Theor Appl Genet (1996) 93:672–678

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CMV protecton in transgenic cucumber plants with an introduced CMV-O *cp* gene

Received: 8 December 1995 · Accepted: 1 March 1996

Abstract We introduced the CMV-O coat-protein gene into cucumber plants, using a Ti-*Agrobacterium*-mediated transformation system, with the aim of producing cucumber plants with CMV resistance. The RNA transcripts from the CaMV 35*s*-*cp* gene could be detected in the leaves of the R_0 transgenic cucumber plants, as well as in the epicotyls containing two cotyledons of transgenic progeny plants, by Northern-blot analysis; but the presence of coat protein originating from the CaMV 35*s*-*cp* gene could not be detected in the cotyledons or leaves of R_0 and transgenic progeny plants by Western-blot analysis. The progenies of a cross between cv "Sharp 1" and transgenic plants (pure line "1021") possessing the *cp* gene displayed strong resistance to inoculation of the CMV-Y strain, although both the control cv "Sharp 1" and segregated cp^- plants displayed many spotted disease symptons on their leaves 5–6 days after CMV-Y inoculation on the cotyledons. The control "1021" had a slight tolerance toward CMV-Y inoculation. The transgenic cucumber plants displayed the absence of resistance to ZYMV. However, transgenic plants showed a reduced degree of disease symptom development following a double inoculation of CMV and ZYMV. The CMV resistance of the present transgenic cucumber plants seems to be due to the synergism of the slight CMV tolerance in the pure line "1021" and the protection against CMV afforded by the introduction of the CMV *cp* gene. This leads to the possibility of producing cucumber plants with the agronomic characteristics of very strong CMV resistance by the combination of genotypes of cucumbers and the CMV *cp* gene. The transgenic plants possessing the *cp* gene should thus be useful as a genetic source for producing cucumber plants with the agronomic characteristic of CMV resistance.

Key words CMV-*cp* gene · Transgenic cucumber plants · CMV resistance

Communicated by G. Wenzel

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Introduction

One of the goals in plant breeding is to produce useful crops with resistance to diseases caused by viruses, bacteria and fungi. However, in many cases, such diseases resistance cannot be accomplished, due either to genetic barriers, such as sexual incompatibility between donor and acceptor plants, or to the absence of plants with the characteristics of disease resistance for breeding purposes.

Using gene-engineering technologies, single dominant agronomic characteristics, including virus resistance, can be introduced into crops in a short time without changing other desirable characteristics, whereas the introduction of desirable genes by conventional plant breeding techniques requires much time and is often accompanied by such changes . Powell-Abell et al. (1986) first proposed the possibility of plant breeding for disease resistance in transgenic tobacco plants, into which a TMV (tobacco mosaic virus) coat-protein gene was introduced, using *Agrobacterium tumefaciens*. The strategy of using coat-protein genes has since been applied for plant protection against various viruses, and transgenic plants with the characteristics of delayed symptom appearance or resistance to the respective virus have been produced (review of Beachy et al. 1990).

Cucumber is one of the important vegetable crops in America, Asia and European countries. It suffers from viral diseases such as CMV (cucumber mosaic virus), ZYMV (zucchini yellow mosaic virus), WMV (watermelon mosaic virus), etc., which cause serious damage to both the plant body and to fruit yield. However, there is no useful genetic source for virus resistance in some types of cultivars of cucumbers, such as the pickle type and vegetable type. Gonsalves et al. (1992) produced transgenic cucumber plants of the vegetable type into which the CMV-C *cp* gene was introduced. These transgenic cucumber plants were 36% symptomless on exposure to CMV inoculation in the greenhouse. However, viral symptoms appeared in all of the remaining plants including the symptom-delayed plants.

In order to produce cucumber plants of the vegetable type with CMV resistance which can be cultivated in field from spring to summer, we introduced a CMV-O coat-protein gene into one cultivar of cucumber and obtained two transgenic plant lines which displayed strong resistance to CMV inoculation. We report here the characteristics of these transgenic cucumber plants, including an analysis of the expression of the CaMV 35*s*-*cp* gene and the coat protein, the characteristics of resistance to CMV inoculation, and the response to ZYMV inoculation and a double inoculation of CMV and ZYMV.

