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Introduction and Expression of Foreign Genes in Rice Cells by Particle Bombardment

Jeon, Jong-Seong, Hou-Sung Jung, Soon-Kee Sung, Jong Seob Lee[1], Yang Do Choi[2], Han-Jip Kim[3] and Kwang-Woong Lee*

Department of Biology and

[1]*Department of Molecular Biology, Seoul National University, Seoul;*

[2]*Department of Agricultural Chemistry, Seoul National University, Suwon; and*

[3]*Department of Biology, Ajou University, Suwon*

For establishing a transformation system of rice, an efficient introduction of foreign genes into embryogenic cell suspension by particle bombardment was conducted. The particle inflow gun based on the acceleration of DNA-coated tungsten particles using pressurized helium was constructed for delivery of DNA into rice cells. Several bombardment parameters were optimized using the transient expression of GUS gene. The conditions that gave the highest GUS gene expression of about 1000 blue spots per g fresh weight of bombarded cells include treatment of the cells with 0.5 M osmotic pressure, and use of the 410 kPa helium, 110 mm target distance, 13 mm syringe filter holder and 5 μL DNA/tungsten mixtures. It was also confirmed that rice actin promoter-intron construct gave the highest expression of all promoter-sequences studied. Eight weeks after the bombardment, stably transformed calluses were obtained on the selection medium containing 100 mg/L G418 and showed the strong activity in *in situ* GUS assay.

Key words: *Oriza sativa* L., particle bombardment, GUS, transformation

For dicots, the *Agrobacterium*-mediated transformation system can be used to generate many transgenic plants but for monocots, which include the agronomically important cereal crops, the use of this method has been hindered by the limited host range of the bacterium. For this reason, methods to deliver foreign genes into protoplasts of cereal plants by polyethylene glycol-facilitated DNA uptake and electroporation have been used (Hayashimoto *et al.*, 1990; Battraw and Hall, 1992). However, wide application of these methods is limited by the need of a system for regenerating plants from protoplasts which is generally genotype-dependent, time-consuming and labor-intensive.

In order to circumvent the problem of manipulating protoplasts, the particle bombardment method based on the accelerated particles was developed to deliver foreign genes into intact cells with cell walls (Klein *et al.*, 1987; Christou *et al.*, 1988; Oard *et al.*, 1990; Sautter *et al.*, 1991). Thereafter, the particle bombardment system has been used for obtaining transgenic plants in several plants such as maize (Fromm *et al.*, 1990), sugarcane (Bower and Birch, 1992) and wheat (Vasil *et al.*, 1993). However, expansion of particle bombardment technology is still limited by the accessibility of devices due to the high cost and complexity. Recently, simple devices that accelerate particles directly in a stream of helium have been developed (Finer *et al.*, 1992; Takeuchi *et al.*, 1992; Vain *et al.*, 1993a). In this study, we constructed a particle bombardment apparatus called a particle inflow gun which is based on a timer relay-driven solenoid system and evaluated some parameters of bombardment, for example, helium pressure, target distance and so on, which are crucial for the efficient delivery of foreign genes into embryogenic cell suspension

*Corresponding author : Fax 82-2-872-6881

of rice.

It is necessary to increase the transient expression of a reporter gene in rice cells for production of many transgenic plants. It was reported that treatment of target cells with high osmotic pressure increased about 3-fold in maize (Vain *et al.*, 1993b) and 60-fold in tobacco (Russell *et al.*, 1992) for transient expression. In this paper, we first report the effect of osmotic treatment on transient expression of foreign genes in rice cells.

In addition to the need to determine the optimal physical parameters and culture conditions of target cells in particle bombardment, it is important to select the DNA construct which gives the high level of gene expression. In our laboratory, it was reported that a new construct (pLS201), inserting a deleted maize alcohol dehydrogenase (*Adh* 1) intron 1 between cauliflower mosaic virus (CaMV) 35S promoter and β-glucuronidase (GUS) gene, increased 30- to 40-fold in GUS activity of transgenic potatoes when compared to construct (pBI121) containing GUS gene alone (Lee and Sung, 1992). It has also been reported that the 5' region of the rice *Act* 1 gene directed high levels of GUS gene expression in transformed rice protoplasts (McElroy *et al.*, 1990) and barley cells (Chibbar *et al.*, 1993). We compared here the effect of various constructs on transient GUS gene expression in rice cells.

