

Comparative Susceptibility of Transgenic Tobacco Plants and Protoplasts Expressing the Coat Protein Gene of Cucumber Mosaic Virus to Infection with Virions and RNA

Tetsuro Okuno, Masaharu Nakayama, Shigeru Yoshida, Iwao Furusawa, and Takeya Komiya

First and fourth authors: Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-01, Japan. Second author: Agricultural Laboratories, Takeda Chemical Industries Ltd., Wadai, Tsukuba Science City 300-42, Japan. Third and fifth authors: Research Laboratories of Integrated Division, Takeda Chemical Industries, Ltd., Ichijoji, Sakyo-ku, Kyoto 606, Japan.

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ABSTRACT

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A cDNA clone encoding the coat protein (CP) gene of cucumber mosaic virus (CMV, Y strain), linked to the cauliflower mosaic virus (CaMV) 35S transcript promoter, was introduced into tobacco tissues by either electroporation or *Agrobacterium*-mediated gene transfer. The inoculated and upper systemic leaves of transgenic tobacco plants expressing the CMV-CP gene were highly protected from infection with CMV, even when the concentration of CMV-virion inoculum was 100 $\mu\text{g}/\text{ml}$. Inoculated leaves, however, often showed greater susceptibility to CMV RNA than to CMV virions. Protoplasts isolated from these transgenic plants also were protected from infection when inoculated with CMV virions but were as susceptible as the control protoplasts when inoculated with

CMV RNA and unrelated viruses, such as tobacco mosaic virus and brome mosaic virus (BMV), and their respective RNAs. The results suggest protection from virus infection in transgenic protoplasts expressing the CMV-CP gene was specific to infection by CMV virions, and interference with an early event in the infection process, such as uncoating virus particles, is probably involved in the resistance of transgenic tobacco plants expressing the CMV-CP gene. The observed resistance against CMV RNA in whole plants suggests virus replication may occur in primary infected cells, but the initiation of the replication process in adjacent cells may be inhibited by the resident CMV-CP gene if RNA encapsidation or binding to the CP is required for cell-to-cell and long-distance transport.

Many studies have demonstrated the great potential of genetically engineered plants with virus-related genes to control virus diseases (2,4). Numerous researchers have shown the resistance of transgenic plants expressing a viral coat protein (CP) gene to infection by a homologous virus. Examples include tobacco mosaic virus (TMV) (25), alfalfa mosaic virus (AIMV) (12,33,36), cucumber mosaic virus (CMV) (6,18,26), potato virus X (PVX) (9), tobacco streak virus (TSV) (37), tobacco rattle virus (TRV) (35), potato virus Y (11), potato leafroll virus (10), and tomato spotted wilt virus (TSWV) (13). Protection has been characterized by a decrease in the number of primary infection sites on inoculated leaves and by a delay or lack of systemic symptom development. Transgenic plants expressing the CP gene of TMV, AIMV, TSV, or TRV showed greater susceptibility to infection with viral RNA than to infection with corresponding virions (12,19,36,37).

These observations of whole plants and similar results obtained from protoplast research with AIMV (12), TMV (27), and TRV (1) suggest an early event in the infection process, such as the uncoating of virus particles, is affected mainly by the endogenously expressed CP gene. On the other hand, protection from viral RNA has been reported in transgenic plants expressing the corresponding CP gene in PVX (9) and AIMV (34).

CMV CP-mediated protection has been reported in transgenic tobacco plants expressing the CP gene of CMV-D, -WL, and -C strains (6,18,26). However, research on susceptibility to infection with CMV RNA and on the susceptibility of protoplasts has not been done. We produced transgenic tobacco plants expressing the CMV-CP gene and compared the susceptibility to infection with CMV virions and CMV RNA in both whole plants and protoplasts. Virus accumulation was monitored by ELISA (enzyme-linked immunosorbent assay) and fluorescent antibody staining. Our results show transgenic plants and protoplasts expressing the CMV-CP gene were highly protected from infection

with CMV virions and RNA. However, protoplasts isolated from the transgenic plants, unlike whole plants, were susceptible to infection with CMV RNA. Resistance to CMV-RNA infection in whole plants might be explained by the fact that RNA encapsidation or binding to the CP is required for cell-to-cell and long-distance transport. Thus, virus replication may occur in primary infected cells, but the initiation of the replication process in adjacent cells may be inhibited by the resident CMV-CP gene. The properties of genetically engineered cross-protection with the CP gene of CMV are compared with those of other virus systems and are discussed.

