Transfer and Expression of Cucumber Mosaic Virus Coat Protein Gene in the Genome of Cucumis sativus

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Abstract. Cotyledon explants of cucumber (Cucumis sativus L. cv. Poinsett 76) seedlings were cocultivated with disarmed Agrobacterium strain CS8Z707 that contained the binary vector plasmid pGA482GG/cpCMV19. The T-DNA region of this binary vector contains plant-expressible genes for neomycin phosphotransferase II (NPT II), β-glucuronidase (GUS), and the coat protein of cucumber mosaic virus strain C (CMV-C). After infection, the cotyledons were placed on Murashige and Skoog medium containing 100 mg kanamycin/liter. Putative transformed embryogenic calli were obtained, followed by the development of mature embryos and their germination to plants. All transformed R, cucumber plants appeared morphologically normal and tested positive for NPT II. Southern blot analysis of selected cucumber DNAs indicated that NPT II, GUS, and CMV-C coat protein genes were integrated into the genomes. Enzyme-linked immunosorbent assay and Western blot analysis indicated that the CMV-C coat protein is present in the protein extracts of progeny plants. These results show that the Agrobacterium-mediated gene transfer system and regeneration via somatic embryogenesis is an effective method for producing transgenic plants in Cucurbitaceae.

Materials and Methods

T-DNA vector. The T-DNA region of the Agrobacterium Ti plasmid-derived binary plasmid pGA482 (An, 1986) contains a kanamycin-resistance-selectable marker gene, NPT II, driven by the nopaline synthase (NOS) promoter. The T-DNA region also contains a multiple-cloning site, the cauliflower mosaic virus (CaMV) 35S promoter-driven GUS gene (Jefferson et al., 1987), and the coat protein gene from CMV-C (Quemada et al., 1991). To facilitate the transfer of this binary vector into various Agrobacterium strains, a bacterial gentamicin gene (Allmansberger et al., 1985) was cloned outside of the T-DNA region to obtain the plasmid referred to as pGA482GG/cpCMV19 (Fig. 1).

Plant material. Seeds of ‘Poinsett 76’ cucumber (Asgrow Seed Co., Kalamazoo, Mich.) were soaked in tap water for 15 min. The seedcoats were removed manually. The decoated seeds were surface-sterilized with 70% alcohol for 1 min. A 25-min treatment with a 25% (v/v) solution of commercial bleach (5.25% sodium hypochlorite) followed. The seeds were rinsed four times with sterile distilled water and germinated in darkness at 28°C on 0.8% water agar (Difco Laboratories, Detroit) for 3 to 5 days. Unless otherwise stated, all media were supplemented with 3% sucrose and solidified with 0.8% Phytagar (Gibco, Gaithersburg, Md.). The pH of all media was adjusted to 5.8 before autoclaving at 121°C for 20 min.

Transformation. Transformation of cucumber was performed according to Chee (1990b). Three- to 5-day-old in vitro-grown seedlings were used as donors of cotyledons, which were cut into 5-mm explants. The explants were submerged overnight in a diluted culture (2 × 10⁶ cells/ml) of the strain C58Z707/ pGA482GG/cpCMV19 (Hepburn et al., 1985; Quemada et al., 1991) (Fig. 1). This strain was grown in LB medium containing 25 mg kanamycin (Km)/liter. After gentle shaking to ensure that all edges were infected, the explants were blotted dry and cultured axillary side down in a sterile 100 × 20-mm petri dish (20 pieces/dish) containing Murashige and Skoog (MS) medium

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Abbreviations: Cb, carbenicillin; CMV, cucumber mosaic virus; GUS, β-glucuronidase; Km, kanamycin; MS, Murashige and Skoog NPT II, neomycin phosphotransferase II; NOS, nopaline synthase.

medium, they were transplanted to 0.5-liter pots containing Pro-

plantlets had developed an extensive root system on the latter

generation to plantlets under the same environment. When

transformed to MS medium + 1.0 mg NAA + 0.5 mg kinetin

on initiation medium, the emerging cotyledon callus pieces were

after 4 days of growth in dark-

development. The environment for this stage was 2 weeks at

covered with plastic storage bags for acclimation. Subse-

Fig. 1. Structure of the binary plasmid pGA482GG/cpCMV19. The

+ 2.0 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter + 0.5

mg kinetin/liter (Chee, 1990a). After 4 days of growth in dark-

ness at 26°C, the infected cucumber explants were transferred to

petri plates containing MS medium + 500 mg carbenicillin

(Cb)/liter + 100 mg Km/liter and cultured as before for 5 ad-

ditional weeks at 26°C in darkness.