At the beginning of transforming rice cells with gene containing the transit peptide of rubisco small subunit, we estabilished the optimized conditions of a particle delivery related to some physical parameters, osmotic treatment of target cells and selection of promoter using a particle inflow gun. The main differences between our procedures and those previously published for rice transformation by particle bombardment include the use of particle inflow gun and osmotic treatment of target cells.

MATERIALS AND METHODS

Cell suspension

Calluses of *Oryza sativa* cvs. Seomjinbyeo and Dobongbyeo were induced from anther (Ryu, 1992) and seed (Jeon and Lee, 1992), respectively. Embryogenic cell suspensions were developed from these calluses and cultured in AA_2 liquid media (Müller and Grafe, 1978) containing 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/L kinetin and 0.1 mg/L gibberellic acid. All cell suspensions were maintained for more than 4 months on the gyratory shaker at 120 rpm in the dark at 27℃.

Target cell preparation

Before the particle bombardment, embryogenic cell suspensions were filtered through a 620 μm stainless steel mesh and 0.5-1 g of the cells was evenly dispersed on a 55 mm filter paper (Whatman #4). To determine the optimal concentration of osmotic treatment of target cells, filters carrying target cells were placed on a N6D medium [N6 medium (Chu *et al.*, 1975) containing 2 mg/L 2,4-D] supplemented with various concentrations (0, 0.25, 0.5, 0.75 and 1.0 M) of mannitol plus sorbitol for 4 h.

Particle bombardment device

The particle bombardment device was constructed using equipments that were readily available according to Finer *et al.* (1992). The vacuum chamber consisted of a 6 mm steel plate and measured 160×160×300 mm^3. Inside the vacuum chamber, to control the target distance, acryl box which contains grooves at every 15 mm was inserted. A 2-way solenoid valve (2 JIP Pipe, CKD) which is controlled by a timer relay (H3BA, Omron) was connected on the vacuum chamber. Timer duration always set for 50 msec. The 10 mm thick glass was attached in the front of the vacuum chamber. The vacuum gauge and vent assembly were connected in both sides of the box. The vacuum set down to about 700 mmHg in all experiments.

Plasmid

Plasmids used in the experiments for transient expression include pAct1D (Zhang *et al.*, 1991), pLS201 (Lee and Sung, 1992) and pBI121 (Clontech). Figure 1 shows schematic diagrams of all constructs. Plasmid pAct1D was mainly used for the optimization of particle bombardment through transient GUS assay, and pLS201 which contains neomycin phosphotransferase II (NPTII) coding region was used for a stable transformation. Plasmids were isolated and

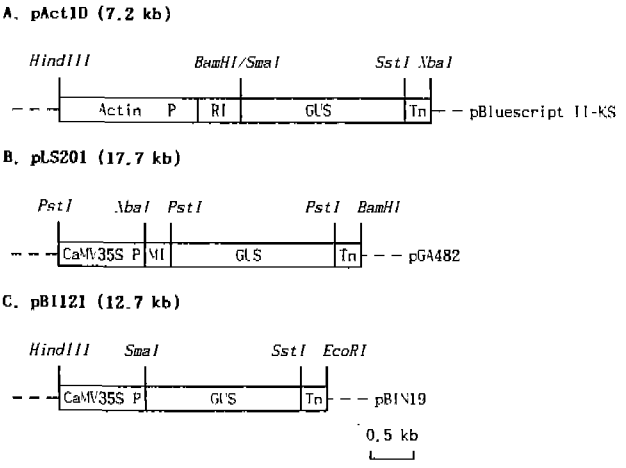

Fig. 1. Structure of gene fusions with GUS in plasmid pAct1D (A), pLS201 (B) and pBI121 (C). The abbreviations used are: Actin P, rice actin promoter; RI, rice *Act* 1D intron; GUS, coding region for β-glucuronidase; Tn, nopaline synthase (NOS) terminator; CaMV35S P, cauliflower mosaic virus 35S promoter; MI, deleted maize alcohol dehydrogenase (*Adh*1) intron. pLS201 (B) and pBI121 (C) contain the neomycin phosphotransferase (NPTII) coding region flanked by the CaMV 35S promoter and NOS terminator.

purified according to Sambrook *et al.* (1989).

DNA coating and particle bombardment

Tungsten particles (M10, Bio-Rad) were kept overnight in absolute ethanol. After washing three times with sterile water, the particles were resuspended in water. The mixture used for bombardment consisted of 10 μL of tungsten (1 mg/10 μL), 20 μL of DNA (1 μg/μL), 25 μL of 2.5 M $CaCl_2$ and 10 μL of 100 mM spermidine (free base). The DNA/tungsten mixtures stained with 10 μg/mL of 4,6-diamidino-2-phenylindole (DAPI) were observed under the fluorescence microscope (UV-2 filter, main wavelength 365 nm) to confirm the DNA coating on the tungsten surface.

