Selection of Transformed Callus in a Liquid Medium and Regeneration of Transgenic Plants in Cucumber (*Cucumis sativus* L.)

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Summary

The effects of three antibiotics and culture methods were investigated to find out reliable selection procedure for *Agrobacterium*-mediated transformation in cucumber (*Cucumis sativus* L.). When cotyledonary explants without inoculation of *Agrobacterium* were cultured on agar medium containing kanamycin, geneticin (G 418) or hygromycin, callus formation and greening of explants were suppressed more effectively by G 418 or hygromycin than by kanamycin. However, G 418 and hygromycin could suppress effectively callus formation only at the part of explants contacting with an agar medium. In order to expose the whole surface of explants to antibiotics, tissues were cultured in a liquid medium. Combination of liquid culture and addition of G 418 or hygromycin were highly effective in suppressing callus formation from uninfected explants. Explants infected with *Agrobacterium* were cultured in a liquid medium with each antibiotic. Hygromycin or G 418 resistant calli were selected under the presence of 20-80 mg/l hygromycin or 20 mg/l G 418, respectively. Calli formed at 20 mg/l hygromycin were transferred to a regeneration medium, and regenerated plants were obtained via embryogenesis. PCR analysis demonstrated integration of hygromycin phosphotransferase (HPT) gene to all regenerated plantlets and transmission of HPT gene to their progeny.

Key Words : *Agrobacterium tumefaciens*, *Cucumis sativus* L., transformation, kanamycin, G 418, hygromycin.

Introduction

Genus *Cucumis* includes 24 species, and some of which are known to have resistance to diseases or pests, such as *Fusarium* wilt, gummy stem blight, nematode (Igarashi *et al.*, 1987) and cucumber mosaic virus (Providenti and Robinson 1974). Because of interspecific incompatibility of cucumber (*Cucumis sativus* L.) to other species in genus *Cucumis* except for *C. hardwickii* (Deakin *et al.*, 1971), it is very difficult to introduce these disease or pest resistances into cucumber by con-

ventional cross-breeding.

Therefore, genetic transformation is considered to be the only reliable way for introducing disease resistances to cucumber. Transformation of *Cucumis* has been reported (Chee 1990 ; Frag and Grumet, 1990 ; Dong *et al.* 1991 ; Yoshioka *et al.* 1992), where they selected transformants by kanamycin resistance. We tried to select transgenic plants by kanamycin resistance using cotyledons of mature seeds as explants. However, kanamycin was not effective on suppressing callus formation and greening of cotyledons, thus many escape plants were regenerated on a selection medium containing kanamycin. In order to get a reliable procedure for selection of transformants in cucumber, we evaluated suitable antibiotics, their concentration and culture condition.

Materials and Methods

Plant materials and *Agrobacterium* : Peeled cucumber seeds (cv. Shinhokusei No. 1, TOKIWA KENKYUJO Co., LTD., Japan) were sterilized with sodium hypochlorite solution containing 1 % active chlorine for 15 minutes and then rinsed three times with sterile distilled water. Two strains of *Agrobacterium* were used. One was disarmed *A. tumefaciens* GV 3101 harboring co-integrated plasmid pGV 3850 HPT (Kato *et al.* unpublished) which has a chimeric gene of cauliflower mosaic virus 35 S promoter, hygromycin phosphotransferase (HPT) gene and the terminator of nopaline synthase. The other was LBA 4404 having a binary vector plasmid, pBI 121 (Clontech, USA).

Effects of antibiotics in agar medium on callus formation of uninfected cotyledonary explants : Effect of three antibiotics, kanamycin, geneticin(G 418), and hygromycin, on callus growth and greening of cotyledonary explants uninfected with *Agrobacterium* was tested. An excised cotyledon from a sterilized seed was cut into 5×5 mm pieces. These explants were cultured for 5 days on TC-1 medium (MS medium (Murashige and Skoog 1962), 1 mg/l NAA (α -naphthylacetic acid), 0.5 mg/l BA (benzyladenine), 3% sucrose and 0.8% agar) (Tabei and Kanno 1989) without antibiotic, pH 5.8, in the dark at 25 °C. Then they were transferred onto the same medium con-

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taining 500 mg/l carbenicillin and three concentrations of each antibiotic. Antibiotics were added to TC-1 medium kept in a water-bath of 50 °C after autoclaving. Media were changed every two weeks. After 1 month culture, number of explants greening and producing callus was evaluated.