MATERIALS AND METHODS

Construction of transformation vectors. A cDNA clone containing the CMV-CP gene was characterized previously (8). This cDNA clone was partially digested with *AccI* and then was digested with *HaeII* to release a DNA fragment (903 bp) containing the CP gene but not the two ATGs present approximately 20 and 24 nucleotides from the 5' to the transcription-initiation site of CMV RNA4. The fragment was gel purified and was blunt ended with T4 DNA polymerase. Both ends of the fragment were converted into *BamHI* sites using synthetic linkers, 5'-pd(CGGATCCG)-3'. This fragment was digested with *BamHI* and was subcloned into the *BamHI* site of the pBluscript SK⁻ mutant (Stratagene, La Jolla, CA) to provide a plasmid pCCP containing an *XbaI* site 5' to the CP-gene initiation codon. To obtain a transformation vector for electroporation, a plasmid containing the CMV-CP gene and neomycin phosphotransferase II (NPT II) gene-expression cassettes in the same orientation were constructed using pCAM35 (14). The *XbaI-KpnI* fragment (993 bp) containing the CMV-CP gene was excised from pCCP, was gel purified, and was inserted between the CaMV 35S transcript-promoter and -terminator regions using the corresponding enzyme sites of pCAM35 to create pCAM35-CCP (Fig. 1). The DNA fragment (1,003 bp) containing the NPT II gene was excised from pNEO (Pharmacia, Inc., Biotechnology Group, Piscataway, NJ) by *SmaI* and *BglII* digestion and was inserted into the corresponding enzyme sites between the CaMV 35S promoter and terminator regions in pCAM35 to create pCAM35-N. The *EcoRI-HindIII* fragment (1,703bp) containing the NPT II gene-expression cassette was excised from pCAM35-N and was gel purified. Both ends of the fragment were blunt ended by T4 DNA polymerase and were converted into *EcoRI* sites using synthetic linkers, 5'-pd(CCGAATTCGG)-3'. This DNA fragment was digested with *EcoRI* and was subcloned into the *EcoRI* site of pUC19. The *EcoRI* fragment of the plasmid containing the NPT II gene-expression cassette was inserted into the *EcoRI* site of pCAM35-CCP to create pCAM35-CCPN. To obtain a binary vector for *Agrobacterium*-mediated plant transformation, the *XbaI-KpnI* fragment containing the CMV-CP gene was excised from pCCP, was gel purified, and was inserted between the CaMV 35S promoter and terminator regions of a binary vector, pBIC35 (16), using the corresponding enzyme sites to create pBIC35-CCP.

Production of transgenic plants. The CMV-CP gene was inserted into tobacco plants by either electroporation or *Agrobacterium*-mediated gene transfer. For gene transfer by electropor-

ation, pCAM35-CCPN and pCAM35-N (for vector control) were linearized by *ApaI* digestion and were used for transformation of tobacco protoplasts (*Nicotiana tabacum* L. 'Bright Yellow'). Protoplasts were prepared as described (21) and were suspended in 0.4 M mannitol to produce a concentration of 5×10^5 cells per milliliter. Protoplast suspension (1 ml) was mixed with an equal volume of 0.4 M mannitol containing 12 μ g of plasmid DNA per milliliter. The mixture (800 μ l) was subjected immediately to electroporation using a square wave pulse generator (SSH-1, Shimadzu Ltd., Kyoto, Japan). The pulse length was 60 μ s, and the field strength was 500–1,500 v/cm. Protoplasts were diluted with 7.2 ml of K3 medium (17) and were cultured at 25 C for 2 wk. Protoplast-derived colonies were embedded in K3 medium containing 0.4% agarose and were cultured in MS medium (16) containing 0.5 μ g/ml of benzylaminopurine (BAP) and 50 μ g/ml of kanamycin sulphate. Kanamycin-resistant colonies were placed on 0.8% MS medium agar containing 0.5 μ g/ml of BAP and 100 μ g/ml of kanamycin sulphate (shooting medium), and kanamycin-resistant shoots were transferred to 0.8% MS medium agar containing 0.5 μ g/ml of naphthalen acetic acid, 0.025 μ g/ml of BAP (rooting medium), and 100 μ g/ml of kanamycin sulphate. After 2–3 wk, plantlets with root systems were transferred to soil and were grown in a greenhouse at 28 C during the day and 22 C during the night.

