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Regeneration of Fertile Transgenic Rice Plants from a Korean Cultivar, Nakdongbyeo

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한국 재배종 낙동벼에서 임성 형질전환식물체의 재분화

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Rice is one of the most successful monocots in regenerating fertile and genetically stable transgenic plants. However, there is no report of a rice line developed in Korea that can be used for regeneration of fertile and genetically stable transformants. In this paper, we first demonstrate that a Korean variety, Nakdongbyeo, is suitable to obtain transgenic rice plants. Protoplasts from embryogenic suspension cultures were co-transformed with HPT (hygromycin phosphotransferase) and GUS (β-glucuronidase) genes in seperate plasmids in the presence of PEG (polyethylene glycol). In 5 independent experiments, the average frequency of calli showing hygromycin resistance were 1.73%. Plantlets were regenerated from the Hyg^R calli. The average efficiency of plantlet regeneration was approximately 27%. Based on the GUS activities of hygromycin resistant calli, ca. 35% of the resistant calli carried active GUS genes. The R0 transgenic plantlets were grown to maturity and R1 seeds were obtained. By examining the *in situ* activity of GUS in R1 seeds and seedlings, we confirmed that the GUS transgene driven by a CaMV 35S (cauliflower mosaic virus) promoter, showed proper expression patterns. We also confirmed Mendelian segregation of the HPT transgene in the R1 generation.

Key words: co-transformation, fertile rice transformants, GUS, HPT, protoplasts

Rice is one of the most important crops in the world. It is a major source of nutrition, especially for people living in Asia countries. Conventional breeding methods and tissue culture systems have been greatly contributed in enhancing the quality and productivity of rice. Due to advances in biotechnology, it is now possible to introduce genetically modified foreign and endogenous genes into rice plants. A variety of transformation methods have been developed to introduce genes of interest into rice cells and to regenerate transgenic rice. These gene-delivery methods include PEG (Uchimiya et al. 1988), electroporation-mediated uptake of DNA by protoplasts (Shimamoto et al. 1989), particle bombardment of immature embryos (Christou et al. 1989), and co-cultivation with *Agrobacterium tumefaciens* (Hiei et al. 1994). Even though there are variations in the efficiency of transformation and regeneration, those transformation methods have been successfully applied to generate fertile and genetically stable transgenic rice. Such advanced transgenic technologies certainly are powerful tools for researchers, not only in the study of the biological aspects of rice, but also to improve the agronomical quality of rice. By being transformed with agronomically useful genes, rice could easily acquire new traits, such as insect-, virus-, and herbicide- resistance. For example, Fujimoto et al. (1993) introduced a modified δ-endotoxin gene of *Bacillus thringiensis* (B. t.), which has a specific biological activity against *lepidoptera* insects, into a japonica rice. Hayakawa et al. (1992) reported that rice plants resistant to rice stripe virus was generated by the introduction of a viral coat protein gene. Rathore et al. (1993) used a *bar* gene to develop herbicide-resistant rice plants.

However, all the rice lines that have been successfully used

for genetic transformation are either of Indica type, such as Taipei 309 or IR72, or of Japonica type which is being cultivated in Japan. To expand genetic resources of rice in Korea, it is important to find Korean rice lines that are easy to be transformed and regenerated. If only foreign rice lines are used for genetic transformation in Korea, it might be necessary that transgenic plants are bred again to adapt them to Korean climate or to meet Korean specific needs. Here, we report a Korean rice variety, Nakdongbyeo, was successfully used to obtain fertile transgenic rice plants. GUS and HPT genes in separate plasmids were introduced into protoplasts by PEG-direct gene transfer technique. Fertile and genetically stable transgenic rice plants were obtained. In this report, we presented the data not only on the efficiency of plantlet regeneration but also on the frequency of the coexpression of the transgenes, GUS and HPT, in R_0 generation. We also presented the genetic and histochemical data of GUS and HPT transgenes in R_1 generation.