Plant regeneration. Putative transformed embryogenic calli

were regenerated according to Chee (1990a). After 5 to 6 weeks

on initiation medium, the emerging cotyledon callus pieces were

transformed to MS medium + 1.0 mg NAA + 0.5 mg kinetin

+ 100 mg Km + 500 mg Cb, all per liter, for somatic embryo

development. The environment for this stage was 2 weeks at

26°C with a 16-h photoperiod under diffuse cool-white fluores-

cent lamps (80 µmol·m⁻²·s⁻¹). The tissues were then trans-

ferred to hormone-free MS medium + 50 mg Km/liter for

germination to plantlets under the same environment. When

plantlets had developed an extensive root system on the latter

medium, they were transplanted to 0.5-liter pots containing Pro-

Mix (peat-lite/soilless mix; Florist Products, Schaumburg, 111.)

and covered with plastic storage bags for acclimation. Subse-

sequently, the regenerated plants were potted in 8-liter pots con-

taining soil mixture (Metro Mix 300R; Florist Products) and
grown in a greenhouse.

NPT II enzyme assay. NPT II enzyme activity was detected

by the in situ gel assay procedure (Chee et al., 1989; Reiss et

al., 1984). About 100 mg of cucumber callus or regenerated

fresh leaf tissue was mixed with 20 µl of extraction buffer in a

1.5-ml Eppendorf tube. The tissue was macerated with a Konte

pestle and centrifuged at 10,000 × g for 15 min at 4°C to remove

cell debris. From each sample tested, a 35-µl aliquot of the

supernatant solution was electrophoresed on a 10% nondena-
turing polyacrylamide gel, which was then exposed to Km sul-
fate and [³²P-γ]-ATP. Phosphorylated Km produced in those

regions of the gel containing the modified bacterial NOS-NPT

II enzyme activity was blotted onto phosphocellulose paper and
detected by autoradiography.

Qualitative fluorometric GUS assay. GUS expression was
determined by the qualitative fluorometric procedure of Jeffe-

son et al. (1987). About 50 mg of tissue was mixed with 200-

µl of lysis buffer (50 mM NaPO₄, pH 7.0, 10 mM EDTA, 0.1%

sarkosyl, 10 mM β-mercaptoethanol) containing 1 mM-4-methyl

umbelliferyl glucuronide. The tissue was macerated and centri-
fuged for 15 min at 4°C. Fifty microliters of each supernatant

sample was transferred to a 96-well plate and incubated over-
night at 37°C. The reactions were stopped with 125 µl of 0.25

M Na₂CO₃. The blue fluorescence was observed visually with a

long-wavelength ultraviolet (UV) light box.

Genomic DNA isolation. Total nucleic acids were extracted

cucumber leaves using the cetyltrimethylammonium bromide

procedure described by Saghai-Maroof et al. (1984). These

extracted DNAs were not subjected to any further purification

steps as they were readily digested by the restriction enzymes.

Genomic blot hybridization. Genomic DNA from individual

cucumber plants (between 5 to 10 µg) was digested with a 5-
fold excess of either BamHI and HindIII or only HindIII en-

zymes, then electrophoresed in a 0.7% agarose gel. The gel was

then electroblotted onto nylon fiber (Reed and Mann, 1985).

Blotted filters were fixed by exposure for 3 min to a UV light

source, then prehybridized in Denhardt (1966) solution [6x

hybridization solution contains 0.9 M NaCl: 0.09 M Na citrate,

pH 7.2; and 0.02% bovine serum albumin, PVP-40 (Sigma, St.

