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Transformation of cucumber (*Cucumis sativus* L.) plants using *Agrobacterium tumefaciens* and regeneration from hypocotyl explants

Soryu Nishibayashi [1,*], Hiroyasu Kaneko [2], and Takahiko Hayakawa [1]

[1] Plantech Research Institute, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227, Japan
[2] Saitama Genshu Ikuseikai, Shobu-machi, Minami Saitama-gun, Japan
* *Present address:* Mitsubishi Chemical Corp., Agricultural Chemicals Laboratory, Yokohama Research Center, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227, Japan

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Summary. Transgenic cucumber (*Cucumis sativus* L.) plants were successfully obtained from hypocotyl explants inoculated with *Agrobacterium tumefaciens*, which harbored a binary vector plasmid with NOS-*nptII*, CaMV 35S-I-*gus* and CaMV 35S-*hph* genes. Acetosyringone enhanced the efficiency of transformation at the cut surface cells of hypocotyl explants during five days of co-cultivation. Transformed cells were more effectively selected using 20-30 mg/l hygromycin B than using 50-100 mg/l kanamycin. Shoot regeneration occurred within 4-6 wks, and 12 of 21 regenerated plantlets displayed strong GUS expression in the very young leaves. All of 8 GUS-positive R0 plants examined showed single or a few positive bands by Southern blot analysis. The expression of the CaMV 35S-I-*gus* gene was observed in various tissues and organs of R0 and R1 transgenic cucumber plants.

Introduction

Cucumber (*Cucumis sativus* L.) is one of the major vegetable crops in America, Asian, European countries. Recent advances in plant biotechnology have enabled the introduction of various foreign genes into cells and plants by regeneration from them. Using the transformation technology, single dominant agronomical characteristics such as insect resistance and virus resistance (Beachy et al. 1990; Lal and Lal 1993) can be introduced into crops in a short time without changing desirable characteristics, although the introduction of desirable genes by conventional plant breeding techniques requires much time and is often accompanied by changes in other desirable characteristics.

The transformation of cucumbers was first achieved by Trulson et al. (1986), and then the transgenic cucumber plants were regenerated from roots that were induced by the inoculation of inverted hypocotyl explants with *Agrobacterium rhizogenes*, which harbored a binary vector plasmid with a NOS-*nptII* gene. Hence, several transgenic plants were obtained. Chee (1990) co-cultivated cucumber cotyledonary explants with *Agrobacterium tumefaciens*, which harbored a binary vector plasmid with a NOS-*nptII* gene and obtained many transgenic plants by the regeneration via somatic embryogenesis from the cotyledonary explants. Sarmento et al. (1992) also used *Agrobacterium tumefaciens*, which harbored various binary vector plasmids with a CaMV 35S-*nptII* gene and achieved the transformation of cucumbers with the use of the regeneration from the petiole and leaf explants, and several transgenic plants were obtained. In the previous reports, only a *nptII* gene was used as a selectable marker gene, and then the transgenic cucumber plants were regenerated from the tissue culture by the cell selection of kanamycin.

We found out the regeneration from the hypocotyl explants in one cultivar of cucumber (*Cucumis sativus* L.) and established an *Agrobacterium*-mediated-transformation system with the use of the regeneration from the hypocotyl explants and *Agrobacterium tumefaciens*, which harbored a binary vector plasmid with NOS-*nptII*, CaMV 35S-I-*gus* and CaMV 35S-*hph* genes. We then found out the enhancement of transformation efficiency at the cut surface cells using acetosyringone treatment during co-cultivation and the effective cell selection using hygromycin B. We also analyzed the GUS expression at various tissues and organs of the transgenic cucumber plants.

Material and methods

Plant material. A pure line 1021 of cucumber cultivar (*Cucumis sativus* L.) was used as the experimental material. The seed coats were removed. The peeled seeds were sterilized in 1.2% (v/v) sodium hypochlorite solution for 10-15 min, then rinsed several times with sterile distilled water, and placed on hormone-free MS medium (pH 5.8) for 7-10 days. All media which were used in the transformation experiments including the media described below were supplemented with 3% sucrose and solidified with 0.2% gellite. All media were autoclaved at 120 °C for 15 min. The culture conditions in all media were constant at 23 °C under a 16 h (light)-8 h (dark) photoperiod in a culture room.