MATERIALS AND METHODS

Plasmids

Two plasmids (Fig. 1), pGL2 and pBI221, were used for transformation of rice protoplasts. pBI221 carries the β-glucuronidase (GUS) gene fused to a CaMV35S promoter. pGL2 contains the hygromycin phosphotransferase (HPT) gene fused to a CaMV35S promoter. The plasmids used for protoplast transformation were of a super-coiled form.

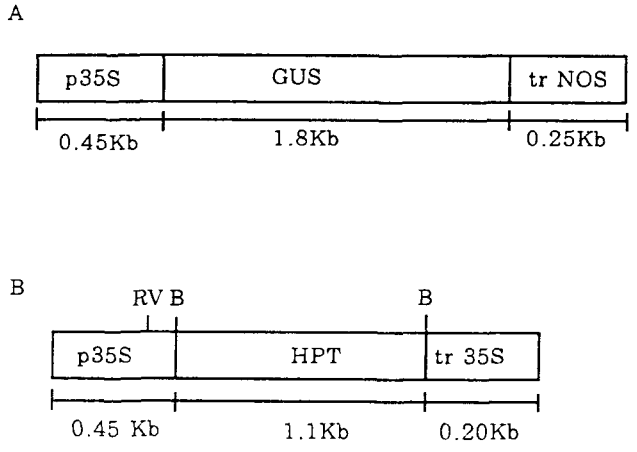

Figure 1. Diagrams of pBI221 (A) and pGL2 (B) plasmids used in transformation experiments. pBI221 carries a 35S CaMV promoter driving the expression of GUS gene. pGL2 contains a 35S CaMV promoter and a HPT gene. B: *Bam*HI, RV: *Eco*RV, p35S: 35S CaMV promoter, tr 35S: 35S CaMV terminator, tr NOS: NOS terminator,

Cell Suspension Culture and Protoplast Isolation

Cell suspension lines of *Oryza sativa* L. variety Nakdongbyeo were maintained essentially as described in Kyozuka et al. (1987). The cells were cultured in R2S medium supplemented with 2.0 mg/l 2,4-D and 3% sucrose. Suspension cultures were subcultured every week. Protoplasts were isolated from the suspension cultures. The cultured cells were incubated in a 12 cm plastic plate containing 20 ml of an enzyme mixture [(4% Cellulase RS (Kinki Yakult, Japan), 1% Macerozyme R10 (Kinki Yakult, Japan) and 0.4 M mannitol)] without shaking at 30° C. The plate was further incubated for 4-5 hrs to develop suspension culture. After incubation, the cell culture was passed through 30, 45, and 64 μm nylon meshes sequentially, and were suspended in 4 × volume of KMC solution (0.35 M KCl, 0.245 M $MgCl_2$, 0.254 M $CaCl_2 \cdot 2H_2O$, pH 6.0). Protoplasts were collected by centrifugation for 7 min at 750 rpm, and were washed twice in the KMC solution by centrifugation (750 rpm, 7 min).

Transformation

Ten to twenty μg of plasmid DNA in TE buffer were mixed with protoplasts suspended in 0.5 ml incubation solution [(0.1 M $Ca(NO_3)_2$, 0.45 M Mannitol, 20 mM HEPES, pH 6.0)] and 1.0 ml of 25% PEG (Boeringer mannheim, MW 1,500). The incubation mixture was kept at room temperature for 25 min and gradually diluted with washing solution [(0.275 M $Ca(NO_3)_2$, 44 mM Mannitol, 20 mM HEPES, pH 6.0)] and sea water (4% Sea Salt, 2.5% Mannitol, 2.5mM $CaCl_2 \cdot 2H_2O$, pH 6.0)].