Agrobacterium-mediated transformation of tobacco was carried out as described by Mori et al (15). Approximately 50 kanamycin-resistant plants were obtained by either of the methods described above. These plants (R₀) were tested for susceptibility to virus infection by inoculating them with 3 μ g of CMV virions per milliliter and were tested for the expression of the CMV-CP gene by Western blot analysis. Those plants that did not develop systemic symptoms and had detectable levels of CP were selected. All transgenic plants (R₀) that did not contain detectable levels of CMV-CP developed systemic symptoms. Seeds from selfed transgenic plants were germinated on 0.8% MS medium agar containing 100 μ g of kanamycin sulphate per milliliter, and kanamycin-resistant plants (R₁) were tested further for resistance to CMV infection and for the expression of the CMV-CP gene, as described above. After two more cycles of similar selection, seeds were harvested from transgenic plants that were protected from CMV infection and that expressed the CMV-CP gene. Three high-expressor transgenic plants, designated CP-E11, CP-E18 (obtained by electroporation), and CP-A3 (obtained by *Agrobacterium*-mediated transformation), were selected. Seeds obtained from progeny plants (R₃) were used for further experiments without antibiotic selection. Vector-control kanamycin-resistant transgenic plants were similarly selected and were designated KR-E (obtained by electroporation) and KR-A (obtained by *Agrobacterium*-mediated transformation).

RNA analysis. Total RNA was extracted from leaves of transgenic and control tobacco plants, as described by Chomczynski and Sacchi (5). Leaves (5 g) were frozen with liquid nitrogen and powdered with a mortar and pestle. RNA (20 μ g) extracted from the powder was denatured in formaldehyde/formamide, was fractionated on a 1.2% agarose gel containing 0.67 M formaldehyde, and was transferred to a nylon membrane (Hybond N) according to the procedure of Sambrook et al (28). CMV-related

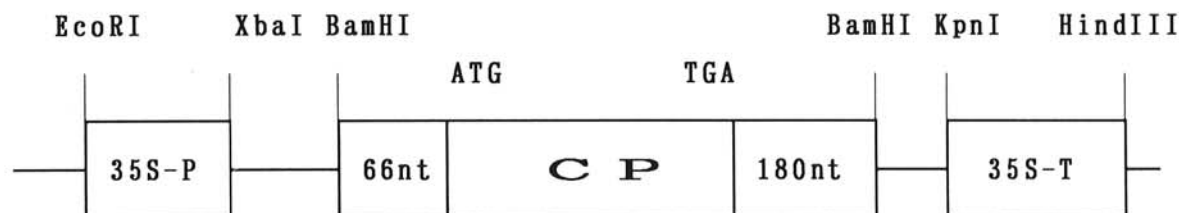


Fig. 1. Schematic representation of the chimeric gene constructed to express the coat protein (CP) gene of cucumber mosaic virus (CMV). The cDNA fragment (903 bp) containing the CMV-CP gene consists of a 66-nucleotide untranslated sequence 5' to the open reading frame (654 nucleotide) and 180-nucleotide 3' to the stop codon of the open reading frame. The fragment was inserted between the CaMV 35S transcript-promoter (35S-P) and -terminator (35S-T) regions in a binary vector, pBIC35, which was used for *Agrobacterium*-mediated transformation and in pCAM35, which was used to construct a vector plasmid for electroporation.

RNA was detected by hybridization with a ^{32}P -labeled DNA probe (7) prepared from the *Xba*I-*Kpn*I fragment of pCAM35-CCP using a random primer DNA labeling kit (Takara Shuzo Co., Kyoto, Japan).

Protein analysis. Accumulation of the CMV-CP gene in transgenic plants and protoplasts was analyzed by the Western blotting method. Leaves of transgenic and untransformed tobacco plants were homogenized with a mortar and pestle in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 10 mM 2-mercaptoethanol. The leaf sap was clarified by centrifugation, and the protein concentration was determined according to the method of Bradford (3). Immunoblot analysis was carried out as described by Towbin et al (32). Proteins (20 μg) were electrophoresed on a 12.5% polyacrylamide gel containing 0.1% SDS (sodium dodecyl sulfate) as described by Okuno and Furusawa (21) and were transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA).