Correspondence to: S. Nishibayashi

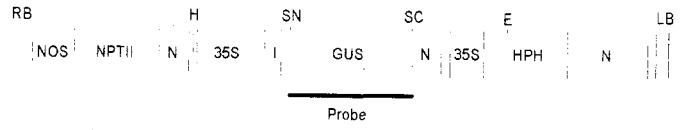

Fig. 1. Schematic representation of T-DNA region of a binary vector plasmid pIG121-Hm used in the transformation of cucumber plants. 35S: CaMV 35S promoter; E: EcoR1; GUS: β-glucronidase gene; H: HindIII; HPH: hygromycin phosphotransferase gene; I: first intron of catalase from castor bean; N: nopaline synthase polyadenylation site; NOS: nopaline synthase promoter; NPTII: neomycin phosphotransferase II gene; SC: SacI; SN: SnaBI; LB: left border; RB: right border.

Agrobacterium strain and vector plasmid. We used the EHA101 strain of *A. tumefaciens* (Hood et al. 1986) harboring a binary plasmid pIG121-Hm which is a derivative of pBI121 (Jefferson et al. 1987). The pIG121-Hm (Fig. 1) has the neomycin phosphotransferase II gene (*nptII*), hygromycin phosphotransferase (*hph*) gene and the β-glucronidase (*gus*) gene (Akama et al. 1992). The first intron sequence of the catalase gene of castor bean (Ohta et al. 1990) is inserted between the CaMV 35S promoter and the β-glucronidase gene.

Inoculation and co-cultivation. The *Agrobacterium* strain was grown in YEB medium containing 50 mg/l kanamycin, 20 mg/l hygromycin B and 25 mg/l chloramphenicol at 23 °C for 3-7 days. The suspension of the *Agrobacterium* strain was diluted to 1/100 concentration with a liquid MS medium. Segments of hypocotyls of seedlings cut with a razor blade were inoculated with the 1/100 diluted bacterial suspension, blotted on filter papers, then transferred to a solidified MS co-cultivation medium (pH 5.2) supplemented with 4 mg/l indole-3-acetic acid (IAA), 1 mg/l N6-(2-isopentyl)adenine (2iP) and 50 or 100 μM 3', 5'-dimethoxy-4'-hydroxyacetophenone (Aldrich) of a derivative of acetosyringone , and cultured for 5 days.

Cell selection and plant regeneration. The co-cultivated hypocotyl explants were transferred to the solidified MS regeneration medium (pH 5.8) supplemented with 1.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.23 mg/l 6-benzylaminopurine (BAP), 1% casein hydrolysate, 500 mg/l carbenicillin, 50 and 100 mg/l kanamycin or 20, 25 and 30 mg/l hygromycin B and cultured for 4-6 wks. The tissues were transferred to solidified hormone-free MS medium supplemented with 500 mg/l carbenicillin and cultured for 2-3 months. The medium was changed, once or twice. Regenerated plantlets were transferred to the hormone-free MS medium supplemented with 500 mg/l carbenicillin in Plantaboxes and then gradually acclimated for 1-2 months. The acclimated plants were transplanted into pots and grown in a closed greenhouse.

Histochemical analysis. Leaves and cotyledons were cut into thin sections with a razor blade, and male and female flowers were cut in two halves. The tissue sections and calli were stained with X-glucronide solution (X-gluc) at 37 °C for 1-18 hr, according to Jefferson et al. (1987). After staining, the chlorophyll of the leaves and cotyledons or the pigment of the flowers was removed with 70% ethanol at room temperature overnight for detailed observations of tissue sections.

GUS enzyme assays. Assay of β-glucronidase (GUS) activity of crude cell extracts from the leaves was done according to Jefferson et al. (1987), using 4 methyl umbelliferyl glucronide (MUG) as the substrate.

Southern blot analysis. Southern blot analysis was performed by methods supplied by Amersham. DNAs were prepared from the leaves of GUS-positive cucumber plants, according to Richards (1987). Twenty five μg samples of DNAs digested with a combination of HindIII and SacI or EcoRI alone were separated on 1% agarose gels. After denaturation, the DNAs on the agarose gels were blotted onto a nylon membrane (Hybond-N, Amersham) under alkaline conditions, using a vacuum blotting system (Vacugene, LKB-Pharmacia). An SnaBI-SacI fragment (Fig. 1) containig the ORF of the GUS gene (1.4 kb) was labelled with ^{32}P-dCTP (111 TBeq/mol) by a multiprime labelling kit (Amersham) and used as a probe for hybridization.