Materials and methods

Construction of plasmids

The full-length cDNA or RNA4 was synthesized by PCR amplification of the cDNA of CMV-O RNA 3 (Hayakawa et al. 1989) and inserted into the *Sma*I site of pUC19, resulting in pUCRN4 (Hayakawa et al. 1990; Nakajima et al. 1993). The *Hin*dIII-*Pst*I fragment containing the CaMV 35s promoter, and the *Sac*I-*Eco*RI fragment containing the NOS polyadenylation signal of pBI22I (Jefferson et al 1987), were both transferred into pUCRN4, resulting in pUCCP. The plasmid pUCCP was digested with *Sac*I and inserted into the *Sac*I site of pIG121-Hm (Akama et al 1992; Nishibayashi et al. 1996), as shown in Fig. 1. The plasmid produced was named pIG121-HmCP and was transferred to *A. tumefaciens* EHA101 (Hood et al. 1986) by a direct transformation method (Hofgen and Willmitzer 1988).

Plant transformation

The transformation experiments were performed according to the previous report of Nishibayashi et al. (1996). Segments of 7-day-old hypocotyls, which were germinated from sterile seeds, were infected by *A. tumefaciens* containing the plasmid pIG121-HmCP. The hypocotyl segments were placed on 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 100 μM 3', 5'-dimethoxy-4'-hydroxyacetophenone (Aldrich), a derivative of acetosyringone, and cultured for 5 days. 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 and 20 mg/l hygromycin B, and then cultured for 4–6 weeks. 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 culture conditions in all media were maintained constant at 23°C under a 16 h (light)-8 h (dark) photoperiod in a culture room. The acclimated plants were transplanted into pots and grown in a closed greenhouse.

Histochemical analysis

Leaves, cotyledons and flowers were cut into thin sections with a razor blade. The tissue sections were stained with X-glucuronide solution (X-gluc) at 37°C for 1–18 h, according to Jefferson et al. (1987). After staining, the chlorophyll of the leaves and cotyledons was removed, with 70% ethanol at room temperature overnight, for detailed observations of the tissue sections

GUS enzymes assays

Assay of the β-glucuronidase (GUS) activity of crude cell extracts from the leaves and cotyledons was done according to Jefferson et al. (1987), using 4-methyl umbelliferyl glucuronide (MUG) as the substrate.

PCR

Genomic DNAs were prepared from the leaves of R_0 plants and progeny plants, according to Richards (1987). Primer 1 (5'-GTTATTGTCTCTACTGATTGTA-3') and Primer 2 (5'-ACGGAATCAGACTGGGAG-3') were synthesized and used for detection of the *cp* gene in the transgenic plants by PCR. The full-length (739 bp) of 5'-untranslated residues and CP coding residues was detected by the two primers (Fig. 1). The PCR amplification was carried out in 50 μl of reaction mixture with 1 μM of each primer, 100 ng cucumber DNA or 100 pg pIG121-HmCP as control, 0.2 mM dNPTs, 2 mM $MgCl_2$, 1.25 U *AmpliTaq* DNA polymerase (Perken-Elmer) and 1/10 reaction buffer (Perkin-Elmer). Amplification was for 32 cycles consisting of 30 s at 94°C, 30 s at 50°C and 60 s at 72°C, with a minimum transition time between each temperature. PCR products were separated on 1% agarose gels and visualized under UV light after staining with 0.5 μg/ml ethidium bromide.