After 5-10 min on ice, 50 μL of the supernatant was removed. Two or 5 μL of the remaining DNA/tungsten mixtures were loaded per shot on the screen of 13 mm Swinney plastic syringe filter holder (#4317, Gelman Sci.) or 25 mm syringe filter holder (#4320, Gelman Sci.). Target cells were covered with a baffle containing 230 μm stainless steel mesh and bombarded using 275-550 kPa helium at 110 or 160 mm distance between the loaded particle and target cells. Two to 6 parallel samples within one experiment were bombarded.

Culture conditions after bombardment

After the particle bombardment, bombarded cells were incubated on an osmoticum-containing medium for 24 h, and then transferred on a N6D medium. Selection of G418-resistant cell lines was initiated 3-5 days after bombardment by placing the filter papers on a N6D medium containing 100 mg/L G418. Filters were transferred to a fresh medium containing antibiotics every 14 days and resistant calluses were isolated after about 8 weeks. Before being transferred on a regeneration medium, isolated G418-resistant calluses were cultured further on N6D solid media and in N6D liquid media containing 25 μM $AgNO_3$ for 2 weeks, respectively.

GUS assay

To determine the optimal incubation time for transient GUS assay of bombarded cells, GUS activity was examined 24, 48, 72 h, 1 week and 2 weeks after bombardment according to Jefferson (1987). A 300 μL solution containing 10 mM sodium phosphate (pH 7.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 10 mM EDTA, 0.1% Triton X-100 and 0.1% 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc) was sprayed onto the filter papers carrying cells, and the selected calluses were immersed in this solution. Incubations were for 2 h at 37℃, followed by incubations at 27℃. After 24-48 h, the number of blue cells was counted under a microscope. To make comparison of experiments possible, blue spot counts were estimated per g of fresh weight.

PCR assay

Genomic DNA was isolated using the procedure of Gordon-Kamm *et al.* (1990). 5'-CTACACCACGCCGAACACCT-3' (+527 from ATG) and 5'-CAGGCACAGCACATCAAAGA-3' (+1391 from ATG), representing sequences on the GUS gene, were used as primers (Omirulleh *et al.*, 1993). Genomic DNA (1 μg) was subjected to 30 cycles of amplification of three steps each (94℃, 1 min; 60℃, 2 min; 72℃, 3 min) in PCR buffer [10 mM Tris-HCl (pH 8.3),

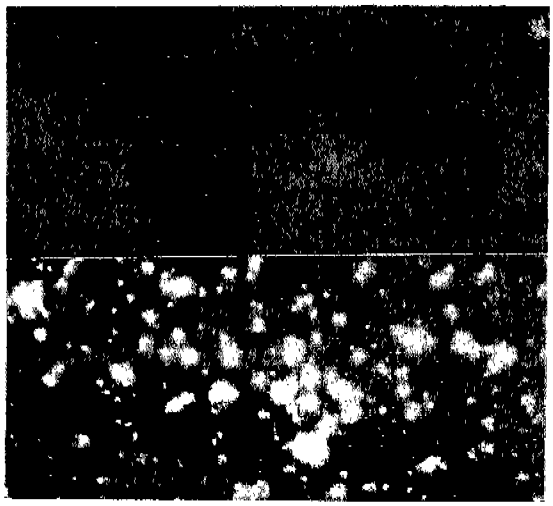

Fig. 2. Photographs of the DNA/tungsten mixtures stained with DAPI. A, Instead of $CaCl_2$ and spermidine, distilled water was added to the mixtures; B, The mixtures were treated with $CaCl_2$ and spermidine. Bar=100 μm.

50 mM KCl, 1.5 mM $MgCl_2$ and 100 μg/mL gelatin] containing 200 μM dNTPs, 1 μM each primer and 2.0 units of AmpliTaq (Perkin Elmer Cetus). Ten μL of PCR products were electrophoresed in a gel of 0.8% agarose.

Scanning electron microscopy

Immediately after particle bombardment, the cells were fixed with 5% glutaraldehyde in 50 mM phosphate buffer, pH 7.0. After 24 h, samples were fixed again with 1% osmium tetroxide in the same buffer for 24 h, dehydrated in ethanol and coated with gold (6 mA). Specimens were examined in an Akashi SX-40 instrument at 15 kV.