Results and Discussion

Effect of acetosyringone on transformation

The effect of transformation using the acetosyringone treatment during co-cultivation was investigated. Each 48 hypocotyl explants was co-cultivated without and with 50 and 100 μM acetosyringone for 5 days. Immediately after co-cultivation, each 16 of the 48 explants without and with the acetosyringone treatments was treated with X-gluc. Without acetosyringone, the GUS expression was not observed at the cut surface cells of all of the 16 hypocotyl explants (Fig. 2A). On the contrary, with the acetosyringone treatments, all of the 16 hypocotyl explants displayed strong GUS expressions at the cut surface cells (Fig. 2B). The same result was obtained in another experiment under the same conditions. These results show that acetosyringone treatment during co-cultivation enhances the efficiency of transformation at the cut surface cells of hypocotyl explants.

Further, each 100 explants was co-cultivated without and with 100 μM acetosyringone for 2 and 5 days and transferred to the regeneration medium containing 20 mg/l hygromycin B for 2 wks. In the treatment without acetosyringone, the frequency of callused hypocotyl explants which indicated hygromycin B resistance was 4% in the 2-day-co-cultivation and 88% in the 5-day-co-cultivation. In the treatment with acetosyringone, the frequency of callused hypocotyl explants was 60% in the 2-day-co-cultivation and 100% in the 5-day-co-cultivation. In both treatments, the size (3-6 mm) of the callused hypocotyl explants in the 5-day-co-cultivation was