Effects of antibiotics in liquid medium on callus formation of uninfected cotyledonary explants : In order to expose the whole surface of explants to antibiotics, the effect of liquid culture was tested. The liquid medium and culture procedure used were those of Bergervoet *et al.* (1989). After initial culture for 5 days on TC-2 medium (MS medium, 1.11 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 1.13 mg/l BA and 3 % sucrose) with 0.8 % agar in the dark at 25 °C, explants were cultured in TC-3 medium (MS medium containing 0.93 mg/l NAA, 1.13 mg/l BA and 3 % sucrose) with 500 mg/l carbenicillin and each antibiotic in a rotary shaker, at 120 r. p. m., 16 hrs photoperiod at 25 °C. Liquid media were changed every week. They were cultured in TC-3 for 3 weeks, then cultured in TC-2 for a week.

Effects of antibiotics on callus formation of explants co-cultivated with Agrobacterium : After cultured on TC-2 agar medium for 3 days in the dark at 25 °C, explants were dipped in suspension of *Agrobacterium* (diluted ten times of overnight cultured) for 5 minutes, blotted dry with sterile filter paper to remove excess bacteria. Then they were placed back onto the sterile filter paper on the same medium. After co-cultivation for 2 days, explants were transferred into TC-2 liquid medium containing 500 mg/l carbenicillin and each antibiotic. These liquid media were changed every week.

Plant regeneration : Hygromycin resistant calli were transferred onto regeneration medium (MS medium with 20 mg/l NAA, 0.5 mg/l BA, 3 % sucrose and 0.8 % agar) (Tabei and Kanno 1989) containing 500 mg/l carbenicillin and 20 mg/l hygromycin. The medium was changed every two weeks. Regenerated somatic embryos were transferred to MS agar medium with 500 mg/l carbenicillin and 20 mg/l hygromycin to stimulate germination and elongation. Elongated shoots (ca. 10 cm) were maintained by cutting *in vitro*.

DNA analysis : Genomic DNA of hygromycin resistant plants and untransformed plants were extracted from young leaves using the procedure described by Edward *et al.* (1991). Plasmid pGV 3850 HPT was isolated from *Agrobacterium* GV 3101 according to Maniatis *et al.* (1982) and used as a positive control. Integrated DNA was detected by polymerase chain reaction (PCR) using two oligonucleotide primers : 5'-GATGTGATATCTCC-ACTGACGTAAG-3' homologous to cauliflower mosaic virus 35 S promoter and 5'-CTCATAAATAACGTCA-TGCATTACA-3' homologous to the terminator of nopaline synthase. DNA was amplified by 40 cycles of 1 min at 92 °C, 1 min at 55 °C and 2 min at 74 °C using 'Gene Amp' Kit (Perkin Elmer Cetus). Amplified DNA were electrophoresed on 1.5 % agarose gel. To examine inheritance of the introduced HPT gene, the progeny of

regenerated plants were obtained by self pollination. Genomic DNA of the progeny were extracted from cotyledon and integrated HPT gene was detected by PCR described above.

Results

All uninfected explants cultured on shoot regeneration medium containing 100-400 mg/l kanamycin produced calli and greened. All explants cultured with 20-40 mg/l G 418 also produced calli and greened, while 80 mg/l G 418 suppressed callus formation of 31 % explants and completely inhibited greening of explants. Under the presence of hygromycin, all uninfected explants produced calli, but greening of explants was inhibited more effectively than by the other antibiotics (Table 1). The percentages of callus formation were the same as that of the control except in the medium with 80 mg/l G 418. But the size of calli growing on the medium with G 418 or hygromycin was smaller than that with kanamycin. Especially in part of explant contacted with the medium containing G 418 or hygromycin, callus formation was suppressed, and the part were also bleached remarkably. These results suggested that the contact of whole surface with the antibiotics was necessary for reliable selection of transformants.

In liquid medium containing 100-200 mg/l kanamycin, all uninfected explants greened, and formed calli. Inhibition of callus formation was found in 20 % of explants with 400 mg/l kanamycin. In 20 mg/l G 418, the percentages of greening and callus formation were 90 % and 65 %, respectively. Greening and callus formation were completely inhibited by 40-80 mg/l G 418. Hygromycin inhibited callus formation completely at all concentrations, and greening was observed in 25 % and 10 % of

Table 1. The effects of antibiotics and their concentrations in agar medium on callus formation and greening of explants uninfected by *Agrobacterium*.