Purification of virus and viral RNA. CMV-Y strain was propagated in *N. tabacum* cv. Samsun NN and was purified as described by Takanami (30). TMV-OM strain was propagated in *N. tabacum* cv. Bright Yellow and was purified by the same method used for CMV. BMV-ATCC66 strain was propagated and purified as previously described by Okuno and Furusawa (20). Viral RNA was isolated from purified virus as described by Okuno et al (22).

Infectivity assays. Tobacco plants at the four- to five-leaf stage were used for assays of virus infection. The third leaf from the top of each untransformed, vector-transformed, and transgenic plant expressing the CMV-CP gene was dusted with Carborundum (600 mesh) and was mechanically inoculated with CMV virions or CMV RNA at concentrations of 3–100 $\mu\text{g}/\text{ml}$. TMV was applied at a concentration of 3 $\mu\text{g}/\text{ml}$ in 50 μl of 0.01 M phosphate buffer, pH 7.2, containing 2 mM EDTA. The inoculated leaf surface was immediately rinsed with tap water, and the plants were grown in the greenhouse. Two transgenic control plants and two untransformed control plants were mock-inoculated with buffer. Symptom development on the inoculated and systemic leaves was monitored daily, and virus accumulation was estimated by ELISA, as described below.

Protoplast experiments. Mesophyll protoplasts were enzymatically isolated in 0.5 M mannitol, as described by Okuno and Furusawa (20). Protoplasts were inoculated with virions or viral RNAs in 10 mM citrate buffer, pH 5.3, containing 0.5 M mannitol, essentially as described before (20), except poly-L-ornithine was replaced by polyethyleneimine (31). Protoplasts were incubated for 24 h, and infection was determined by fluorescent antibody staining (20) and by ELISA.

ELISA. Indirect ELISA was used to assess the accumulation of virus in leaves and protoplasts. Leaf samples were homogenized

with 1:20 (w/v) of 0.05 M carbonate buffer, pH 9.6, and duplicates or triplicates of each sample (200 μl), including standards and blanks, were placed in microtiter wells and were incubated overnight at 7 C. Protoplasts (approximately 2×10^5) in 1.5-ml tubes were pelleted in a microcentrifuge and were suspended in 1 ml of 0.05 M carbonate buffer, pH 9.6. After vortexing for 1 min, 200 μl of the sap, after appropriate dilution, was placed into the microtiter wells. Other procedures and conditions were the same as those described by Okuno et al (22). Absorbance at 405 nm was determined with a microplate reader (Model 450, Bio-Rad Laboratories, Richmond, CA) after 60 min of incubation with the substrate.

RESULTS

Expression of CP in transgenic plants. Expression of the CMV-CP gene was examined by immunoblot analysis of proteins extracted from transgenic and untransformed tobacco plants (Fig. 2). A protein that migrated to the same position as CMV-CP and reacted with CMV antiserum accumulated in leaves of CP-E11, CP-E18, and CP-A3 plants but was not detected in leaves of untransformed and vector-transformed KR-E tobacco plants. Although CMV antiserum reacted with some host components, such nonspecific reactions were not observed at the CMV-CP position in control plants. The level of CP was estimated to range between 0.05 and 0.2% of the extracted protein (data not shown) by comparing band intensities of transgenic, vector-transformed, and untransformed tobacco plants to band intensities of purified CMV mixed with untransformed tobacco-plant extracts (Fig. 2), without considering some proteolysis by host enzymes.

Expression of RNA in transgenic plants. In Northern blots, the CP gene-specific probe hybridized to RNA extracted from transgenic CP-E11, CP-E18, and CP-A3 tobacco plants but not to RNA extracted from vector-transformed KR-E and untransformed tobacco plants (Fig. 3). The size of the RNA, approximately 1,300 nucleotides, is the approximate size expected of the CMV-CP gene transcript from the chimeric DNA.

Susceptibility of transgenic plants to infection with CMV virions and RNA. Inoculation of control plants with either CMV virions or CMV RNA elicited typical systemic symptoms 5–7 days after inoculation, and symptom development was indistinguishable between inoculation with CMV virions or with CMV RNA. In contrast, no symptoms were observed on any of the CP-E11 or on most of the CP-E18 plants when inoculated with CMV virions. Approximately 70% of the transgenic plants expressing the CMV-CP gene did not develop systemic symptoms 25 days after inoculation with CMV RNA (Table 1). To assess the level of transgenic plant resistance to infection with CMV virions and