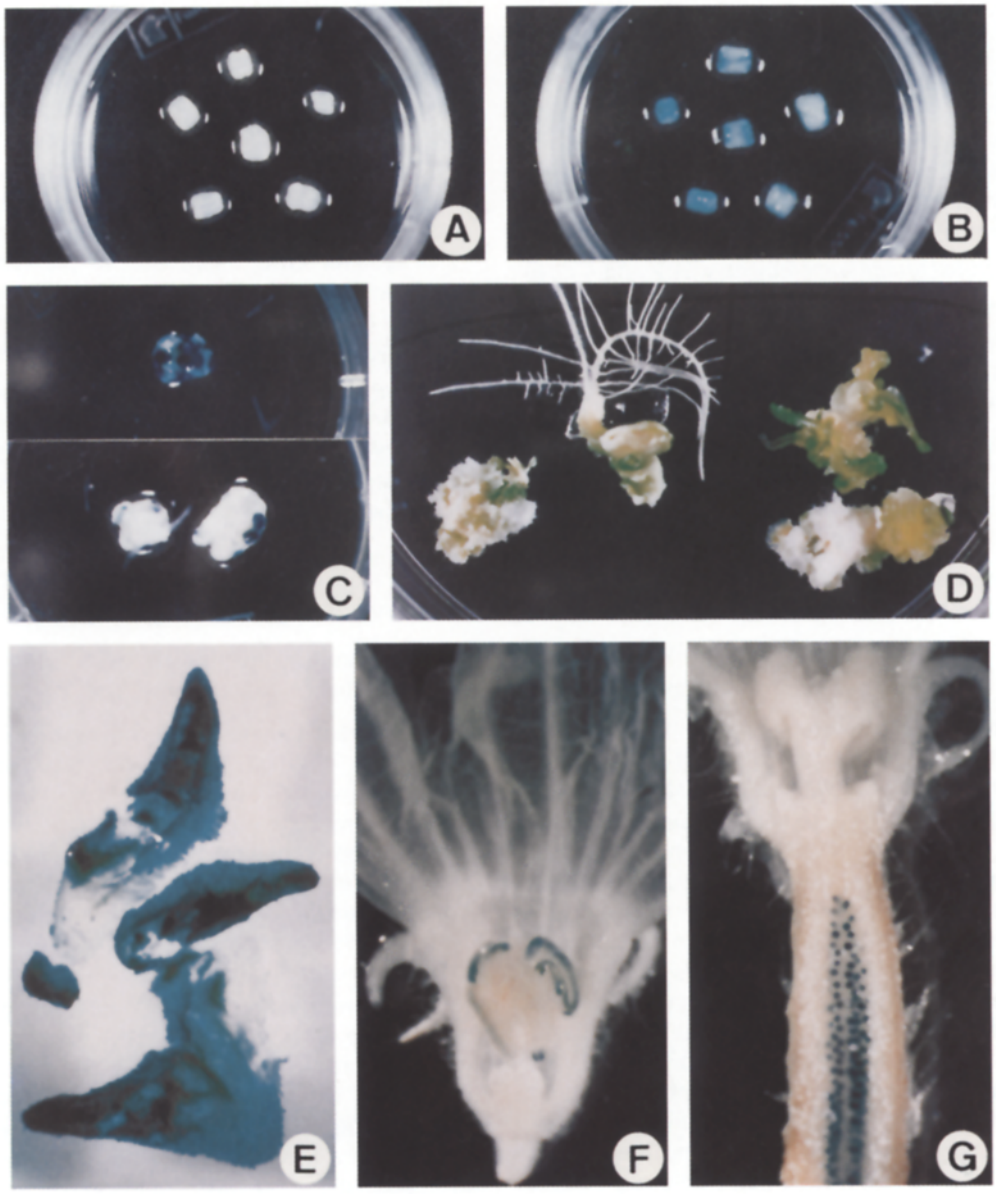

Fig. 2. GUS expression at different stages of culture and in different tissues and organs of R_0 transgenic plants in cucumber (*Cucumis sativus* L.). A: Non-detectable GUS expression in hypocotyl explants immediately after co-cultivation without acetosyringone. B: Strong GUS expression in the cells on cut surface of hypocotyl explants immediately after co-cultivation with 100 μM acetosyringone. C: Callus derived from hypocotyl explant about 2 wks after transferring it to regeneration medium with 100 mg/l km (lower) and 20 mg/l hygromycin B (upper). Transformed cells are effectively selected by hygromycin B. D: Regeneration from calli about 2 wks after transferring it to hormone-free MS medium. E: Strong GUS expression in young leaves of a regenerated plantlet. F: Strong GUS expression in anthers of a male flower of a transgenic cucumber plant. The male flower was immersed into 70% ethanol overnight. G: Strong GUS expression in ovules of a female flower of a transgenic cucumber plant.

larger in width or in length than that (2-3 mm) in the 2-day-co-cultivation.

Sarmento et al. (1992) have reported that exposing the petiole segments of cucumbers to a bacterial suspension, which contained 20 μM acetosyringone for 5 min, had no significant effect in enhancing the frequency of kanamycin resistant calli. In the present study, the acetosyringone treatment that was used during co-cultivation was effective in enhancing the efficiency of transformation at the cut surface cells of hypocotyl explants. In the previous reports (Chee 1990; Sarmento et al. 1992), the period of co-cultivation with *Agrobacterium tumefaciens* was 2 and 4 days at 25-26 °C. From the comparison between the 2-day- and 5-day-co-cultivations, the longer period (5 days) of co-cultivation particularly with acetosyringone treatment was effective in enhancing the frequency of hygromycin B resistant calli.

Comparison of kanamycin and hygromycin B as selectable agents

The selection comparison using kanamycin and hygromycin B was investigated in the hypocotyl explants without and with *Agrobacterium* infection (Fig. 2C). Each 100 hypocotyl explants without both *Agrobacterium* infection and co-cultivation was placed on the regeneration medium that contained 50 and 100 mg/l kanamycin. All of the explants grew during 2-3 wks and the whole of all explants changed to white or yellow callus cells. The size of the callused hypocotyl explants was 3-4 times larger in width or in length than that which was first placed on the regeneration medium containing kanamycin. The size of the callused hypocotyl explants was similar between the 50 and 100 mg/l kanamycin. The change in size was not found in the 3- and 4-wks-cultured hypocotyl explants in either 50 and 100 mg/l kanamycin. In the *Agrobacterium* infection, each 100 explants was co-cultivated with the 100 μM acetosyringone treatment for 5 days and transferred to the regeneration medium that contained 50 and 100 mg/l kanamycin. The hypocotyl explants gradually changed to white or yellow callus cells during the culture. Sixteen of the 100 explants that were selected using 50 and 100 mg/l kanamycin were treated with X-gluc at 9 and 14 days after initiation of selection. In the callused hypocotyl explants, only a part of the callus cells, which derived from the cut surface cells, displayed strong GUS expression. However, many escape cells were observed in the calli that were selected using 50 and 100 mg/l kanamycin (Fig. 2C). Then, the size of the callused hypocotyl explants was similar between the 50 and 100 mg/l kanamycin.