Antibiotics	Concentrations (mg/l)	The percentages of callus formation	The percentages of greening of explants
Kanamycin	100	100	100
	200	100	100
	400	100	100
G418	20	100	94
	40	100	100
	80	69	0
Hygromycin	20	100	57
	40	100	25
	80	100	0
Control		100	100

Explants were cultured for 5 days on TC-1 medium (MS medium containing 1 mg/l NAA, 0.5 mg/l BA, 3 % sucrose, 0.8 % agar), and transferred to the same medium containing 500 mg/l carbenicillin and each antibiotic. Control means that explants were cultured on the shoot regeneration medium containing 500 mg/l carbenicillin. The percentages of explants greening or producing calli in the total explants cultured were shown.

explants at 20 mg/l and 40 mg/l hygromycin, respectively. All explants were bleached at 80 mg/l hygromycin (Table 2). From these results, combination of liquid culture and selection by G 418 or hygromycin was found to be highly effective to inhibit callus formation and greening of untransformed tissue.

After co-cultivation with *Agrobacterium* LBA 4404 having pBI 121, 45 % of cotyledonary explants formed green calli in 20 mg/l G 418, but their sizes were small. No callus was induced in 40-80 mg/l G 418. Explants cultured in 20-80 mg/l hygromycin after co-cultivation with GV 3101 harboring pGV 3850HPT formed hygromycin resistant green calli within 2 weeks (Table 3). The other parts of explants were bleached completely (Fig. 1-A-b). The percentage of callus formation was 80-85 %. Hygromycin was considered to be a more suitable antibiotic for the selection of transformed calli than G 418.

Hygromycin resistant calli selected by 20 mg/l hyg-

Table 2. The effects of antibiotics and their concentrations in liquid culture on callus formation and greening of explants uninfected by *Agrobacterium*.

Antibiotics Concentrations (mg/l)		The percentages of callus formation	The percentages of greening of explants
Kanamycin	100	100	100
	200	100	100
	400	80	100
G418	20	65	90
	40	0	0
	80	0	0
Hygromycin	20	0	25
	40	0	10
	80	0	0
Control		100	100

Explants were cultured for 5 days on TC-2 medium (MS medium containing 1.11 mg/l 2,4-D, 1.13 mg/l BA, 3 % sucrose) with 0.8 % agar, and cultured for three weeks in TC-3 medium (MS medium containing 0.93 mg/l NAA, 1.13 mg/l BA and 3 % sucrose) with 500 mg/l carbenicillin and each antibiotic. Then explants were cultured for one week in TC-2 with antibiotics. Control means that explants were cultured under the presence of 500 mg/l carbenicillin. The percentages indicated explants greening or producing calli in the total explants cultured.

romycin were transferred to regeneration medium. After 2-3 months, 20 somatic embryos were produced (Fig. 1-B). They were transferred to MS agar medium with no growth regulators to stimulate embryogenesis and germination. Six embryos germinated and grew normally (Table 3). After transfer of elongated shoots (ca. 10 cm) to MS agar medium with 500 mg/l carbenicillin and 20 mg/l hygromycin, elongated shoots formed adventitious roots (Fig. 1-C) and grew to normal plantlets (Fig. 1-D). G 418 resistant calli were not transferred to a regeneration medium, because calli were formed on not only infected explants but uninfected ones in the medium with 20 mg/l G 418.

Amplified DNA of hygromycin resistant plants had the same size as one of plasmid pGV 3850 HPT, and the amplified DNA was not detected in an uninfected control (Fig. 2-A). All hygromycin resistant plantlets had the chimeric gene, and there was no escaped plant (Table 3). These results suggested the integration of the chimeric genes (35S promoter, HPT gene and terminator of nopaline synthase) in the hygromycin resistant plants. PCR analysis also detected the amplified DNA in the progeny of the hygromycin resistant plant (Fig. 2-B). This result confirmed the integration of the chimeric gene to the chromosome of the hygromycin resistant plant.

Discussion

Since cotyledonary explants of mature seeds or young seedlings have high ability of organogenesis and embryogenesis (Oridate and Oosawa 1986, Driks and Buggenum 1989, Tabei and Kanno 1989), they were used as explants in tissue culture of *Cucumis*. Agar medium containing each antibiotic (100-400 mg/l kanamycin, 20-80 mg/l G 418 or 20-80 mg/l hygromycin) could not inhibit callus formation and greening of uninfected cotyledonary explants completely. Chee (1990) reported 25 mg/l kanamycin did not allow regeneration of escape plants, and Frag and Grumet (1990) reported 75 mg/l kanamycin completely inhibited growth of uninfected explants using cotyledonary explants 3-5 days after germination. However, Dong *et al.* (1991) and Yoshioka *et*

Table 3. The effects of G418 and hygromycin in liquid medium on callus formation and greening of explants infected by *Agrobacterium*.