RESULTS AND DISCUSSION

DNA coating assay

A calcium-spermidine method (Klein *et al.*, 1987) that has been used widely for DNA coating on the surface of tungsten particles was utilized in this study. To examine whether plasmid DNA was coated on the surface of tungsten particles by addition of calcium and spermidine, we compared DNA/tungsten mixtures containing calcium and spermidine with those containing distilled water under a fluorescence microscope after staining with DAPI (Fig. 2). It was certified that fluorescence appeared around the particle in case of DNA/tungsten mixtures with calcium and spermidine (Fig. 2B) when compared to DNA /tungsten mixtures without both (Fig. 2A). This fact supports that DNA was coated on tungsten by calcium-spermidine method. Calcium ions have been considered to have a major role in the binding of DNA to the tungsten particles, and the addition of spermidine was recommended in the view of its involvement in the DNA binding mechanism (Vasil *et al.*, 1993).

Scanning electron microscopy of bombarded cells

The penetration of cell wall by accelerated tungsten particles was validated by the observation of the surface of cells collected immediately after particle bombardment. The number of the uptake of tungsten particles into narrow areas of about 10 μm length of cell surface varied from 1 to 7 (the number of diameter 1 μm pore which is equal to the size of a tungsten particle) according to the position of cells placed on a filter paper (Fig. 3B and 3C). However, no pore existed on the surface of the cells which were not bombarded (Fig. 3A). In this study, we did not examine whether the cells containing pores showed expression of the GUS gene or not.

Influences of osmotic treatment on transient expression

To determine the optimal concentration of osmotic treatment of target cells, we tested different concentrations of mannitol plus sorbitol in the bombardment medium. Embryogenic cells placed on a medium containing 0.5 M osmoticum (0.25 M sorbitol and 0.25 M mannitol) gave the highest number of blue spots (Table 1). Placement of cells on an osmoticum-containing medium before, during and after bombardment resulted in a statistically significant 20-fold increase in the transient GUS gene expression. Under this condition, an approximately 700 blue spots were obtained from g fresh weight of bombarded embryogenic cells. It is suggested that the osmotic enhancement of transient expression of GUS gene in rice cells was facilitated through plasmolysis