Further, each 100 explants without both *Agrobacterium* infection and co-cultivation was placed on the regeneration medium that contained 20, 25 and 30 mg/l hygromycin B for 9-14 days. In all concentrations of hygromycin B, the callus cells were not formed in all of the 100 explants. On the contrary, in the *Agrobacterium* infection with 100 μM acetosyringone during co-cultivation, green-callus cells were formed on the cut surface in the frequency of almost 100% of each of the 100 hypocotyl explants selected using 20, 25 and 30 mg/l hygromycin B. The same result of selection using hygromycin B was obtained in another experiment as compared to the selection using kanamycin. Sixteen of the 100 hypocotyl explants that were selected using 20, 25 and 30 mg/l hygromycin B were then treated with X-gluc at 9 and 14 days after the initiation of selection. Most of the callus cells on the cut surface of each of the 16 explants displayed strong GUS expressions, and the area of the callus cells that indicated GUS expression increased between 9 and 14 days. The difference of the area of the GUS expression within the calli was not found between 20, 25 and 30 mg/l hygromycin B. The area of GUS expression in the calli of 16 explants selected using 20 mg/l hygromycin B was visually two- to several-fold larger than that selected using 100 mg/l kanamycin at 14 days after the initiation of selection (Fig. 2C).

That is, the cell selection using hygromycin B with use of a CaMV 35S-*hph* gene was decreased the escape cells and more effective than it using kanamycin with use of a NOS-*nptII* gene in the transformation of hypocotyl explants of cucumbers.

Plant regeneration

Twenty mg/l hygromycin B was used in the present transformation experiments. The co-cultivated hypocotyl explants were transferred to the regeneration medium that contained 20 mg/l hygromycin B. Shoot regeneration occurred about 4-6 weeks later from the calli that derived from the hypocotyl explants. Shoot regeneration and plantlet formation were enhanced with the transfer to a hormone-free MS medium (Fig. 2D). Twenty-six plantlets were regenerated from the calli of 832 hypocotyl explants in a total of three transformation experiments. Eight of 26 regenerated plants were acclimated in Plantaboxes. Further, 3 of 8 acclimated R0 plants were transplanted into pots and grown in a closed greenhouse. They grew into fully mature plants and all of them flowered. Only one plant set three seeds by self pollination. This may be the result of pollination between different stages of male and female flowers, because in another transformation experiment we obtained many seeds by pollination between transgenic and non-transgenic cucumber plants.

GUS activity in R0 plants

Twelve (No. 1-12 plants) of 21 plantlets that were examined displayed strong GUS expressions in very young leaves with the treatments of X-gluc (Fig. 2E) and MUG (Table 1). However, No. 1-5 plants, which had grown to about 20 cm in height in the course of acclimation, did not display GUS expression in the mature leaves, although in one of their transgenic plants, the vascular bundle system of the petiole was stained with X-gluc. Upon detailed examination, the GUS expression also could not be detected in the 10-20 cm long leaves of transgenic No.1 plant (about 1.5 m in height), which had displayed strong GUS expression in the regenerated young leaves. Further, the GUS expression could not be detected in old roots, petioles, tendrils, and

various tissues of the male and female flowers of the transgenic No.1 plant. However, in the roots of the transgenic No. 3 plant that was treated with X-gluc, the root meristematic regions were strongly stained. On the contrary, the GUS expression could be detected in the cells of ovules and anthers, which probably included pollens, in the transgenic No. 1-3 plants (Figs. 2F and G). The GUS activity in the cells of ovules of the transgenic No. 1 plant decreased with its development and finally diminished. However, the cells of anthers in the control plants sometimes displayed GUS expression with the treatment of X-gluc, although the cells of ovules and other tissues or organs in the control plants did not display GUS expression.

Southern blot analysis in R0 plants

Southern blot analyses were performed on the 8 GUS-positive R0 plants (No. 1-8 ones). The probe of the GUS coding sequence, which was digested with the two restriction enzymes of SnaBI and SacI, did not hybridize with the total genomic DNA from the control plants that were doubly digested with the HindIII and SacI but hybridized with the total genomic DNA from the 8 GUS-positive R0 plants that were digested with the two restriction enzymes (Fig. 3A). The 8 GUS-positive R0 plants displayed single Southern positive band, which was identical with the expected 2.8 kb band (the result of No. 8 plant is not shown). Further, using EcoR1, which only cuts on one site of the *hph* gene within the T-region, the variable hybridization patterns with single or a few Southern-positive bands were observed in the No. 4-7 plants (Fig. 3B). Judging from the number of the Southern-positive bands, it was suggested that the copy number of introduced T-region in the transgenic plants was single or a few.