Antibiotics Concentrations (mg/l)		The percentages of callus formation	No. of calli transferred to regeneration medium	No. of calli with somatic embryos	No. of plantlets regenerated	No. of plantlets having HPT gene
G418	20	45	NT ¹⁾			
	40	0	NT			
	80	0	NT			
Hygromycin	20	80	64	20	6	6
	40	85	NT			
	80	80	NT			

Explants were pre-cultured on TC-2 for 3 days. Then they were inoculated with *Agrobacterium* for 5 minutes and co-cultivated for 2 days on TC-2. The following culture was described in Table 2.

¹⁾ NT : not transferred to regeneration medium.

Fig. 1. A : Cotyledonary tissues of mature seeds cultured in liquid medium with 20 mg/l hygromycin, a; Control explant uninfected by *Agrobacterium*, b; Explants infected by GV3101+pGV3850HPT. The cultures were photographed at four weeks after inoculation. B : Germinated somatic embryos on MS medium with 500 mg/l carbenicillin and 20 mg/l hygromycin. C: Rooting in the MS medium with 500 mg/l carbenicillin and 20 mg/l hygromycin, a ; untransformed control, b ; transformant. D : A transgenic cucumber planted in a pot.

Fig. 2. Detection of the chimeric genes (CaMV 35S promoter+HPT gene+terminator of nopaline synthase) in regenerated cucumber plants and their progeny by PCR.

- A : Detection of chimeric gene in regenerated hygromycin resistant cucumber. Lanes M; size marker λ /Hind III, P; pGV3850HPT, N; untransformed control, 1-6; regenerated hygromycin resistant cucumber (KN-1~6).
- B : Transmission of the chimeric gene to the progeny of a regenerated hygromycin resistant cucumber (KN-5). Specific amplified DNA was detected at lane, 1, 3 and 4, but it was not detected at lane 2 and 5. Lanes M; size marker λ /Hind III, P; pGV3850HPT, K; KN-5, 1-5; progeny of a hygromycin resistant cucumber (KN-5).

al. (1992) observed regeneration of escape plants under the presence of kanamycin at 100 mg/l and 50 mg/l, respectively. Yoshioka *et al.* (1992) used cotyledonary explants 1-2 days after germination, while Dong *et al.* (1991) and we used cotyledonary explants of mature seeds. Cotyledonary explants of mature seeds or germinated seeds may have higher tolerance to antibiotics than those of seedlings.

The use of G 418 instead of kanamycin was effective for selecting transformants in rice (Toriyama *et al.* 1988), and HPT gene was an effective marker for selection of stable transformants in rice (Shimamoto *et al.* 1986). In cucumber, G 418 and hygromycin were also more effective antibiotics for inhibition of callus formation and greening than kanamycin. But calli were formed from uninfected as well as infected explants under the presence of 20 mg/l G 418, and higher concentration of G 418 suppressed callus formation from both explants completely. While hygromycin suppressed callus formation and greening of uninfected explants and allowed the formation of hygromycin resistant calli from infected explants at 20-80 mg/l. Therefore, hygromycin was considered to be more suitable antibiotic than G 418.

Liquid culture was essential for the reliable selection of transformed calli in cucumber. In direct DNA transfer to protoplasts, transformed cell colonies were selected in a liquid medium containing antibiotics. In this selection procedure, little or no background colony

growth was observed in control experiments (Shimamoto *et al.* 1986, Toriyama *et al.* 1988).

In reported transformation experiments of genus *Cucumis* (Chee, 1990 ; Frag and Grumet, 1990 ; Dong *et al.*, 1991; Yoshioka *et al.*, 1992), somatic embryos or adventitious shoots were developed in the medium with 25-100 mg/l kanamycin, and regenerated plants were selected by the ability of producing adventitious roots under the presence of kanamycin. The percentages of plants rooting were 30 % (Frag and Grumet, 1990) and 6 % of regenerated shoots (Dong *et al.*, 1991). These reports suggest that secondary selection is necessary to obtain true transgenic plants. Since all regenerated plants were transformants in our experiment, secondary selection is not necessary in our selection procedure. Liquid culture in the medium with hygromycin is considered to be the most reliable procedure for selection of transgenic plants in *Agrobacterium*-mediated transformation of cucumber.

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