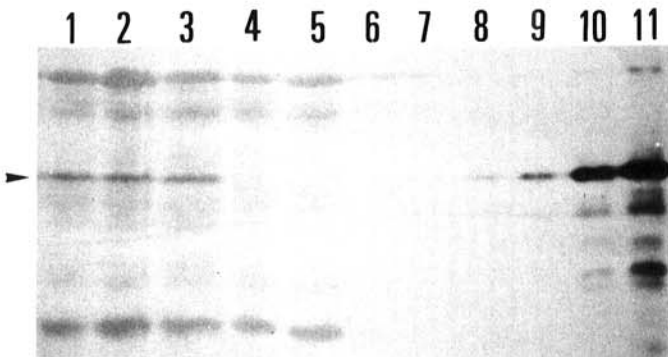


Fig. 2. Immunoblot analysis of the accumulation of the coat protein (CP) of cucumber mosaic virus (CMV) in transgenic tobacco plants. Proteins were extracted from leaves of individual tobacco plants. The lanes illustrate proteins from transgenic CP-E11, CP-E18, and CP-A3 (lanes 1–3, respectively), vector-transformed KR-E (lane 4), and untransformed tobacco plants (lane 5). As a standard, protein from 5, 10, 20, 50, 100, and 200 ng of purified CMV particles (lanes 6–11, respectively) is included. An arrow indicates the position of CMV CP.

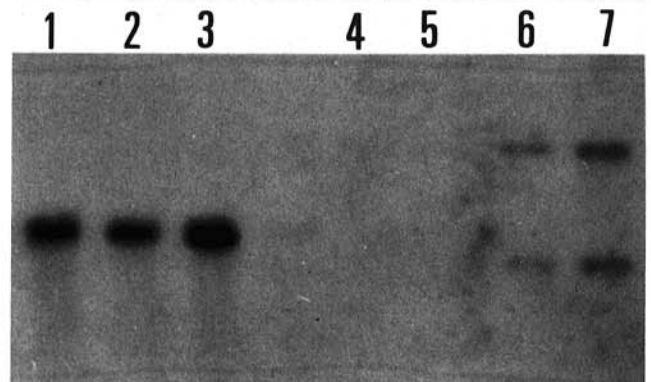


Fig. 3. Northern blot analysis of total RNA extracted from leaves of transgenic tobacco plants expressing the coat protein (CP) of cucumber mosaic virus (CMV). RNA extracted from transgenic CP-E11, CP-E18, and CP-A3 (lanes 1–3, respectively), untransformed (lane 4), and vector-transformed KR-E (lane 5) plants. As a standard marker, CMV RNA from 0.1 ng (lane 6) and 1 ng (lane 7) of purified CMV particles is included. The positions of CMV RNA 3 and 4 are indicated. The blot was probed with ^{32}P -labeled cDNA of the CMV-CP gene.

RNA, transgenic plants expressing the CMV-CP gene and vector-control KR-E plants were inoculated with increasing concentrations of inoculum, and symptom development was monitored daily after inoculation. Transgenic plants were highly protected from infection with both CMV virions and RNA at concentrations as high as 100 µg/ml (Table 2). Because transgenic plants in most reported cases of CP-mediated protection showed greater susceptibility to infection with viral RNA than to virions, monitoring the relative accumulation levels of CMV in leaves after inoculation with CMV virions and RNA, using ELISA, was of great interest. After inoculation with CMV RNA at a 10 µg/ml concentration, negligible or small amounts of CMV were present in the leaves of CP-E18 plants, and accumulation levels were not significantly different from levels detected in CMV virion-inoculated leaves (3 µg/ml; Table 3, experiment 1).

The results show that the transgenic plants were resistant equally to infection with CMV virions and RNA. However, these results seemed to contradict those obtained with protoplasts from transgenic plants that were highly susceptible to CMV RNA. As a result, we repeated these experiments using different concentrations of inoculum to assess whether there is any difference in virus accumulation between virion-inoculated and RNA-inoculated leaves in transgenic plants expressing the CP gene. Although it was evident that virus-accumulation levels were extremely low in the CP expressors compared to nonexpressors after inoculation with virions or RNA, statistical analysis indicated that the levels of virus accumulation in the RNA-inoculated leaves were significantly higher than those in the virion-inoculated leaves (Table 3, experiments 2 and 3). The difference could be more distinct because in control plants the number of chlorotic spots was always less on RNA-inoculated leaves than on virion-inoculated leaves at a similar inoculum concentration (data not shown).