Southern-blot analysis

Southern-blot analysis was performed by the methods supplied by Amersham. Genomic DNAs were prepared from the leaves of R_0

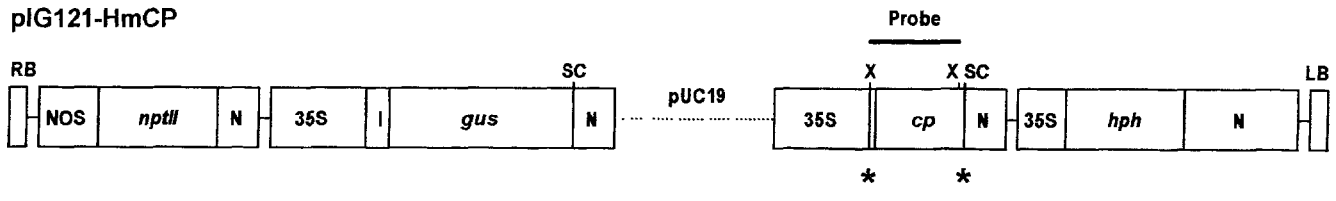


Fig. 1 Schematic representation of the vector plasmid used in the transformation of cucumber plants. *RB* right border; *NOS* nopaline synthase promoter; *nptII* neomycin phosphotransferase II gene; *N* nopaline synthase polyadenylation site; 35*s* CaMV 35*s* promoter; I first intron of catalase from castorbean; *gus* β-glucuronidase gene; *cp* CMV-O coat protein gene; *hph* hygromycin phosphotransferase gene; *LB* left border; *SC Sac*I; *X Xba*I. * positions of primers used for PCR

plants, according to Richards (1987). Ten-microgram samples of genomic DNAs digested with *Xba*I were separated on 1% agarose gels. After denaturation, the genomic DNAs on the agarose gels were blotted onto a nylon memebrane (Hybond-N, Amersham) under alkaline conditions, using a vacuum blotting system (Vacugene, LKB-Pharmacia) An *Xba*I-*Xba*I fragment (Fig 1) of the cDNA containing 5′-untranslated and CP-coding regions (732 bp) was labelled with [^{32}P]-dCTP (111 TBeq/mol) using a multiprime labelling kit (Amersham) and used as a probe for hybridization.

Northern-blot analysis

Genomic RNAs were isolated from young leaf tissue according to the method of Chomczynski and Sacchi (1987). Northern-blot analyses were performed by the methods supplied by Amershem. A *Xba*I-*Xba*I fragment of the cDNA containing 5′-untranslated and CP-coding regions was labelled with [^{32}P]-dCTP (111 TBq/mmol) using a multiprime labelling kit (Amersham), as in the Southern-blot analysis, and was then used as a probe for hybridization.

Western-blot analysis

Cotyledon and leaf tissues were ground in Tris buffer (50 mM Tris-HCl pH 7.5, 2% SDS, 2 mM 2-mercaptoethanol and 0.1 M PMSF) and boiled for 1 min. After centrifugation, extracted proteins were separated on 10% SDS-polyacrylamide gels and transferred onto a nylon membrane (Immobilon, Millipore). Non-specific binding to the membrane was blocked by 3% skimmed milk in TBS buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl), and the membrane was treated with rabbit antibody to the CMV coat protein or to the ZYMV coat protein provided by the Society of Japan Plant Protection. The bound protein was detected using alkaline phosphatase-conjugated goat anti-rabbit antibody (Perbal 1988). Protein (20 µg/lane) was quantified as described by Bradford (1976).

Virus inoculation

CMV-Y and ZYMV were provided by the Society of Japan Plant Protection, multiplied in infected leaves of "Sharp 1", and purified by centrifugation. The concentrated CMV-Y and ZYMV were diluted to the respective concentration (1–1000 µg/ml) with a solution of 100 mM K_2HPO_4 (pH 7.0). Peeled seeds of control and transgenic F_1F-2, F_1F-7 plants were soaked in Petri dishes for 1 day and transferred to soil in pots. The control and transgenic plants, which were grown from the peeled seeds, were mechanically inoculated on both cotyledons in 4–6-day-old seedlings or the first and second leaves in 2-week-old plants with the respective virus. In the experiment with ZYMV inoculation and double inoculation of CMV and ZYMV, the transgenic plants were inoculated with 100 µg/ml ZYMV diluted with 100 mM phosphate buffer or tissue extracts (1 g leaf tissue/10 ml phosphate buffer) of ZYMV-infected leaves of cv "Sharp 1". The multiplication of CMV or ZYMV in the leaves of the control and transgenic plants was examined by Western-blot analysis.

