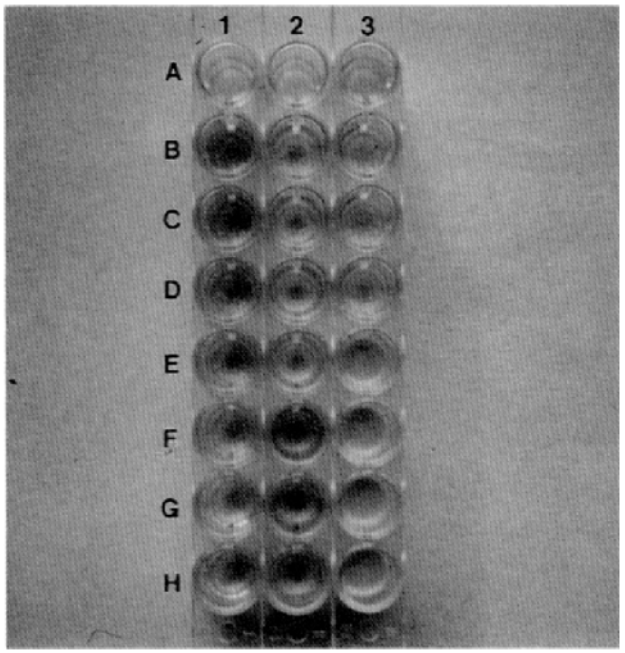

Fig. 5. ELISA of NPTII activity. 1B-1F, Namyang inoculated with LBA4404 harboring pTOK233; 1H-2D, Namyang inoculated with EHA101 harboring pSMABuba; 2F-3B, Nipponbare inoculated with LBA4404 harboring pTOK233; 3C and 3D, Nipponbare inoculated with *Agrobacterium* only; 1A, 1G and 2E, negative control contained only dilution buffer.

bean catalase gene in the GUS gene cassette of pTOK233, which was also designed to prevent unnecessary expression of the GUS reporter gene in *Agrobacterium* (Ohta *et al*, 1990). In an early assay, only a part of the callus was densely stained. However, the callus tissues at 4 weeks were nearly entirely stained, indicating rapid multiplication of transformed calli (Fig. 3). This result suggests that this reporter gene has been integrated in rice chromosomes.

To identify the stable gene integration, transformant tissues were subjected to PCR-Southern blot analysis, which requires a small quantity of sample. Positive signals appeared in all transformants as expected sizes, 310 bp for the *bar* gene and 590 bp for the *hph* gene (Fig. 4). The result strongly indicated that all the survived calli on the second selection medium were stably transformed, and foreign genes were integrated into the genomic DNA.

ELISA (enzyme-linked immunosorbant assay) was carried out to manifest transgene expression at the protein level. This colorimetric method has sensitivity comparable to radioisotopic methods, detecting as low as 100 pg of NPTII protein per mL of crude cellular extract. The NPTII ELISA kit, a so-called sandwich immunoblot system in which the rabbit polyclonal antibody raised against the NPTII protein was coated onto polystyrene microwells and the biotinylated secondary antibody double-tipped NPTII in the sample, was used. Callus tissues of cvs. Nipponbare and Namyang transformed with pTOK 233 were compared to negative controls both of cv. Namyang transformed with pSMABuba, and cv. Nipponbare inoculated with *A. tumefaciens* without vectors (Fig. 5). The specific yellow color development was not detected in extracts from control tissues even when the amount of protein was increased ten-fold. Of the 4 tissue extracts tested, all of the hygromycin-resistant and GUS positive transformants inoculated with pTOK233 displayed positive reaction for NPTII. This confirmed that plasmid pTOK233 was successfully integrated into rice genome and expressed *in vivo*. The confirmation of transgene expression at the protein level has greater implication in the point that many reports from transgenic studies revealed the malfunctioning of introduced genes mostly by gene silencing, even after stable transformation (Kumpatla *et al*., 1998). Right now, we are harvesting the seeds from the putative transgenic plants in the greenhouse. We observed relatively normal plant types and better seedling

rates from the anther-derived transgenic plants (data not shown), revealing that anther-derived calli might have better potential to grow into normal plant types. The germination test of the available seeds on hygromycin media showed the presence of various number of T-DNA insertion among transgenic plants; so far, 16 segregating lines and 6 non-segregating lines out of 22 lines. The result confirmed stable transformation and its expression at the next generation.

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