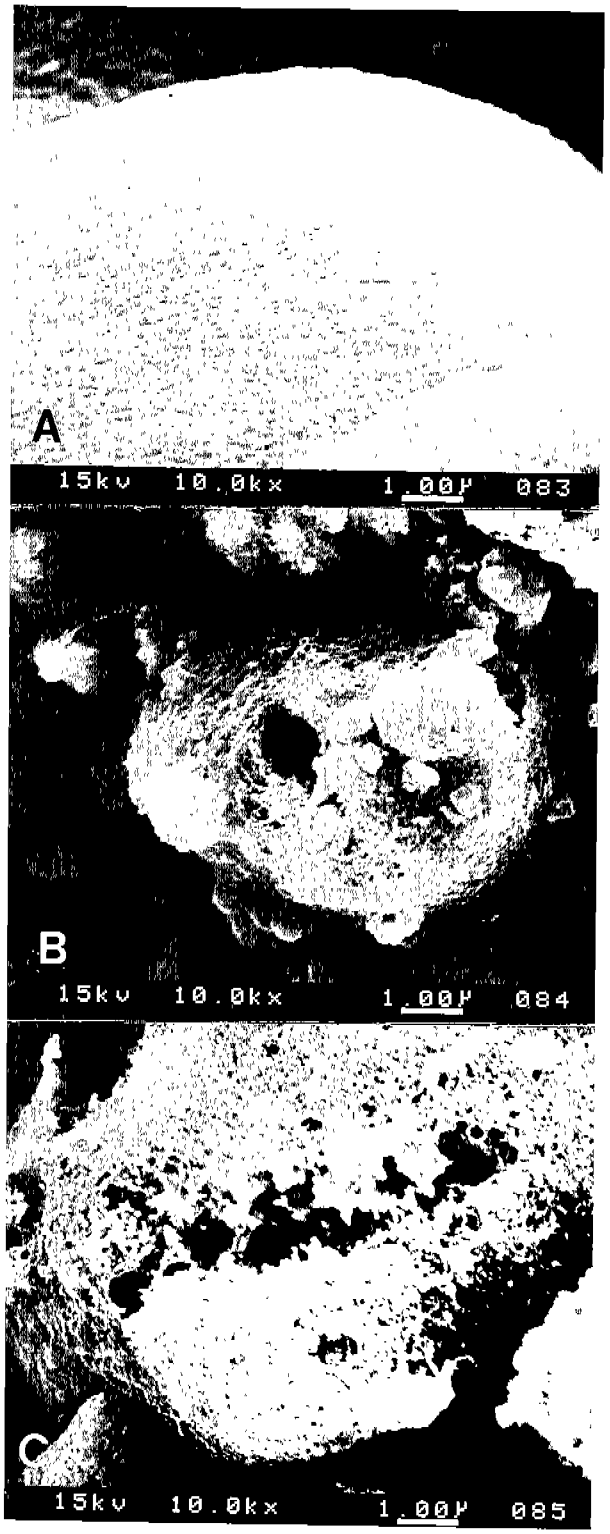

Fig. 3. Scanning electron micrographs of the surface of non-bombarded (A) and bombarded rice cells (B and C).

of the target cells. The plasmolysed cells may be less likely to extrude their protoplasm following the penetration of the cell wall by a particle (Russell *et al.*, 1992; Vain *et al.*, 1993b).

Yamashita *et al.* (1991) reported that the majority of GUS expressing cells received DNA-coated particles in their nucleus after bombardment. This fact suggests that the removal of the vacuole or the reduction of its volume in cells can increase the gene expression efficiency by the particle bombardment. Therefore we also surmise that the osmotic enhancement of GUS gene expression may be due to the reduction of vacuole volume in cells treated with high osmotic pressure.

Table 1. The effect of various concentrations of osmoticum on transient expression. The pAct1D and embryogenic cell suspensions of Seomjinbyeo were used in all experiments for optimization of particle bombardment. The number of blue spots is given as the mean number± standard error of the mean. The number of replicate experiments is given in parenthesis

Osmotic treatment	No. of blue spots/g fr wt
0 M	33 ± 12 (n=4)
0.25	423 ± 126
0.50	704 ± 297
0.75	274 ± 79
1.00	82 ± 14

Table 2. The effect of helium pressures and target distances on transient expression. The number of blue spots is given as the mean number± standard error of the mean. Means were based on two replicate experiments

Distance (mm)	Helium pressure (kPa) 275	410	550
110	149 ± 110	852 ± 474	440 ± 197*
160	25 ± 4	46 ± 22	107 ± 14

*No. of blue spots/g fr wt

Optimization of bombardment parameters

The effect of different helium pressures and bombardment distances were evaluated for transient expression of the GUS gene in rice cells. Generally, the transient expression of the GUS gene increased with increasing pressure (Table 2). However, at the highest pressure (550 kPa) and the closest distance (110 mm), transient expression of the GUS gene decreased because of the displacement of cells by helium burst. Hence, the particle bombardment experiments were performed at 410 kPa and 110 mm target distance

Table 3. The effect of two syringe filter holders on transient expression. The number of blue spots is given as the mean number± standard error of the mean. The number of replicate experiments is given in parenthesis

Description	No. of blue spots/g fr wt
13 mm Swinney filter holder	1043 ± 57 (n=4)
25 mm syringe filter holder	<3

Table 4. The effect of the volume of loaded mixtures on transient expression. The number of blue spots is given as the mean number± standard error of the mean. The number of replicate experiments is given in parenthesis

Volume of DNA/ tungsten mixture (μL)	No. of blue spots/ g fr wt
2	513 ± 101 (n=6)
5	1005 ± 481

Table 5. The effect of incubation times on the level of transient expression. The number of blue spots is given as the mean number± standard error of the mean. The number of replicate experiments is given in parenthesis

Interval between transformation and GUS assay	No. of blue spots/ g fr wt
24 h	1077 ± 481 (n=2)
48 h	1189 ± 493
72 h	330 ± 167
1 wk	86 ± 27
2 wk	12 ± 3

from syringe filter to cells.

The use of a baffle was indispensible for bombarding the embryogenic cells. The baffle reduced not only the dislodgement of the cells, but also cell damage by helium burst.

A comparison between the 13 mm and 25 mm plastic syringe filter units gave very significant differences in the number of blue spots obtained per g fresh weight. The use of 13 mm Swinney filter holder increased about 350-fold for transient expression when compared to 25 mm filter holder (Table 3). It is suggested that this result is due to a different velocity of particles in the outlet of between two syringe filter holder. We believe that in the outlet of 13 mm filter holder, velocity of the DNA-coated particle is higher than that in the outlet of 25 mm filter holder.