Protoplast Culture and Selection for Hygromycin Resistant Colonies

Protoplasts were cultured with the agarose-bead method (Shillito et al. 1983) using nurse cells. One ml of protoplast suspension (10^6 protoplasts/ml) in the R2P medium (R2 basal salts, 2 mg/l 2,4-D, 0.4 M sucrose, pH 5.6) was mixed with an equal volume of melt agarose medium (3.2% Sea Plaque agarose) in a 3 cm plastic plate. The solidified medium containing protoplasts was cut into blocks and transferred into plastic plates containing 20 ml of a protoplast culture medium. Nurse 'Oc' cells (ca. 100 mg/plate) were

added in a liquid part of the culture. Suspension cultures of the rice 'Oc' cells (Baba et al., 1986) were gift from Dr. K. Shimamoto at Plantech Research Instituite. Plates were shaken slowly (ca. 30 rpm/min) in the dark at 30°C. At the 14th day of the protoplast culture, hygromycin B was added to the medium at the concentration of 20 μg/ml, and after 2 weeks, the agarose blocks were transferred to R2SA medium (R2 basal salts, 2 mg/l 2,4-D, 6% sucrose, 0.25% agarose) containing hygromycin B (20 μg/ml).

Plant Regeneration

Three weeks later, callus colonies of 1-2 mm in diameter were transferred to MSKN medium containing MS basal salts supplemented with 3% sucrose, 0.4% agarose, 2 mg/l kinetin and 0.5 mg/l NAA, and were cultured under the light of ca. 3,000 lux at 27°C. Plantlets with shoots of 2 cm or longer, were transferred onto MSN1.5 medium containing 0.4% agarose, MS basal salts, 3% sucrose, and 1.5 mg/l NAA to promote root development. Regenerated plants longer than 10 cm were transferred to soil pots and were grown in a growth chamber.

Southern Blot Analysis

Genomic DNA was extracted from leaf blades of transgenic R_1 progenies according to Dellaporta et al. (1983). Restriction enzyme digestion, electrophoresis and Southern blot analysis were performed according to Maniatis et al. (1982). A DNA probe used for hybridization to detect a HPT gene, was an 1.1 kb *Bam*HI fragment of pGL2.

GUS Assays

Fluorometric assay. GUS activities in the various tissues of transgenic plants were measured as described by Jefferson et al. (1987). Using 4-methylumbelliferyl glucuronide as a substrate, 4-methylumbelliferone production was fluorometrically detected with a Hoeffer Mini Fluorometer (TKO 100). Specific GUS activities were calculated as picomoles of 4-methylumbelliferone produced per minute per milligram of total soluble protein.

Histochemical assay. Histochemical staining was performed according to the method described by Jefferson (1987). Rice tissues were hand-sectioned with a razor, and were fixed in a 10 mM MES (pH 5.6) solution containing 0.3% formaldehyde and 0.4 M mannitol for 45 min at room temperature. The tissue samples were washed 2 times with 50 mM phosphate buffer (pH 7.0), and were incubated in a staining solution of 1 mM of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and 50 mM phosphate (pH 7.0) at 37°C overnight.

Analysis of Hygromycin Resistance among the R_1 Progeny

The R_1 seeds from selfed R_0 plants were sterilized in 1.0% $NaClO_4$, and were washed twice in sterile distilled water. The sterilized seeds were placed onto MSO medium containing 20 μg/ml hygromycin B. The seeds were left in the MSO medium for ten days at 27°C under constant light.

RESULTS AND DISCUSSION

Selection of Hygromycin-Resistant Calli and Regeneration of Plants

Protoplasts transfected with plasmid DNA in the presence of PEG were grown in a mixture of R2P medium and 'Oc' feeder cells during the first 2 weeks. In the next 2 weeks, calli were developed and were screened for hygromycin resistance. Most calli stopped growing on the hygromycin plates, however a few putative transformants continued to grow (Fig. 2A). Calli derived from protoplasts treated with only PEG, were used as a control in this experiment. The control calli failed to grow when subjected to hygromycin selection (Fig. 2A). The frequencies of Hyg^R calli in 5 independent experiments were uniform, from 1.29 to 2.20% (Table 1). An average frequency was 1.73%. After transferred to regeneration medium (MSKN medium), hygromycin-resistant calli

Table 1 The frequency of Hyg^R transformants from generated calli.