Results

Transgenic plants

Four transgenic R_0 plants were obtained using an *Agrobacterium*-mediated transformation system, and three (R_0-2, 5, 7) of them produced many seeds following crosses between the transgenic R_0 plants (pure line "1021") and cv "Sharp 1" (F_1 hybrid). In none of the three transgenic plants could seeds be obtained by self fertilization. Their progenies (hybrids between R_0-2, 5, 7 and cv "Sharp 1") were called F_1F-2, F_1F-5, F_1F-7.

GUS analysis

GUS expression, which indicates the introduction of the CaMV 35*s*-I-*gus* gene, was detected in the ovule cells of R_0-2, 5, 7 and in the cotyledons of the progeny plants (F_1F-2, F_1F-5, F_1F-7), as noted in a previous report (Nishibayashi et al. 1996). The segregation ratio of the plants with and without GUS expression, using X-gluc and MUG treatments in the cotyledons, was about 1:1 in the F_1F-2 (37:42) and F_1F-7 (48:52) plants.

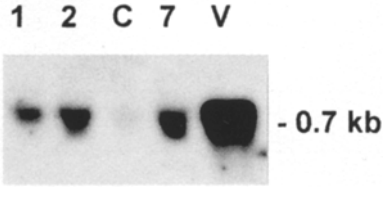
DNA analysis

The four transgenic R_0 plants were analyzed by PCR to detect the presence of the introduced *cp* gene. The expected approximately 0.7-kb band, which corresponds to the size of the 5′-untranslated residues and CP coding residues, was detected in all four transgenic plants (data not shown). As in the analysis of GUS expression, the segregation ratio for the presence and absence of the *cp* gene by PCR analysis was about 1:1 in both F_1F-2 (37:42) and F_1F-7 (48:52) plants. Judging from the these ratios, it appears that a single *cp* gene was introduced into R_0-2 and R_0-7 plants. Three such plants (R_0-2, 5, 7) were analyzed by Southern blotting and showed a single approximately 0.7-kb band with the [^{32}P]-labelled probe of vector plasmid hybridized with the *Xba*I-*Xba*I fragment containing the CP-coding region from genomic DNA (Fig 2).

RNA analysis

The genomic RNAs from leaves of the transgenic R_0-1 and R_0-2 plants were analyzed by Northern blotting. No hybridization bands with the [^{32}P]-labelled probe were detected in the control parent plant. A band of about 1.1 kb which included 5′-untranslated residues, CP-coding residues, 3′-untranslated residues and polyadenylated residues was detected (Fig 3 A), as in previous reports on transgenic tobacco plants into which the same CMV-O *cp* gene had been introduced (Hayakawa

Fig. 2 Southern-blot analysis of genomic DNAs from R_0 transgenic cucumber plants. The DNA blots in the transgenic plants were hybridized with a *Xba*I-*Xba*I fragment containing the CP coding region of pIG121-HmCP. *Lanes* (*1, 2, C, 7, V*). R_0-1, R_0-2, control (pure line "1021"), R_0-7, pIG121-HmCP. *Lane* (*V*), the approximately 0.7-kb *Xba*I-*Xba*I fragment containing the CP coding region from pIG121-HmCP (100 pg)