CMV was not detected in the systemic leaves of any plants that did not show systemic symptoms when assayed 25 days after inoculation (data not shown).

To test the susceptibility of transgenic plants expressing the CMV-CP gene to an unrelated virus, five CP-E18 plants were inoculated with TMV, 3 µg/ml. All plants tested developed typical systemic symptoms. No difference in symptom development was observed between transgenic and untransformed tobacco plants (data not shown).

Susceptibility of protoplasts to virus infection. Protoplasts from transgenic plants expressing the CMV-CP gene were tested for susceptibility to infection with CMV virions and RNA. Protoplasts isolated from untransformed and vector-transformed tobacco plants were used as controls. The protoplasts from transgenic plants were resistant to infection with CMV virions, as evidenced by the lower number of fluorescing protoplasts and the reduced accumulation of virus, as assessed by fluorescent antibody staining and ELISA, respectively (Table 4).

On the other hand, transgenic protoplasts expressing the CMV-CP gene were as susceptible as the control protoplasts when inoculated with CMV RNA (Table 4). To confirm the results and to determine the susceptibility of transgenic protoplasts to infection with an unrelated virus and its RNA, protoplasts were inoculated with a mixture of CMV and TMV virions or their RNAs. The results in Table 5 show that protoplasts isolated from transgenic plants expressing the CMV-CP gene were protected only from infection with CMV virions, not from TMV, TMV RNA, or CMV RNA. Transgenic protoplasts expressing the

TABLE 1. Development of systemic symptoms in control and transgenic tobacco plants expressing the cucumber mosaic virus-coat protein (CMV-CP) gene after inoculation with CMV and CMV RNA^a

Plant	Inoculum	Plants with systemic symptoms (%)					
		Days after inoculation					
		5	7	10	15	20	25
Untransformed (23) ^b	Virion	65	91	100	100	100	100
Untransformed (23)	RNA	48	65	100	100	100	100
KR-E (23)	Virion	43	61	100	100	100	100
KR-E (23)	RNA	22	52	100	100	100	100
CP-E18 (24)	Virion	0	0	0	0	0	0
CP-E18 (24)	RNA	0	0	25	25	29	29
CP-E11 (25)	Virion	0	0	12	12	20	20
CP-E11 (25)	RNA	0	16	16	24	28	28

^a Plants were inoculated with CMV or CMV RNA at a 10 µg/ml concentration.

^b Numbers in parentheses are the total number of plants used for the tests, repeated four times using 5–7 plants in each treatment.

TABLE 2. Development of systemic symptoms in control and transgenic tobacco plants expressing the cucumber mosaic virus-coat protein (CMV-CP) gene after inoculation with CMV and CMV RNA^a

Plant	Inoculum Type	Conc. (µg/ml)	Plants with systemic symptoms (%)					
			Days after inoculation					
			6	7	8	10	15	20
KR-E (20) ^b	Virion	10	60	100	100	100	100	100
KR-E (20)	RNA	10	0	80	100	100	100	100
CP-E11(19)	Virion	50	0	0	11	11	11	11
CP-E11(20)	Virion	100	0	0	0	15	15	15
CP-E11(18)	RNA	50	0	11	11	11	11	22
CP-E11(18)	RNA	100	11	11	11	28	28	28

^a Plants were inoculated with CMV or CMV RNA at indicated concentrations (Conc.).

^b Numbers in parentheses are the total number of plants used for the tests, repeated three times using 5–7 plants in each treatment.

TABLE 3. Virus accumulation in inoculated leaves of control and transgenic tobacco plants expressing the cucumber mosaic virus-coat protein (CMV-CP) gene after inoculation with CMV and CMV RNA^a

Plant	Inoculum	Conc. (µg/ml)	Plants with symptoms ^b (%)	Virus accumulation by ELISA
				(<i>A</i> _{nm405}) ^c
Experiment 1				
Untransformed	Virion	3	100	1.08 ± 0.04
CP-E18	Virion	3	0	0.04 ± 0.01
Untransformed	RNA	10	100	1.41 ± 0.14
CP-E18	RNA	10	0	0.14 ± 0.05
Experiment 2				
Untransformed	Virion	10	100	1.61 ± 0.04
CP-E18	Virion	10	0	0.13 ± 0.07
Untransformed	RNA	10	100	1.45 ± 0.10
CP-E18	RNA	10	0	0.45 ± 0.14
Experiment 3				
KR-E	Virion	100	100	1.89 ± 0.16
CP-E11	Virion	100	0	0.09 ± 0.06
KR-E	RNA	40	100	1.37 ± 0.14
CP-E11	RNA	40	0	0.34 ± 0.06

^a Seven, six, and six plants (experiments 1–3, respectively) were used for each treatment in the tests; approximately one-fourth of the inoculated leaves from each test plant were cut off 8 days (experiment 1 and 3) and 7 days (experiment 2) after inoculation. Virus accumulation was determined by ELISA. The values at *A*_{nm405} after 60 min of incubation with the substrate are presented.