Experiment	No. of treated protoplasts	No. of generated calli($\times 10^3$)[a]	No. of Hyg^R calli[b]	The frequency of Hyg^R transformants(%)
1	1×10^6	5.8	83	1.43
2	1×10^6	5.6	106	1.89
3	1×10^6	5.0	110	2.20
4	1×10^6	6.5	120	1.84
5	1×10^6	8.0	103	1.29
				Average 1.73

[a] Calli were two weeks old after PEG treatment
[b] Calli were cultured for 3 weeks on a medium containing 20 μgml^{-1} hygromycin B and survived calli were scored as Hyg^R transformants.

Figure 2. Hygromycin resistance selection in transgenic and control calli(A), a close-up of plantlets produced from a resistant callus, (B) and mature R0 transgenic rice plants (C). A. Hyg^R colonies were grown for 4 weeks on the plate containing 20 μg/ml hygromycin B. The calli were 6 weeks old after transformation. In the left plate of the Figure, the calli were originated from protoplasts treated with only PEG solution. In the right plate, the calli were developed from protoplasts transformed with GUS and HPT plasmids. B. Plantlets were produced from Hyg^R calli 5 weeks after the protoplasts were plated. The plantlets produced shoots and roots after being transferred onto a root regeneration medium. C. Mature and fertile transgenic rice plants. Plantlets derived from Hyg^R calli were grown to maturity.

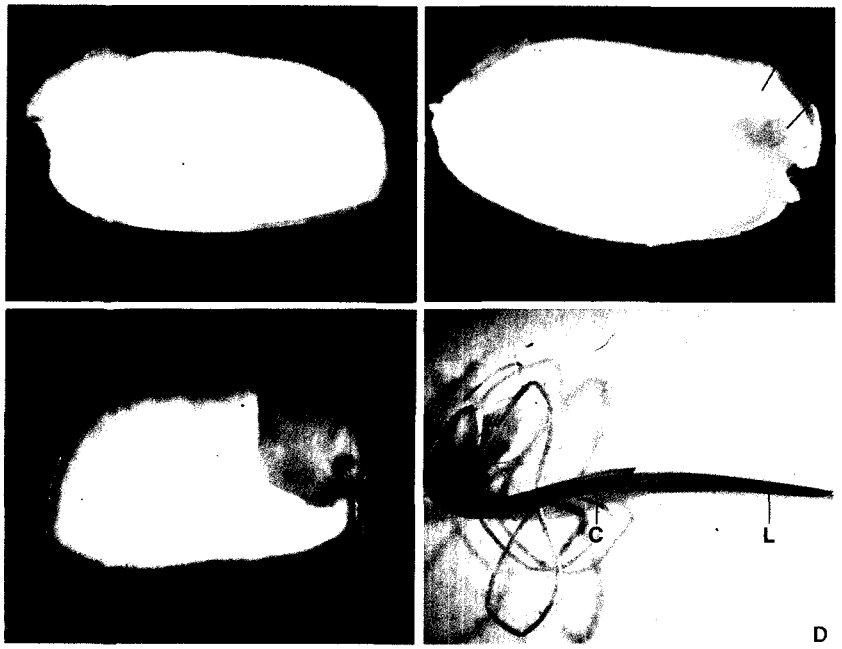

Figure 3. Expression of the CaMV35S-GUS gene in transformed R1 seeds. A. A cross-section of the untransformed seed treated with X-Gluc to visualize GUS activity B and C. Cross-sections of the transformed seed after 24 h imbibition. Early detection of GUS stains 24 hrs after imbibition (B). Later, more intense GUS stains were developed in scutellum and aleurone. D. The staining pattern of a germinating seedling of 5 days old. Most of the plant body showed strong GUS activity.
AL, aleurone layer: SC, scutellum: SH, shoot: R, radicle: L, leaf: C, coleoptile.