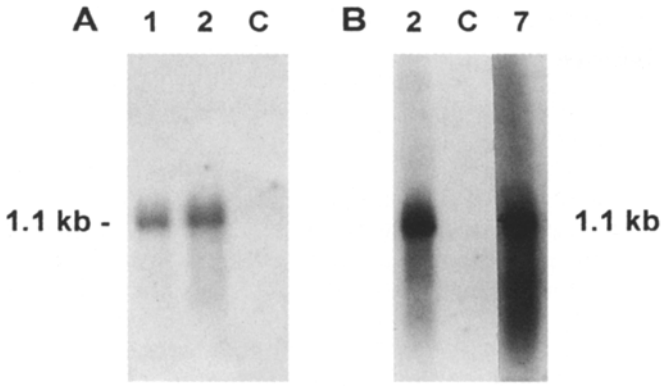


Fig. 3A,B Northern-blot analysis of genomic RNAs from leaves of R_0 transgenic cucumber plants (**A**) and from epicotyls of progeny plants (**B**). The RNA blots in the transgenic cucumber plants were hybridized with *Xba*I-*Xba*I fragments containing the CP coding region of pIG121-HmCP. **A** *Lanes* (*1*, *2*, *C*), R_0-1, R_0-2, control (pure line "1021"). **B** *Lanes* (*2*, *C*, *7*), F_1F-2, control (pure line "1021"), F_1F-7

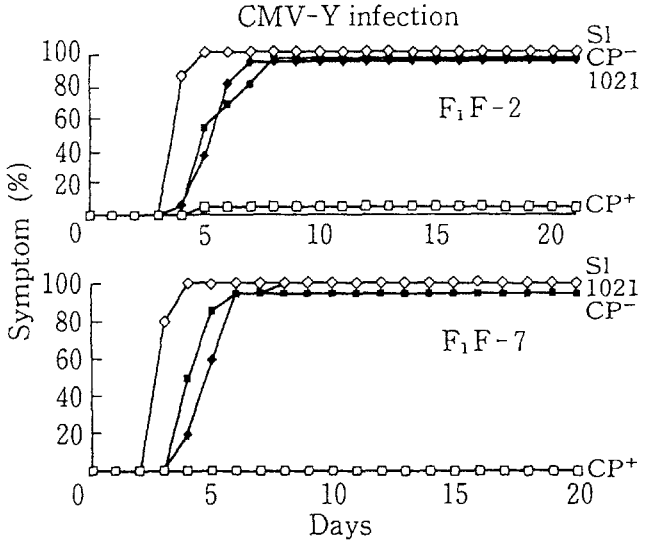


Fig. 4 Symptom developement of F_1F-2 and F_1F-7 transgenic cucumber plants inoculation of 100 µg/ml CMV-Y. Sl (cv "Sharp 1") and pure line "1021" as control plants

et al. 1990; Nakajima et al. 1993). Also in the progeny plants F_1F-2 and F_1F-7 of R_0-2 and R_0-7, a single 1.1-kb band indicative of RNA transcripts of the CaMV 35*s*-*cp* gene was detected (Fig. 3B).

Protein analysis

In several repeated experiments, however, the presence of the expressed coat protein could not be detected in the leaves of R_0-1 and R_0-2, or in both the cotyledons and leaves of the transgenic F_1F-2 and F_1F-7 plants, by Western-blot analysis.

Inoculation of CMV

The inoculation experiment was performed with the transgenic F_1F-2 and F_1F-7 plants in which RNA transcripts of the CaMV-35*s*-*cp* gene could be detected (Fig. 4).

Disease symptoms appeared in the first and second leaves of the control cv "Sharp 1", pure line "1021", and cv "Poinsett 76" plants several days after CMV (1, 10 and 100 µg/ml) was inoculated into the cotyledons. The cv "Poinsett 76" displayed heavier symptoms (necrosis) on the leaves than did cv "Sharp 1" and the pure line "1021", at any concentration of CMV. The CMV inoculation experiment in the transgenic plants was performed at a concentration of 100 µg/ml. Control cv "Sharp 1" plants displayed spotted disease lesions on the leaves at a frequency of 100% (20 plants each), 5–6 days after inoculation (Fig. 4). Control "1021" plants also displayed spotted disease lesions on the leaves at frequencies of 95–100% (20 plants each), 8–9 days after inoculation. The appearance of symptoms in "1021" was slightly delayed, compared with cv "Sharp 1". In the segregated plants of F_1F-2 (26 plants) and F_1F-7 (21 plants) without the CP gene, spotted disease lesions appeared at frequencies of 95–96%, at an intermediate period of 7–8 days after inoculation. In the cp^+ plants of F_1F-2 (21 plants) and F_1F-7 (22 plants), the frequency of appearance of symptom development was 5% and 0% at 3 weeks after CMV-Y inoculation (Fig. 4). The remainder of the cp^+ plants of F_1F-2, and all of the cp^+ plants of F_1F-7, were symptomless in response to CMV-Y inoculation after 3 weeks.