^b No transgenic plants expressing the CMV-CP gene developed systemic symptoms 20 days after inoculation, except for one CP-E18 plant inoculated with CMV RNA in experiment 2, although the plant had no detectable symptoms 14 days after inoculation.

^c Values are means (±SD) after subtracting the mean values of corresponding leaves from mock-inoculated plants: 0.01 for untransformed and 0.05 for CP-18 (experiment 1), 0.02 for untransformed and 0.03 for CP-E11 (experiment 2), and 0.02 for KR-E and 0.13 for CP-E11 (experiment 3). Virus-accumulation level was significantly different (*P* = 0.05) between plants expressing the CMV-CP gene and those not expressing the CMV-CP gene, according to Duncan's multiple range test. When the virus-accumulation level was compared between virion inoculation and RNA inoculation in either the CP-expressor or nonexpressor controls, the accumulation level was significantly different (*P* = 0.05), according to Duncan's multiple range test, except for that of CP-expressors in experiment 1.

TABLE 4. Susceptibility of protoplasts isolated from tobacco plants to infection with cucumber mosaic virus (CMV) and CMV RNA^a

Protoplasts	Inoculum			
	Virion		RNA	
	(%) ^b	<i>A</i> _{nm405} ^c	(%)	<i>A</i> _{nm405}
Experiment 1				
Untransformed	87.3	0.87	97.8	1.02
KR-E	85.4	0.92	98.6	1.06
CP-E18	3.5	0.06	97.1	0.93
CP-A3	2.3	0.03	98.9	0.97
Experiment 2				
KR-A	73.2	1.17	96.2	1.36
CP-E11	4.2	0.09	97.2	1.30
CP-E18	1.1	0.05	95.3	1.31
CP-A3	1.3	0.05	87.5	1.25

^a Protoplasts (approximately 5×10^5) were inoculated with 0.5 $\mu\text{g/ml}$ of CMV or 0.25 $\mu\text{g/ml}$ of CMV RNA together with 0.5 $\mu\text{g/ml}$ of polyethyleneimine; infection was assessed by fluorescent antibody staining and by ELISA after 19 h (experiment 1) and 24 h (experiment 2) of incubation.

^b Fluorescing protoplasts.

^c Mean values of triplicates in each sample after subtracting the mean values of corresponding mock-inoculated protoplasts: 0.02 for untransformed, 0.01 for KR-E, 0.05 for CP-E18, and 0.04 for CP-A3 (experiment 1); and 0.02 for KR-E, 0.08 for CP-E11, 0.05 for CP-E18, and 0.06 for CP-A3 (experiment 2). Values after 60 min of incubation with the substrate in ELISA are presented.

CMV-CP gene also were highly susceptible to infection with BMV and its RNA (data not shown).

DISCUSSION

Numerous reports indicate that transgenic tobacco plants expressing the CP gene of CMV-D, -WL, and -C strains are protected against infection with CMV virions (6,18,26), but the susceptibility to infection with viral RNA has not been tested. Likewise, the susceptibility of protoplasts from such transgenic plants to CMV infection has not been tested. To obtain further insight into the mechanism(s) of CMV-CP gene-mediated protection in transgenic plants, we compared the susceptibility to infection with virus particles and RNA in whole plants and protoplasts. Our results indicated that transgenic plants expressing the CP gene of CMV-Y strain were greatly protected from infection with either CMV virions or CMV RNA but not from infection with unrelated viruses, such as TMV. In most cases of CP-mediated protection, transgenic plants have shown greater susceptibility to infection with viral RNA than to infection with virions (2). Protection from infection with viral RNA has been reported in transgenic plants expressing the corresponding CP of PVX (9) and AIMV (34). In either case, transgenic CP expressors were resistant to infection with PVX and AIMV RNA at concentrations of 20 and 50 $\mu\text{g/ml}$, respectively, as evidenced by a decrease in the number of infection sites and/or the amount of virus accumulation in inoculated leaves. However, it is noteworthy that the protection in one line of lower PVX-CP expressor was overcome by PVX-RNA inoculum at a concentration of 5 $\mu\text{g/ml}$. Furthermore, another report indicated that AIMV-CP expressors were only protected from infection with virions not from AIMV RNA (12). Although our CMV-CP expressors were highly protected from infection with CMV RNA, inoculated leaves of these plants seemed to be more susceptible to infection with CMV RNA than to infection with CMV virions; we often observed that the levels of virus accumulation in leaves inoculated with CMV RNA were significantly higher than in those inoculated with CMV virions, even when these plants did not develop systemic symptoms (Table 3, experiments 2 and 3). Because protoplasts from these transgenic plants were highly susceptible to infection with viral RNA, the observed resistance to infection with CMV RNA in whole plants suggests that the virus may replicate in primary infected cells, but initiation of the replication process in adjacent cells of the inoculated leaves and in cells involved in systemic spread of the