Louis), and Ficoll-400 (Sigma)] for at least 2 h at 68°C. Filters

were hybridized against either a nick-translated 32P-labeled

(Maniatis et al., 1982) 600 bp BgIII-NcoI fragment containing

mostly NPT II coding sequences (Beck et al., 1982), a ³²P-
labeled 600 bp BamHI fragment from pUC1813 that contained

the GUS coding sequences (Jefferson et al, 1987), or a ³²P-
labeled 1700 bp EcoRI fragment from phage λ clone CMV 9.9

that contained the CMV-C coat protein gene (Quemada et al.,

1989). DNA labeling was done using a commercial kit (Be-

gman's, Chicago, Ill.). Hybridizations were done using 7.5 × 10⁵
counts per minute (cpm) of the labeled probe per milliliter of hybridization buffer containing 2% sodium dodecyl sulfate (SDS), and incubating at 68°C for at least 18 h. Hybridized filters were washed in 1 x hybridization solution (Denhardt, 1966) for 1 h at 68°C, dried, and then ex-
posed to film for 12 to 24 h.

Protein extract preparation and coat protein detection. Proteins

were extracted from lyophilized leaf materials by hand-

grinding 100 mg of sample in 1.0 ml of sample extraction buffer

[750 mM Tris-HCl, pH 8.8; 4% SDS (w/v); 4% (3-mercaptopo-
etanol (w/v); and 40% sucrose (w/v)]. After heating for 10 min

at 100°C, cell debris was separated by centrifugation at 10,000 ×
g for 10 min. Protein was quantified by the Bradford assay (Bradford, 1976). Protein extracts were fractionated on 12% SDA-PAGE (Laemmli, 1970) and then transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, Mass.) by electroelution (Towbin et al., 1979). Membranes were blocked at 25°C for 1 h in Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5) that contained 2% nonfat milk, then were incubated overnight at 4°C in TBS containing 3 µg of anti-CMV IgG/ml, followed by protein A gold conjugate solution (Bio-Rad) for 2 to 4 h. The color intensity of stained bands was enhanced by incubating the membrane in 100 ml of enhancement solution (90 ml of citrate buffer containing 0.85 g hydroquinone + 10 ml of silver lactate solution at 11 mg·ml⁻¹). Enzyme-linked immunosorbent assay (ELISA) was performed by the double-antibody sandwich technique, and 200 µl of antigen was quantified using the alkaline phosphatase assay (Clark and Adams, 1977).

**Results and Discussion**

*Regeneration of transformed cucumber plantlets.* After 6 weeks on initiation medium, a characteristic gel-like callus tissue formed! on the surface of the infected explants, particularly at the site where tissue contacted the culture medium. The upper part of this tissue contained small sectors of putative embryogenic tissue (Chee, 1990a). These were selected for transfer to the secondary medium with 100 µg Km/liter. After transfer, such structures developed into embryos with normal morphology and subsequently germinated into plantlets on MS medium without hormones but supplemented with 50 mg Km/liter. More than 100 transgenic plants were obtained from eight separate infection experiments and all flowered and set seeds normally.

**Detection of NPT II and GUS enzyme activities.** More than 100 Rₐ plants passed the selective test for Km resistance. They were identifiable by their ability to form roots on MS medium + 50 mg Km/liter. In contrast, nontransgenic control plants were inhibited from root development on this medium. To determine their transformation status after plant regeneration, each plant was tested for the presence of NPT II enzyme activity. Figure 2 shows an example of this analysis for NPT II enzyme activity in the protein extracts of 16 transformed Rₐ plants. Individual protein extracts from these putative transgenic Rₐ plants all showed some level of NPT II enzyme activity. This NPT II enzyme activity always comigrated with the activity associated with the control bacterial-derived NPT II enzyme. Variation in the level of this activity could be due to differences in the amount of protein extracted or to positional effects of having the nopaline synthase (NOS) promoter inserted at different chromosomal locations in each plant (An et al., 1986).

In contrast, noninfected control plants that resulted from cocultivation with C58Z707 minus the binary plasmid showed no comigrating NPT II activity in leaf extracts. Each plant was also tested for GUS activity. Only 10 NPT II-positive plants did not have GUS activity. These results suggest that not all transformed plants contained the complete T-DNA region, a result that is consistent with the directional transfer (right to left border) (Wang et al., 1984) of the T-DNA region of the vector pGA482GG/cpCMV19 (Fig. 1).