produced coleoptile- or shoot-like structures within 3 weeks (Fig. 2B). Table 2 shows the frequency of plantlet regeneration from Hyg^R calli in 5 different experiments. In all the experiments, 20 μg of HPT plasmids were used for transformation. The regeneration frequency among the different experiments were rather consistent, from 16 to 33%. An average frequency was 27%. The coleoptile- or shoot-like organs were developed into plantlets when transferred to MSN1.5 medium. After plantlets were grown for 5 weeks on the MSN1.5 medium, they were transplanted into the soil and were grown in growth chambers. Twenty hygromycin-resistant calli from five seperate experiments gave rise to at least 1 plantlet. Some calli produced more than 6 plantlets (Table 2). Many plants had been grown to maturity (Fig. 2C).

Stable Co-Transformation

Table 2 Regeneration of transgenic plantlets from hygromycin resistant calli.

Experiment	No. of Hyg^R [a] calli tested for regeneration	No. of Hyg^R calli producing plantlets	The frequency of plantlets regeneration(%)
1	43	14	33
2	66	19	29
3	70	24	34
4	80	13	16
5	63	16	25

[a] Hyg^R: Hygromycin resistant.

Table 3 Hygromycin resistant calli expressing GUS activity 5 weeks after PEG treatment.

Experiment	pBI221+pGL2[a] (μg)	GUS^+[b]/Hyg^R[c]	Co-transformation frequency(%)
1	10+20	16/40	40.0
2	10+20	5/40	12.5
3	20+20	24/40	60.0
4	20+20	14/40	35.0
5	20+20	11/40	27.5

[a] pBI221 carries GUS and pGL2 does HPT.
[b] GUS^+, the number of calli that showed blue stains with X-Gluc.
[c] Hyg^R, the number of hygromycin-resistant calli tested for GUS activity.

GUS and HPT genes in separate plamids were introduced simultaneously into protoplasts in the PEG solution. The resulting calli were screened for hygromycin resistance. Survived calli were assayed for GUS activity using X-Gluc (Table 3). Two different ratios of the amount of GUS plasmid DNA (pBI221) to that of HPT plasmid DNA (pGL2) were used for co-transformation (Table 3). Even though there was a high variation in the number of hygromycin resistant calli expressing the GUS gene, it tends to obtain higher frequency of co-expressing calli when the higher ratio of GUS to HPT plasmids was used for transformation. On average, more than 35 % of hygromycin resistant colonies exhibited GUS activity. In one case, the frequency of resistant calli expressing GUS was as high as 60 %. From 24 out of 40 hygromycin-resistant calli, GUS activities were detected (Exp. 3 in Table 3). Co-transformation of two different genes delivered in separate plasmids has been reported in tobacco (Czernilofsky et al. 1986), tomato (Jongsma et al. 1987), petunia (Tagu et al. 1988), maize (Lyznik et al. 1989), japonica rice (Shimamoto et al. 1989), and indica rice (Peng et al. 1990). Our co-transformation efficiency is comparable to those of the previous reports. Recently, we make efforts to further increase the efficiency of co-transformation and regeneration by using newly established regenerable cell lines and an advanced transformation system such as particle bombardment. Gene transfer technique using separate plasmids has an advantage over one using a single plasmid. Usually, one of the transgenes is used only as a selection marker during plant regeneration. Once fertile transgenic plants are obtained, the selection marker is not needed any more. Also, it should be considered that a selection marker is not harmful to human or animals if transgenic plants are developed for consumption. Therefore, when two transgenes are integrated into a genome at a separable distance, it is easy to screen out plants carrying only a selection marker.

