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Expression of β-Glucuronidase (GUS) Gene in Transgenic Lettuce (*Lactuca sativa* L.) and Its Progeny Analysis

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형질전환된 상추내에서 GUS 유전자의 발현 및 후대검정

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Agrobacterium tumefaciens LBA 4404 harboring binary vector pBI 121 was used for genetic transformation of lettuce (*Lactuca Sativa* L.). Optimal shoot regeneration from cotyledon explants was obtained in MS medium supplemented with 0.1 mg/L NAA and 1.0 mg/L 2ip. In this condition, cotyledon explants were cocultivated with *A. tumefaciens* for 2 days, and then transferred to selection medium supplemented with 50 mg/L kanamycin and 500 mg/L carbenicillin. These explants were subsequently subcultured every 2 weeks on shoot induction medium. PCR analysis indicated that the GUS gene was stably integrated into the nuclear genome of lettuce. Histochemical analysis based on the enzymatic activity of the GUS protein showed that GUS activity was associated with vascular tissue in leaves and roots. Progenies of Ro plants demonstrated a linked monogenic segregation for GUS gene.

Key words ; cotyledon explants, genetic transformation, GUS activity

Lettuce, *Lactuca sativa* L., is one of the major salad vegetables in the world (Anonymous, 1992) and also the demand for lettuce has increased annually in Korea. Conventional breeding methods have required a long time and a large amount expenses to breed a useful variety, however, the recently developed genetic engineering techniques by *Agrobacterium*-mediated DNA transfer (Horsch et al., 1984) and direct DNA transfer (Riggs and Bates, 1986) have facilitated the transfer of foreign genes into plants. *Agrobacterium*-mediated transformation has been proved to be a useful genetic engineering techniques to produce valuable plants in agronomic and scientific area. In order to induce genetic modification via *Agrobacterium* successfully, both a reproducible regeneration system and an effective transformation system are essential.

Up to now, β-glucuronidase (GUS) gene has been transferred into lettuce (Torres et al., 1993; Enomoto et al., 1996) and transgenic plants harboring that gene have been obtained. Choi et al., (1994) also succeeded in the production of transgenic plant with GUS gene, but they did not showed frequency of shoot regeneration, histochemical analysis and progeny test of R0 transgenic plants.

The reports will be presented more advanced methodology for genetic transformation of lettuce via cocultivation of cotyledon with *Agrobacterium* vector, histochemical analysis of R0 transgenic plants, and progeny test of R0 plants.

MATERIALS AND METHODS

Plant Materials and *Agrobacterium* Vector

Seeds of *Lactuca sativa* were soaked in 70% ethanol for 2 minutes, then surface-sterilized in sodium hypochlorite solution containing 0.1% Tween 20 for 20 minutes · Finally, the seeds were washed with sterile distilled water more than tree times, and then transferred to hormone free MS (Murashige and Skoog 1962) medium under the 16h photoperiod at a light intensity of 2,000 lux. After 4 days the cotyledons were excised about 2~3 mm in length above the cotyledonary node. *Agrobacterium tumefaciens* strain LBA4404 containing a binary vector pBI121 (Jefferson, 1987) was used. The binary vector, carries the β-glucuronidase (GUS) gene fused to a CaMV 35S promoter, and also specifies kanamycin resistance. *Agrobacterium* was grown overnight in LB medium (1% Bacto-peptone, 0.5% Bacto-yeast extract, 1% NaCl) supplemented with kanamycin (Km 50 mg/L) at 28°C in the dark.

Plant Regeneration and Transformation

To investigate the optimal shoot regeneration, cotyledons were cultured in a MS solidified medium supplemented with 0.1 mg/L NAA and 1.0, 2.0, 3.0 mg/L 2ip and kinetin. Cotyledons were dipped into the bacterial suspension (A_{600}=0.7) for 10 minutes, and blotted with sterile filter paper and cocultivated for either 24, 48, or 72 hours on MS medium supplemented with 0.1 mg/L NAA, 1.0 mg/L 2ip, 3% sucrose, and 0.2% Gelrite. After cocultivation, the cotyledons were transferred to MS selection medium added with 50 mg/L Km. and 500 mg/L carbenicillin.

In order to induce roots, shoots formed explants on selection medium were moved to hormone free MS medium containing with 25 mg/L Km. and 250 mg/L carbenicillin. After roots formation from shoot, the plants were transferred to potting soil in greenhouse.

PCR Analysis

The 5′primer (5′-AACTGGACAAGGCACTAGCGG-3′) and 3′primer (3′-TGCCACCTGACCGTACTTGAA-5′) which correspond to position 974 to 994 and 2054 to 2074 of the GUS gene, were used in amplification (Jefferson et al., 1986). PCR reaction was carried out with 1 unit Tag polymerase, 0.1 mM dNTP mixture, 5 pM primer, and 50 ng genomic DNA. PCR was performed in a thermal cycler for 35 cycles. The PCR reaction, which consisted of heat denaturation (94°C, 45 second), annealing (55°C, 30 second), extension (72°C, 10 second) and post elongation (72°C, 4 minute) were carried out, and the reaction mixtures were subjected to agarose gel electrophoresis.