**Southern blot analysis of transgenic Rₐ cucumber plants.** Total cucumber DNAs were isolated from 80 NPT II- and GUS-positive plants. These DNAs were first subjected to digestions with both BamHl and HindIII enzymes and all yielded the predicted 2.0 kb NOS-NPT II (An, 1986) hybridizing fragment (data not shown). The Southern blot result for four Rₐ plants are shown in Fig. 3. To determine whether the NOS-NPT II gene is indeed integrated within these genomic DNAs, these four DNAs were subjected to restriction endonuclease digestion with only HindIII (Fig. 3). Integration of the NOS-NPT II can be characterized for each transformation event because this gene is only flanked on its right side by a HindIII site (Fig. 1). Thus, the size of the hybridizing fragments is indicative of the location of the nearest HindIII site derived from the plant genome that flanks the opposite side of the NOS-NPT II gene. The presence of the four different size bands for this digestion indicated that each plant was derived from an independent transformation event (Fig. 3). In most cases, the HindIII digest yielded a single band, as seen for Rₐ plant 1, 36, and 106 (Fig. 3), which suggests that a single T-DNA region has been integrated. However, plant 54 showed the presence of multiple bands (Fig. 3), which suggests that this genome may contain multiple T-DNA insertions.

**Southern blot analyses of transgenic Rₐ cucumber plants.** Analysis of 85 Rₐ plants derived from plant 36 showed that 75% expressed NPT II activities (data not shown). Plant 36 was selected for further analyses because it appeared to contain a
single T-DNA integration (see Fig. 3). Southern blot analysis of six R<sub>1</sub> plants derived from self-pollination of plant 36 are shown in Figs. 4 and 5. The blotted filters show the expected hybridization bands—at 2.0 kb for the NPT II gene (An, 1986) and 5.0 kb for the GUS gene (Jefferson et al., 1987) (Fig. 4). Hybridization of HindIII- and BamHI-digested transgenic cucumber DNAs with the 32P-labeled CMV-C coat protein gene fragment showed the expected 0.75- and 0.9-kb hybridizing bands, while hybridization to this DNA digested with only HindIII showed the presence of a single band at 0.95 kb (Fig. 5). This 0.95-kb band is a doublet because the CMV-C coat protein gene contains an internal HindIII site (Quemada et al., 1989). This doublet can be separated by the BamHI digest because it removes > 200 bp from the 3' end of the engineered CMV-C expression cassette (unpublished data).

**Detection of coat protein polypeptides in R<sub>1</sub> plants.** The results for immunological hybridization of protein extracts of 11 R<sub>1</sub> plants derived from R<sub>0</sub> plant 36 are shown in Fig. 6. The coat protein polypeptides, measuring > 24 kDa, were found in these 11 R<sub>1</sub> plants. This polypeptide size is similar to that found for purified CMV-C coat protein (Fig. 6) and derived from the sequence of the CMV-C coat protein gene (Quemada et al., 1989). The level of coat protein present in 50 R<sub>1</sub> plants derived from R<sub>0</sub> plant 36, as determined by ELISA, is 14 ± 6 ng·mg<sup>-1</sup> protein (Table 1), which is similar to the level of coat protein produced in transgenic tobacco plants (Quemada et al., 1991).

These experiments clearly showed that the Agrobacterium binary vector pGA482GG/cpCMV19, cloned within the disarmed Agrobacterium strain C58Z707, effectively transferred and integrated the CMV coat protein gene into the cucumber genome. The cucumber plant tissues used were from the CMV-susceptible cultivar Poinsett 76. This report also demonstrates that the CMV-C expression cassette is functional not only in tobacco (Quemada et al., 1991), but also in cucumber and is capable of producing about the same amount of CMV-C coat protein as in tobacco. The development of a series of transgenic cucumber plant lines containing and expressing the CMV-C coat protein gene provides plant material to determine whether this level of coat protein will be sufficient to protect cucumber plants from CMV. If so, these plants will be useful in determining the level of CMV resistance between transgenic plants containing
the coat protein gene and natural resistance. Our preliminary field test results from infecting several of these transgenic plants with CMV suggest that CMV coat-protein gene expression does offer protection from CMV infections (Slightom et al., 1990).

**Literature Cited**