GUS Fluorometric Assay

Since a 35S CaMV promoter used to express the GUS gene is highly active in leaves, the expression activity of the transgene, GUS, was examined in leaf tissues. The GUS specific activity of plants was determined by measuring the rate of hydrolysis of the substrate 4-methylumbelliferyl-β-D-glucuronide. In the leaves of the non-transformant and some of the hygromycin resistant R0 plants, GUS activities were very low (10 to 17 pmoles 4-MU/min per mg protein). These non-stained R0 plants presumably did not carry a GUS gene since they showed the similar GUS activity as non-transformants did. The GUS activities of the stained R0 plants were 10 to 100 fold higher than non-transformants (112 to 1195 pmoles 4-MU/min per mg protein) (Table 4). GUS activities were highly variable among the stained plants. Such a variation is not surprising since it has been well documented that the expressivity of the same transgene shows

Table 4. GUS activity in leaf extracts from transgenic plants.

Plant#	GUS activity[a]	Plant#	GUS activity
C1[b]	10	GUS transformants	
C2[c]	17	-4	188
		-5	1,230
GUS transformants		-6	1,105
15-3	1,172	-7	117
-4	382	23 -8	1,100
-5	280	-9	811
-6	789	-10	112
-7	1,126	-11	305
18-3	1,195		

[a] pmoles 4-MU/min per mg protein.
[b] Non-transformant.
[c] Non-stained transformant.

Figure 4. Hygromycin resistant transgenic R1 progeny. Transformed (upper) and untransformed seeds (below) were germinated on hygromycin (20 μg/ml) medium for 10 days. While transgenic seeds showed normal germination, non-transformants failed to produce radicles and died within 2 weeks.

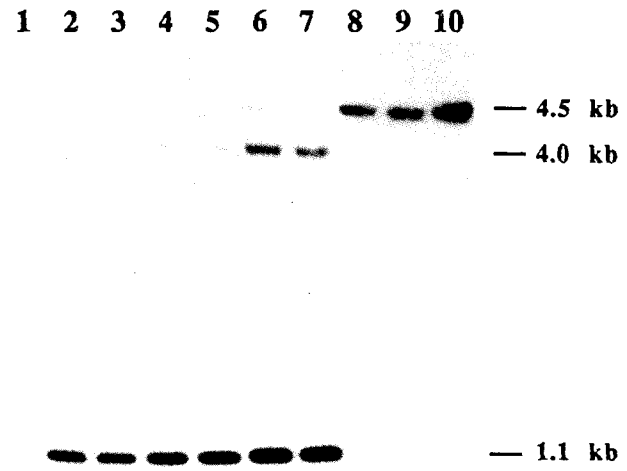

Figure 5. Genomic DNA from nine independent Hyg^R plants digested with *Bam*HI or *Eco*RV.
Lane 1: DNA from non-transformed plants
Lane 2-7: *Bam*HI-digested DNA from 6 independent Hyg^R plants
Lane 8-10: EcoRV-digested DNA from three Hyg^R plants.

large variation, depending upon insertion sites in a genome.

GUS Histochemical Assay.

To test whether the expression of the transgene, GUS, can be stably maintained in the next generation, the expression pattern of GUS was examined in R1 progeny from selfed R0 plants using a histochemical assay. Since a 35S CaMV promoter is constitutively expressed in rice, most tissues in R1 plants should show GUS activity. Sections from various parts of the transgenic R1 plants-seeds, leaves, roots, and germinating seedlings-were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to detect GUS activity. The blue-colored products of GUS activity were visible 5-24 hrs after incubation. No blue-colored products were detected in the tissue sections of untransformed control plants. GUS activity was detected in a leaf blade (data not shown). In a root, the tip was intensely stained, which is characteristics of the expression of a 35S CaMV promoter (data not shown). Both endosperm and embryo were stained. In the endosperm, the aleurone layer showed the most intense activity (Fig. 3B). A change in the localization of GUS activity was observed after germination. Compared to dormant seeds, germinating seeds showed the strong GUS stains at the outermost layer of the scutella and leaf- and root-primordia in the embryos. The coleoptile of the seedling in Fig. 3D, maintained residual GUS activity that was initially observed during germination. The GUS transgene was expressed in most parts of R1 seedling (Fig. 3D). All together, our data showed that the transgene GUS maintained the expression pattern in the next generation. Also, we demonstrated that the co-transformation of a non-selectable gene with a selectable marker could be used to generate fertile transgenic rice.