TABLE 5. Susceptibility of protoplasts isolated from tobacco plants to infection with a mixture of cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) or their RNAs^a

Protoplasts	Inoculum							
	Virion				RNA			
	(%) ^b	<i>A</i> _{nm405} ^c	(%)	<i>A</i> _{nm405}	(%)	<i>A</i> _{nm405}	(%)	<i>A</i> _{nm405}
KR-E	77.1	1.51	43.9	0.47	90.7	1.93	85.0	0.88
CP-E11	1.3	0.04	40.6	0.41	92.3	1.88	88.1	0.92
CP-E18	4.5	0.08	42.2	0.49	89.7	1.79	82.3	0.80
CP-A3	2.1	0.05	45.1	0.49	87.6	1.86	76.3	0.71

^a Protoplasts (approximately 5×10^5) were inoculated with a mixture of CMV and TMV at 0.5 $\mu\text{g/ml}$ concentrations in each and a mixture of viral RNA at 0.25 $\mu\text{g/ml}$ concentrations in each together with 0.5 $\mu\text{g/ml}$ of polyethyleneimine; infection was assessed by fluorescent antibody staining and by ELISA after 24 h of incubation.

^b Fluorescing protoplasts.

^c Mean values of duplicates in each sample after subtracting the mean values of corresponding mock-inoculated protoplasts incubated with CMV antiserum: 0.01 for KR-E, 0.05 for CP-E11, 0.04 for CP-E18, and 0.05 for CP-A3; and 0.01 for all samples incubated with TMV antiserum. Values after 60 min of incubation with the substrate in ELISA are presented.

virus may be inhibited by endogenously expressed CMV-CP gene. Indeed, RNA encapsidation or formation of nucleoprotein with the CP is required for cell-to-cell transport of spherical plant viruses, such as cowpea mosaic virus (38) and CMV (29).

These results also suggest that replication of incoming viral RNA is not inhibited in the cells of transgenic plants expressing the CMV-CP gene, and the infection process prior to release of RNA from virus particles, such as uncoating of virus particles or binding of virus particles to specific receptors, may be inhibited in transgenic plants and in their protoplasts expressing the CMV-CP gene. Others have proposed similar mechanisms to explain the higher susceptibility to infection with viral RNA than to infection with virus particles in transgenic plants expressing the CP gene of TMV (19), AIMV (1,12), or TRV (1) and in their protoplasts (1,12,27).

We did not observe any protection against virus infection in vector-transformed kanamycin-resistant plants; therefore, the reduced susceptibility to CMV infection in the transgenic plants used here is probably not the result of the selection of somaclonal variants during regeneration of transgenic plants expressing the CMV-CP gene. We showed that both CMV-CP and its mRNA accumulated in transgenic plants, so both or either of them could be responsible for virus protection. Evidence for specific involvement of CP in virus protection has been presented in the case of transgenic plants expressing the CP gene of AIMV (37) and TMV (24). Further study is required to identify whether CP and/or CP-mRNA transcripts are responsible for virus protection in transgenic plants expressing the CMV-CP gene.

Although the use of transgenic tobacco protoplasts expressing the TMV-CP gene has provided important information about early events in infection with TMV (23,27,39), little is known about the early processes of infection with other plant viruses. The protoplast system presented here, which provides specific protection against CMV virions, will be useful in studying the early stages of infection by this virus and in developing genetic engineering to control virus disease.

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