Two different volumes of the DNA/particle mixtures for each loading were tested. Results of the experiment are shown in Table 4. The higher volume of the mixture for each bombardment showed 2-fold increase. In subsequent experiments, 5 μL of the DNA/particle mixtures were used.

Table 6. The effect of different DNA constructs on the transient expression of GUS gene in the two embryogenic cell lines. The number of blue spots is given as the mean number± standard error of the mean. Means were based on four replicate experiments

Cell line	pAct1D	pLS201	pBI121
Seomjinbyeo	1104 ± 557	315 ± 107	30 ± 19*
Dobongbyeo	1159 ± 173	260 ± 40	48 ± 14

*No. of blue spots/g fr wt

To determine the optimal incubation time for transient GUS assay of bombarded cells, the number of blue spots was examined at the different time intervals between bombardment and histochemical GUS assay after the particle bombardment (Table 5). Already 24 h after bombardment, the number of blue spots was about the same as the number of blue spots after 48 h. The low number of blue spots observed after two weeks probably represents stably transformed cell clusters or is related to the persistant transient expression in the limited number of cells. In all experiments the histochemical assay was performed 48 h after bombardment.

Effect of different promoter-sequences on transient expression

To determine the strongest promoter-sequences, we tested the effect of three constructs containing different promoter-sequences fused with GUS gene. The blue spots expressing GUS gene were observed in all the constructs (Table 6). The number of blue spots was greater than 1100 with rice actin promoter-intron construct (pAct1D) and as low as 40 with CaMV 35S promoter alone (pBI121). This result agrees with that previously published in barley cells (Chibbar *et al.*, 1993).

Another interesting observation was that the apparent size of the blue spots was much larger with rice actin promoter-intron fusion as compared to other constructions (Fig. 4A, B, C and D). These results support the highest GUS activity of the construct containing rice actin promoter-intron fusion among three constructs, suggesting that the rice *Act*1

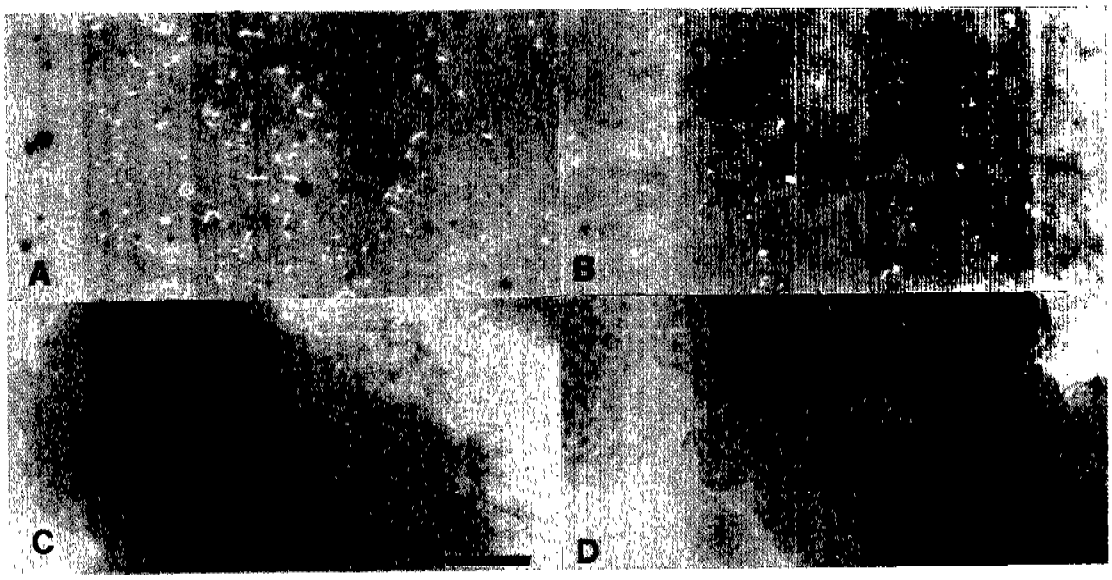

Fig. 4. Transient GUS gene expression in cell suspensions after particle bombardment with pAct1D (A and C) and pLS201 (B and D). Bar=100 μm (A and B) and 50 μm (C and D).

5' region contains an efficient promoter for use in rice transformation.