Transmission of Expressed HPT Genes among the Progeny

We examined the stable transmission of the HPT gene in R1 progeny from selfed R0 plants at the molecular level. Selfed seeds (R1) from 2 hygromycin resistant plants (R0) were germinated in 20 μg/ml of hygromycin medium for 10 days. Under these conditions, non-resistant germinating seeds produced very little radicles and then died within 2 weeks (Fig. 4). Approximate ratios of hygromycin resistant plants to non-resistant ones among the selfed progeny of 2 R0 transformants are shown in Table 5. Family #1 showed 3 to 1 ratio, which implies that the HTP gene is present at a single locus in the genome. However, Family #2 showed 5 to

Table 5. Transmission of hygromycin resistancs to the R1 progeny.

R1 family#	No. of seeds tested	No. of resistant seeds	No. of sensitive seeds
1	18	15	3
2	24	18	6

1 ratio. This family might contain a HTP gene at more than one locus. Since the sample number in this experiment is relatively small, further study is required. To confirm that the drug resistance in R_1 plants was acquired due to the presence of the transgene, HTP, Southern analysis was performed using an 1.1 kb *Bam*HI internal fragment of the HPT gene as a probe (see Fig. 1A). Genomic DNA was obtained from leaf blades of R_1 progeny of transgenic plants and nontransformants. All the resistant R_1 plants carried DNA hydridized with a HPT internal probe, while a non-transformed plant did not show any detectable hybridization signal (Fig. 5). Two high molecular weight DNA hybridized with the probe were detected when digested with *Bam*HI (lane 2 to 7). This is not due to incomplete digestion of the genomic DNA (data not shown). The detection of higher molecular weight DNA might be due to partial methylation of *Bam*HI recognition sites in the genomic DNA. Methylation of transgenes is one of common phenomena in higher plants. Our data demonstrated that HPT and GUS genes integrated in transgenic rice plants were stably transmitted and properly expressed in a subsequent R_1 generation.

적 요

벼는 임성 식물체의 재분화 뿐만 아니라 유전적으로 안정된 형질전환 식물체를 얻을 수 있는 가장 성공적인 단자엽 식물중 하나이다. 그러나 우리나라에서는 아직 유전적으로 안정된 임성 형질전환 벼품종에 대한 보고가 없었다. 본 연구에서 우리나라의 재배종인 낙동벼로부터 임성 형질전환 식물체를 얻을 수 있음을 증명하였다. 현탁배양세포로부터 분리된 원형질체를 이용하여 PEG로 HPT와 GUS plasmids를 함께 형질전환시켰다. 다섯번의 실험을 통하여 hygromycin 저항성 캘러스는 평균 1.73%이였다. 소식물체는 항생제 저항성 캘러스로부터 재분화되었고 식물체 재분화 효율은약 27%이었다. 항생제에 저항성이 있는 캘러스에서 GUS 유전자가 발현되는 캘러스는 평균 35%였다. R_0 형질전환식물체는 성숙 개화하여 R_1 종자를 생성하였다. R_1 종자와 유모의 해부학적 GUS 활성을 분석하여 CaMV35S 프로모터에 의해 GUS 유전자가 적절히 발현되는 것을 확인하였다. 또한 우리는 R_1세대에서 HPT유전자가 멘델 법칙에 따라 유전됨을 확인하였다.

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