Histochemical Assays

Histochemical staining was performed according to the method described by Jefferson (1987). Leaf and root tissues of putative transformants were hand-sectioned with a sharp razor blade, 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 incubated in a staining solution formulated with 1 mM of 5-bromo- 4-chloro-3-indolylgulcuronide (X-Glu) and 50 mM phosphate (pH 7.0) at 37°C overnight.

RESULTS AND DISCUSSION

Before attempting transformation experiments in lettuce, the cotyledons were screened for their shoot regeneration ability. Therefore, cotyledons of lettuce were cultured on MS medium containing different concentration of NAA, 2ip, and kinetin.

As shown in Table 1, the combination of 0.1 mg/L NAA and 1.0 mg/L 2ip produced the highest frequency of shoot formation (56.7%). The combination of NAA and 2ip was better with than that of NAA and kinetin for shoot

Table 1. Effect of plant growth regulations on callus and shoot formation of cotyledon cultures of *Lactuca stativa* L.

Plant growth regulators			No. of cultured cotyledon	Shoot formation(%)
NAA	2ip	kinetin		
0.1	1.0	-	90	51(56.7)
0.1	2.0	-	90	47(52.2)
0.1	3.0	-	81	15(18.5)
0.1	-	1.0	90	21(23.3)
0.1	-	3.0	81	10(12.3)

formation. But Choi et al., (1994) have reported that the combination of NAA and kinetin was more effective for shoot regeneration. It may be attribute to different genotype of donor plants tested.

The effects of kanamycin on shoot formation from lettuce cotyledons were examined. At 50 mg/L kanamycin, green color cotyledon turned white and died within 30 days in

culture. Michelmore et al., (1987) and Torres et al., (1993) also reported that kanamycin concentrations above 25 mg/L inhibited callus formation. Therefore, kanamycin at 50 mg/L was chosen for selection of transformants (Table 2). The NPT II gene has proven useful as a selectable marker in many plant species (Fraley et al., 1986). But kanamycin resistance of doner plant was different in variety of tissue and *Agrobacterium* species (Raffaela et al., 1988: Sheerman and Bevan, 1987). Futhermore, transformation frequency in same phenotype was influenced by kanamycin concentration added to selection medium (Radke et al., 1988).

Table 2. Effect of kanamycin concentrations on shoot formation of cotyledon cultures of *Lactuca sativa* L.

Kanamycin concentration(mg/L)	Shoot formation (%)
0	66.7
10	50.0
25	33.3
50	0
100	0

The period of cocultivation has been reported to affect the frequency of transformation rate in lettuce (Michemore et al., 1987: Enomoto et al., 1990) and other plant species (Fillati et al., 1987: Chabaud et al., 1988). The maxium frequency of green shoot regeneration (34.2%) was obtained from the cotyledons which had been cocultured with *Agrobacterium* for 48 hours (Figure 1). Prolonged cocultivation for 72 hours had an inhibitory effect on shoot formation in the selection medium.

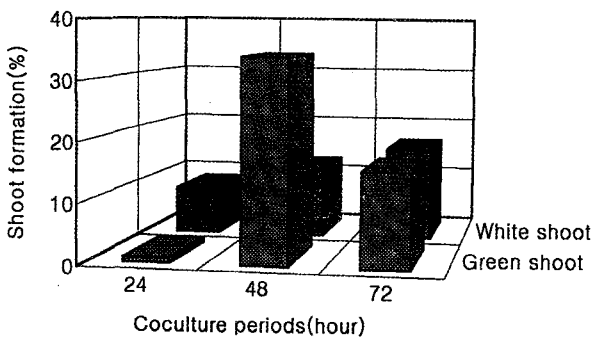

Figure 1. Effect of cocultivation periods on shoot formation from cotyledon cultures of lettuce.

The green shoots regenerated on medium with antibiotics were transplanted into potting soil. The presence of GUS sequences was confirmed by PCR analysis. As shown in Figure 2, the DNA fragments of 1.2 kb size were identified in transgenic plants, whereas there was no detectable GUS DNA fragment in nontransgenic plant. This PCR analysis was

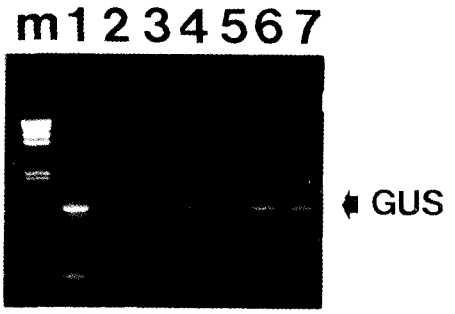

Figure 2. Agarose gel electrophoresis of PCR amplification products. m: marker, lane 1: amplified product from plasmid pBI 121, lane 2: amplified product from genomic DNA of nontransformed plant, lanes 3, 4, 5, 6 and 7 amplified product from genomic DNA of transformed plants. The arrow indicates approximately 1.2kb of amplified GUS product.

suggested that GUS gene was integrated into the genome of lettuce. The GUS specific activity of plants was determined by histochemical assay. Leaf, stem, and root of transgenic lettuce (R0) were stained intensely blue when incubated with the X-Glu substrate, while the control and non transformed tissue were not stained (Figure 3). Torres et al.(1993) also observed expression activity of GUS in different tissues of transgenic lettuces.