The number of spotted disease lesions on the first leaf in control "Sharp 1" plants was 108 and 172, as an average of each experiment, and was 8 and 9 in control "1021" plants. Control "1021" plants showed a reduced number of spotted disease lesions and had slight tolerance toward CMV, compared with the control cv "Sharp 1". In the segregated plants of F_1F-2 and F_1F-7 without the *cp* gene, the number of spotted disease lesions was intermediate (62 and 68 on average) compared with the control cv "Sharp 1" and "1021". One cp^+ plant of F_1F-2 displayed only one lesion on the first leaf. There was no problem with viral symptoms. The multiplication of inoculated CMV virions could be detected in the leaves of the cp^- plants of F_1F-7 4 days after inoculation of 100 µg/ml CMV on the cotyledons, but could not be detected in the cp^+ plants of F_1F-7 by Western-blot analysis. Moreover, following the inoculation of 500 µg/ml and 1000 µg/ml CMV, three or four transgenic plants of F_1F-7 displayed no symptom development during 3 weeks. By contrast, one or two cp^- plants of F_1F-7 did display symptom development at the same concentrations.

Inoculation of ZYMV

Waved leaves, a systemic symptom, clearly developed on the leaves of the control cv "Sharp 1", the pure line

"1021", and both of the cp^- and cp^+ plants of F_1F-2 and F_1F-7 (several plants each) following the inoculation of ZYMV into the cotyledons or the young first and second leaves (Fig. 5A,B). No difference in the degree of symptom development was found between the control cv "Sharp 1" and "1021" and both the cp^- and cp^+ plants of F_1F-2 and F_1F-7.