GUS activity in R1 plants

Two of three R1 plants, which were progenies of the transgenic No. 1 plant, fully grew in a closed greenhouse, but one R1 plant died after seeding in a pot. The GUS expression was strong in the meristematic region of their young roots, cells of cotyledons, anthers and ovules, but could not be detected in the mature leaves of both R1 plants. The control plants did not display GUS expression in the cells of roots, cotyledons, leaves, ovules and various tissues of male and female flowers except for the anthers where the GUS activity was sometimes expressed. Therefore, the GUS gene was inherited from the R0 plant to the R1 progeny plants.

The expression of GUS using histochemical assay has been detected at the cells of embryo, meristem, young tissues or vascular bundle system in the transgenic tabacco (Jefferson et al. 1987; Benfey et al. 1989), melon (Dong et al. 1991) and rice (Terada and Shimamoto 1990) plants, which are introduced the CaMV 35S-*gus* gene. In the transgenic melon plants of *Cucumis* plants, the expression of the CaMV 35S promoter is active at the cells of the vascular bundle system of root, stem, leaf and petal (Dong et al. 1990). On the contrary, the GUS expression was not detected at the

Table 1. GUS activity of regenerated and mature leaves of transgenic R0 cucumber plants by treatment of MUG

Organ	Plant no.	GUS activity (pmol/mg/min)
Regenerated leaf	1	3396.8
	8	3481.2
	9	4437.1
Control leaf	C-1	4.6
Mature leaf	1	1.0
	2	3.1
	3	1.5
	4	0.5
	5	0.4
Control leaf	C-2	0.4

No. 1-5, 8, 9, C-1 and C-2 plants are independent ones. No. 1-5 plants displayed strong GUS expression in the regenerated leaves by treatment of X-gluc. No. 1-5 and 8 plants are Southern positive ones.

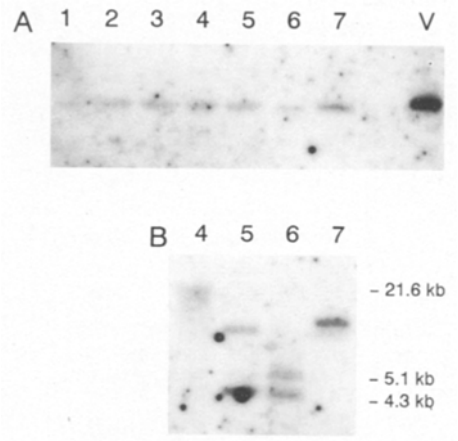

Fig. 3. Southern blot hybridization analysis of genomic DNA from mature leaves of regenerated R0 plants. (A) The DNA was doubly digested with HindIII and SacI. ^{32}P-labelled GUS fragment isolated from pIG121-Hm by double digestion of SnaBI and SacI was used as probe. Lane V: 80 pg of 2.8 kb CaMV 35S-I-GUS fragment isolated from pIG121-Hm by double digestion of HindIII and SacI. Lanes 1-7: genomic DNA (25 μg) extracted from young leaves of individual regenerated R0 plants. Lane C: genomic DNA (25 μg) from the leaf of control cucumber plant. (B) The samples were digested with EcoRI and hybridized with the same probe described above. Genomic DNA (15 μg) was loaded onto each lane.

cells of the vascular bundle system of root, leaf and petal in the transgenic cucumber plants of the same *Cucumis*. However, the GUS expression at the cells of the root meristem, cotyledon and ovule in the transgenic cucumber plants was similar to that in the transgenic melon plants. It is unclear why the GUS expression disappeared in the mature leaves of the transgenic cucumber plants. Further investigation such as the analysis of RNA transcription level of the CaMV 35S-*gus* gene in the transgenic cucumber plants may be necessary to clarify it.

Conclusions

The present transformation study using the GUS gene clearly demonstrated the production of transgenic cucumber plants and also validated the acetosyringone treatments during co-cultivation and the cell selection using hygromycin B. The expression level of CaMV 35S promoter in the transgenic cucumber plants and the cells or tissues where the CaMV 35S promoter actively worked was confirmed by both treatments with X-gluc and MUG. This information on the CaMV 35S promoter in the transgenic cucumber plants should be valid for further transformation experiments such as the introduction of other foreign genes in cucumbers.

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