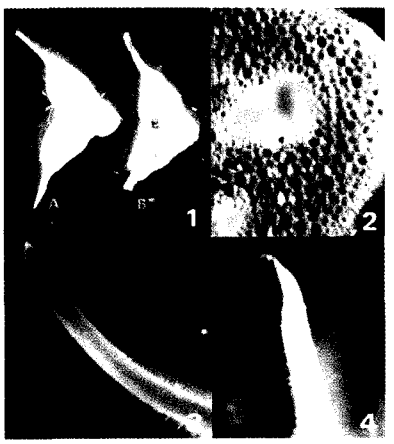

Figure 3. GUS activity in various explants of transformed lettuce. Explants were incubated in X-Glu for 24h at 37°C. 1: Cross section of leaves of nontransgenic (A) and transgenic (B) plant, 2: Cross section of stem, 3: Root of transgenic plant, 4:Root of nontransgenic plant.

Table 3. Segregation of GUS staining in R1 seeds derived from self-fertilization of two independent transgenic plants

R1 plant	Segregation of GUS[a]		X^2 values (p=0.05)
	GUS^+	GUS^-	3:1
R1-1	52	14	0.39
R1-2	48	17	0.12

[a]GUS^+ = GUS positive: GUS^- = GUS negative

To test whether the expression of the GUS gene can be stably maintained in the next generation, GUS activity was examined in R1 progeny of R0 plants using a histochemical assay (Figure 4). R1 seeds having GUS gene were stained prominently blue. The segregation patterns in R1 seeds suggested that inheritance of GUS activity was identified as a single dominant gene. The segregation data from the progenies of two R1 plants are presented in Table 3.

Some studies have supported the idea that foreign genes are inherited in a normal Mendelian pattern (Budar er al. 1986). The number of copies of a foreign gene inserted into the plant cell genome by *Agrobacterium*-mediated transformation can vary from single to multiple inserts. Several recent reports show that there is variabillity in the transmission of foreign genes. Also, the expression of foreign genes has been linked to the presence of extra copies inserted into the genome, methylation, environmental factors, gene interaction, and gene loss (Hobbs et al., 1990: Kilby et al., 1992: Meyer et al., 1992). Genetic analysis of 44 transgenic tobacco plants by Budar et al. (1986) indicated normal Mendelian inheritance of the neomycin phosphotransferase Ⅱ (NPT Ⅱ) gene in 35 plants that had a single gene insert. However, 4 of the plants produced progeny that expected Mendelian ratios did not be showed, and 5 others segregated as expected for two gene copies. In this experiments, our data showed that the transgene GUS inherited in a normal Mendelian pattern in the next generation

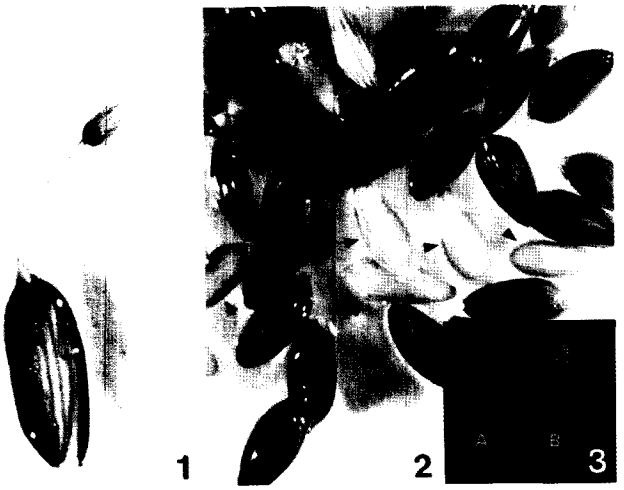

Figure 4. Expression of the GUS gene in transformed R1 seeds. 1: a filament of transgenic R0 plant 2: Segregation patterns of transformed R1 seeds, 3: A transformed R1 seed (A) and a nontransformed R1 seed (B) treated with X-Glu for 24h. Arrows indicate GUS-negative R1 seeds.

적 요

상추의 종자 무균발아후 4일된 자엽조직을 GUS 유전자가 도입된 *A. tumefaciens* LBA 4404와 2일간 공동배양한 다음 0.1 mg/L NAA, 1.0 mg/L 2ip, 50 mg/L kanamycin, 500 mg/L carbenicillin이 첨가된 MS 배지에 배양하여 식물체를 재분화시켰다. PCR 분석결과 GUS 유전자가 형질전환된 식물체의 게놈상에 삽입되어 있음을 확인하였다. 해부학적 GUS 활성을 분석하여 형질전환된 식물체의 줄기, 잎 그리고 뿌리에서 GUS 유전자의 발현을 확인하였다. 형질전환체로 확인된 식물체를 자가수정시켜 얻어진 종자의 GUS 활성을 분석하여 GUS 유전자가 발현되는 것을 확인하였다.

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