In addition to the above fact, a construct (pLS201), inserting a deleted *Adh*1 intron 1 between CaMV 35S promoter and GUS gene with gave about 300 blue spots and increased 7- to 8-fold in the level of GUS gene expression when compared to the construct containing GUS gene alone (pBI121). This result agrees with that published in transgenic potatoes (Lee and Sung, 1992). In this study, we confirmed that pLS201 containing CaMV 35S promoter with a deleted *Adh*1 intron 1 directed high levels of GUS gene expression in transformed cells of monocots as well as those of dicots. Now, it is suggested that higher GUS activity in rice cells transformed with pLS201 may be due to the increase of translational ability. pLS201 has an efficient translation initiation codon (ACTATG) for eukaryotic gene but pBI121 has bacterial ATG codon of GUS gene (CTTATG) [See Lee and Sung (1992) for sequences]. This speculation is supported on the ground of facts that an efficient eukaryotic consensus sequence at the ATG initiation codon consisted of purine nucleotides at -3 (Kozak, 1984) and the sequence context surrounding the translation initiation codon affected remarkably on transgene expression in transformed plant cells (Kato *et al.*, 1991; McElroy *et al.*, 1991).

We also examined the extent of GUS gene expression in two rice cultivars, Seomjinbyeo and Dobongbyeo (Table 6). No significant differences in bombardment efficiency between two cultivars were shown, suggesting that the differences of physiological state of embryogenic cell suspension do not exist between these cultivars. The packed cell volumes of two embryogenic cell lines increased 4 to 5-fold for a week (data not shown).

Selection of stably transformed callus

We bombarded with pLS201 containing GUS gene as a reporter and NPTII gene as a selection marker for a stable transformation. The conditions that gave the highest levels of transient expression were used for stable transformation. After 8 weeks on a N6D medium containing 100 mg/L G418, approximately three independent G418-resistant cell lines were selected from one filter paper carrying bombarded cells (Fig. 5A). Except the selected calluses, all the other calluses failed to grow. Being further cultured on the selection medium for 2 weeks, G418-resistant calluses were grown actively when compared to untransformed calluses (Fig. 5B). Antibiotics G418 (100 mg/L) inhibited entirely growth of control calluses. Transformed calluses transferred on a N6D medium containing $AgNO_3$ proliferated rapidly and showed typical examples of embryogenic calluses (Fig. 5C).

In this study, three stably transformed embryogenic clones from about 300 blue spots per g fresh

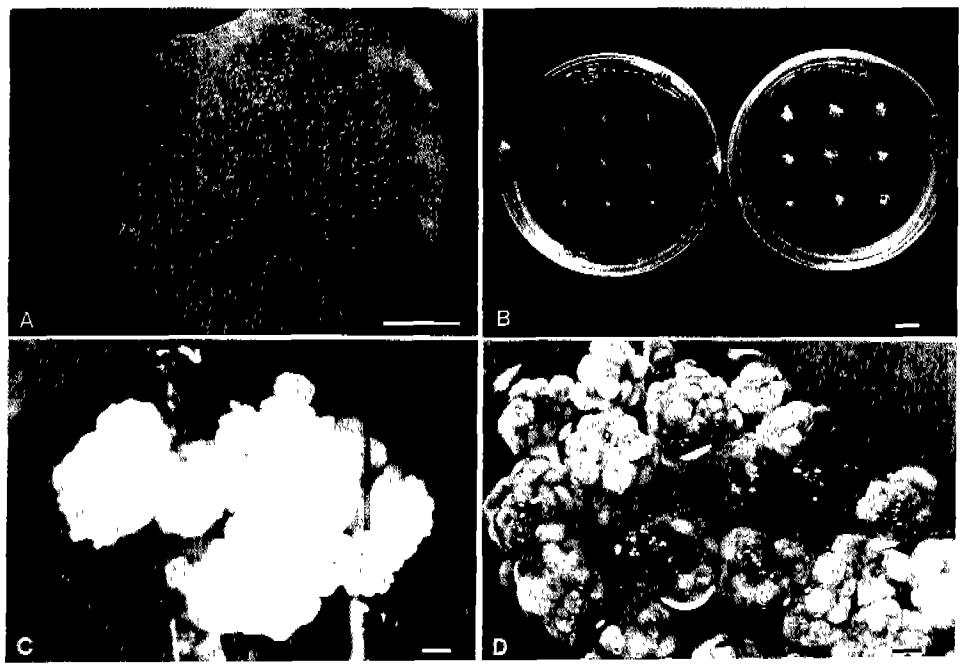

Fig. 5. A, The G418-resistant calluses grown on a selection medium. G418-resistant calluses (arrows) were obtained 8 weeks after transferring on a selection medium containing G418. B, Transformed calluses (right) and untransformed control calluses (left) 2 weeks after transferring on a N6D medium containing 100 mg/mL G418. Transformd calluses grew actively but untransformed calluses did not.; C, Proliferation of transformed embryogenic calluses on a N6D medium containing 25 μM $AgNO_3$; D, GUS staining of transformed embryogenic calluses which were maintained in N6D liquid media containing 25 μM $AgNO_3$ for 2 weeks. Bar=10 mm (A and B) and 1 mm (C and D).