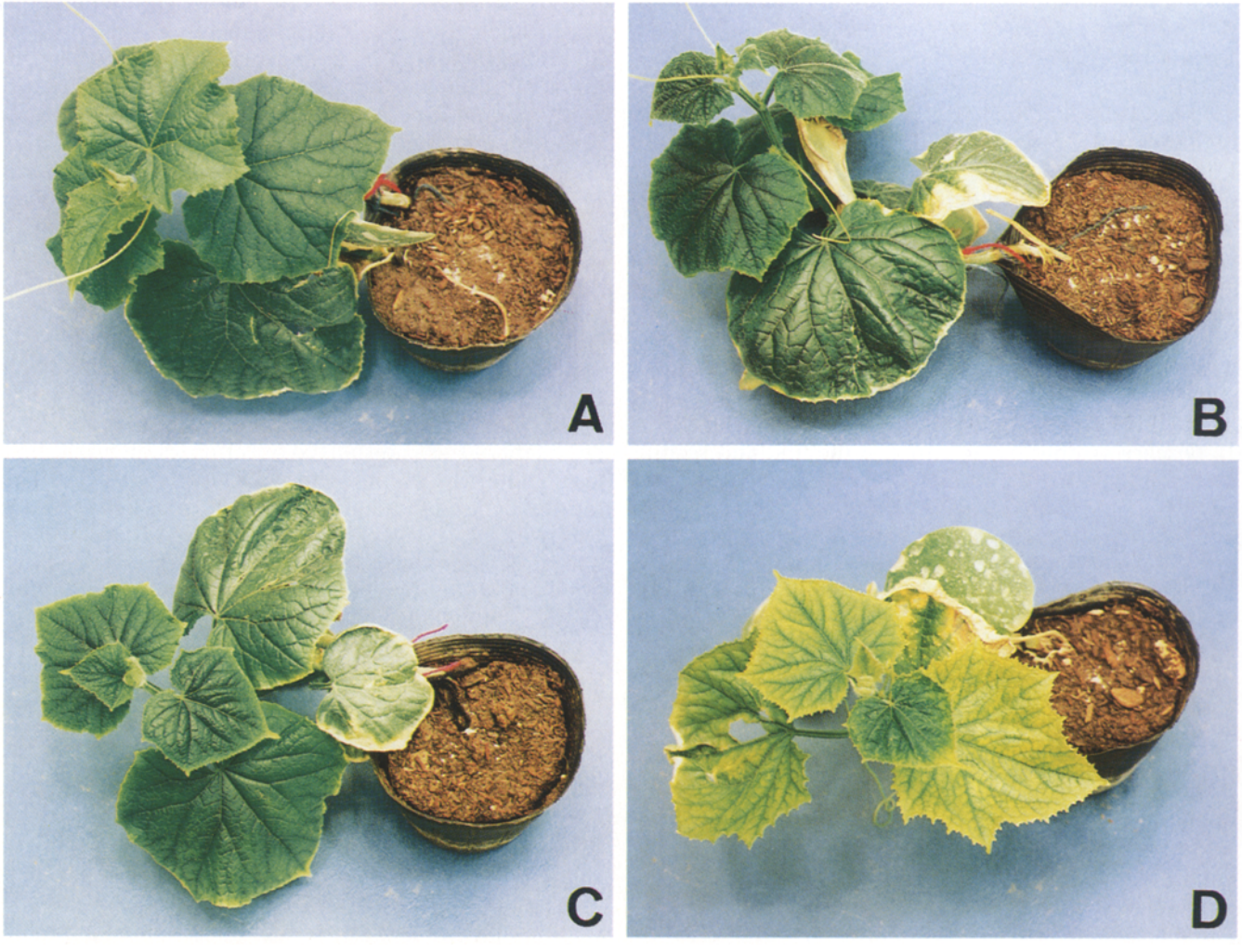
Double inoculation of CMV and ZYMV

Control cv "Sharp 1" and pure line "1021" plants (five plants each), which were inoculated with both CMV and ZYMV, displayed very severe disease symptoms (necrosis) on the leaves 10–14 days after inoculation into the cotyledons or the young first and second leaves. By contrast, each of the three cp^+ plants of F_1F-2 and F_1F-7 displayed fewer symptoms than the control cv "Sharp 1", in the pure line "1021", and the cp^- plants of F_1F-2 and F_1F-7 (3–5 plants each) following double inoculation with CMV and ZYMV (Fig 5C, D). In this case, the cp^+ plants of F_1F-2 and F_1F-7 showed similar symptoms to those caused by ZYMV inoculation (Fig. 5B, C). That is, the cp^+ transgenic plants showed a reduced degree of symptom development in response to double inoculation of CMV and ZYMV which individually caused very severe disease symptoms (necrosis).

Discussion

Though the RNA transcripts from the CaMV 35*s*-*cp* gene could be detected in the leaves of R_0 transgenic cucumber plants, as well as in epicotyls containing two cotyledons of the transgenic progeny plants, by Northern-blot analysis, the presence of the coat protein originating from the CaMV 35*s*-*cp* gene could not be detected in either cotyledons or leaves of R_0 and transgenic progeny plants by Western-blot analysis. GUS expression was observed in the cotyledons of the transgenic progeny plants following with both X-gluc (visual

Fig. 5A–D Virus inoculation experiment in the cp^+ and cp^- plants of F_1F-2. **A** Control (no inoculation). **B** A cp^+ plant inoculated with 100 µg/ml ZYMV alone on the leaves. **C** A cp^+ plant inoculated with both 100 µg/ml CMV and 100 µg/ml ZYMV on the leaves. The disease symptom (waved leaves) is similar to that of the plant (**B**) inoculated with ZYMV alone. **D** A cp^- plant inoculated with both 100 µg/ml CMV and 100 µg/ml ZYMV on the leaves. The disease symptom is very severe (necrotic). The leaves changed from green to yellow



assay) and MUG (enzymatic assay), but could not be detected in the leaves of the R_0 and transgenic progeny plants. In a previous report (Gonsalves et al. 1992), where transgenic cucumber plants (cv "Poinsett 76") were analyzed by ELISA, the presence of expressed coat protein was also not detected. Here, however, Northern-blot analysis was not carried out. Judging from the results of GUS analysis and Northern-blot analysis, it is suggested that the CaMV 35*s* promoter functions actively in the cotyledon cells but only slightly in leaf cells, as noted in 1996 a previous report (Nishibayashi et al. 1996). However, in spite of the expression of CaMV 35*s*-I-*gus* in the cotyledons, the reason why the presence of expressed coat protein could not be observed in the cotyledons is probably due to the fact that the specificity of the immunological assay is not adequate to detect it. Moreover, if there is expressed coat protein in the leaf cells, it would be expected to be only a very small amount.

The strength of resistance to the virus in transgenic plants has been reported both to be parallel to the amount of expressed coat protein (Loesch-Fries et al. 1987) and not to be parallel (Namba et al. 1991).

In previous transgenic tobacco plants into which the same CMV-O *cp* gene was introduced (Hayakawa et al. 1990; Nakajima et al. 1993), a single clear band which showed the expression of the CaMV 35*s*-*cp* gene was detected in leaves on both Northern and Western blots. Although the transgenic tobacco plants displayed symptom appearance at a frequency of 25% with a treatment of 1 μg/ml CMV-Y, they showed a frequency of 70–80% following with 10 μg/ml CMV-Y and a frequency of 100% with a treatment of 50 μg/ml CMV-Y. The transgenic tobacco plants were thus susceptible to the inoculation of a high concentration of CMV-Y. The comparison between the transgenic tobacco and cucumber plants in Western-blot analysis and the virus inoculation experiment shows that the CMV resistance of transgenic plants into which the CMV-O *cp* gene is introduced is not parallel to the amount of expressed coat protein. The same phenomenon was observed in transgenic tobacco plants into which the CMV-WL *cp* gene was introduced (Namba et al. 1991). In this case, the expressed coat protein was detected by ELISA analysis.

In cucumber plants, both of the transgenic plant lines displayed no symptom with a CMV-Y treatment of 100-, or 500- and 1000-times concentration, whereas control plants did display symptom appearance (at a concentration of 1 μg/ml). It is unclear why the transgenic tobacco and cucumber plants gave a different response to CMV inoculation, in spite of the introduction of the same CMV-O *cp* gene. One possibility is that the susceptibility to CMV is different between the tobacco and cucumber plants and that this affects the degree of symptom development in response to CMV inoculation.

As previously reported by Gonsalves et al. (1992), 14% of the transgenic cucumber plants (cv "Poinsett 76") into which the CMV-C *cp* gene was introduced displayed symptoms, 50% displayed delayed symptoms, and 36% were symptomless after inoculation with CMV-CAT in a greenhouse. In this case, therefore, 64% of the transgenic cucumber plants finally displayed virus symptoms in response to the CMV-CAT inoculation. The cv "Poinsett 76" used by Gonsalves et al. (1992) was clearly more susceptible than the cv "Sharp 1" and pure line "1021".

The CMV resistance of the present transgenic cucumber plants seems to be due to the synergism of the slight CMV tolerance, particularly in the pure line "1021", and the protection against CMV caused by the introduction of the CMV *cp* gene. This leads to the possibility of producing cucumber plants with the characteristics of very strong CMV resistance by the combination of cucumber genotypes and the CMV *cp* gene. The present transgenic plants should then be useful as a genetic source for breeding to produce CMV-resistant cucumber plants, which can be cultivated in the field from spring to summer.

It will, however, be necessary to study the plant growth, fruit yield, and CMV resistance in the transgenic cucumber plants which are grown in the field.

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