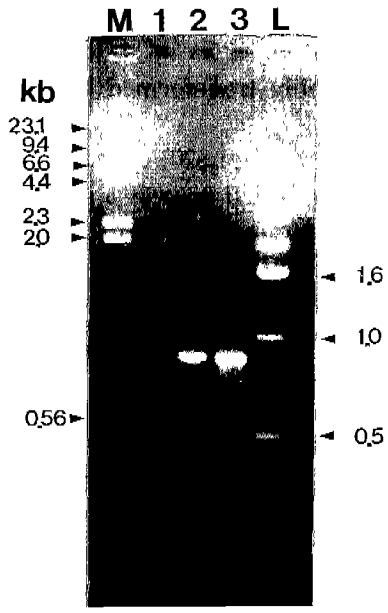

Fig. 6. Agarose gel electrophoresis of PCR-amplified DNA of untransformed and transformed calluses. Lane M, lambda DNA digested with *Hin*dIII; Lane 1, DNA from untransformed callus; Lane 2, DNA from transformed callus; Lane 3, pLS201; Lane L, 1 kb DNA ladder.

weight of bombarded cells were recovered. The transient-to-stable conversion frequency was about 1% and was a few high when compared to the frequency obtained in maize (Vain *et al.*, 1993b). This result suggests that our protocols, optimized in this study, for particle bombardment can be used for subsequent experiments related to rice transformation.

In histochemical GUS assay, X-gluc staining was detected by blue coloration within 30 minutes in most of the transformed calluses (Fig. 5D). Many calluses stained entirely with an intense blue color except for some calluses containing both a white and a blue staining. The latters may be due to the discrepancy of penetration of substrates into cells or originate from cases in which non-transgenic cells survived due to detoxification of G418 by the adjacent transformed cells.

The presence of GUS gene in the genomic DNA from transformed calluses was detected by PCR amplification of the 864 bp fragment, by using primers based on sequences from the coding region of the GUS gene (Fig. 6). This result indicates that G418-resistant calluses are true transformed calluses.

In conclusion, we have established a particle bombardment-mediated transformation system of rice embryogenic cell suspension using a simple, inexpensive and homemade particle inflow gun. This result will open up opportunities for transferring of agronomically beneficial genes into rice. Now, stably transformed calluses are underway to generate transgenic rice plants.

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粒子射出技法에 의한 벼 細胞로의 外來 遺傳子 導入과 그 發現

田鍾聲 · 鄭昊城 · 成淳基 · 李鍾燮[1] · 崔良熹[2] · 金翰集[3] · 李光雄*

서울大學校 自然科學大學 生物學科, [1]分子生物學科, [2]農業生命科學大學 農化學科

[3]亞洲大學校 理科大學 生命科學科

적 요

유용한 유전자가 도입된 벼의 창출을 위하여 입자사출장치를 이용한 형질전환체계를 확립하였다. 입자사출장치는 DNA가 묻혀진 텅스텐 입자를 압축된 헬륨가스의 힘에 의해 가속화시키도록 제작되었다. 제작된 입자사출장치를 이용하여 GUS의 조직화학적 분석을 통하여 형질전환에 영향을 주는 여러 요인들을 최적화하였다. 입자사출된 세포에서 GUS 유전자의 가장 높은 발현(생체중량당 약 1000 blue spots)은 세포의 삼투(0.5 M) 처리, 410 kPa 헬륨압력, 110 mm의 입자사출거리, 13 mm 필터장치 및 5 μL의 DNA/텅스텐 혼합물을 이용하는 조건에서 관찰되었다. 일시적 발현에 미치는 프로모터 부위의 효과 분석을 통하여 벼 액틴 프로모터-인트론이 가장 강한 GUS의 발현을 유도함을 밝혔다. 100 mg/L G418이 첨가된 선별배지에서 8주 후 안정적으로 형질전환된 벼 캘러스는 유도되었으며, 이들은 높은 GUS 활성을 보였다.

주요어 : 입자사출기법, GUS, 형질전환, 벼

*교신저자 : Fax (02) 872-6881