Satellite-Mediated Protection of Tomato Against Cucumber Mosaic Virus: I. Greenhouse Experiments and Simulated Epidemic Conditions in the Field

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ABSTRACT

Montasser, M. S., Tousignant, M. E., and Kaper, J. M. 1991. Satellite-mediated protection of tomato against cucumber mosaic virus: I. Greenhouse experiments and simulated epidemic conditions in the field. Plant Dis. 75:86-92.

Cucumber mosaic virus (CMV) strain S, with its naturally occurring satellite, can be used as a biological control agent to protect tomato (*Lycopersicon esculentum* 'UC82B') plants against disease induced by two severe CMV strains (D and 16). In greenhouse experiments, tomato plants were preinoculated or "vaccinated" with total RNA extracted from CMV-S and when challenge-inoculated after 3 wk with the severe strains, were protected against their effects. No synergistic effects were observed in mixed infections of CMV-S and a number of viruses commonly infecting tomato. Satellite-mediated protection was more protective and could be established sooner after vaccination than conventional cross-protection. In field studies, the fruit yield from protected and challenge-inoculated plants was double that of similarly challenged but not protected plants, whether the challenge was applied mechanically or by aphids.

In recent years, there has been a rapidly growing interest in the use of viral satellites as agents in the biological control of plant viruses (14,30). Satellites are small virus-associated nucleic acids that are sequence-unrelated to, but replicatively dependent upon, the viral genome, with which they essentially have a molecular parasitic relationship (18). Recently, viral satellites have been referred to as "natural inhibitors of crop-damaging viruses" (4). This concept is based on the earliest observations of Kassanis and Nixon (23) with the satellite of tobacco necrosis virus (sTNV), which greatly decreases the accumulation of TNV in infected plants. These observations were further substantiated by the demonstration that satellites of cucumber mosaic virus (CMV) could ameliorate the pathogenicity of their helper viruses (26,32).

CMV occurs worldwide and causes severe damage in many vegetable crops (8) including tomato, peppers, and cucurbits (6,28,29,31). In this country, it was reported that 50–80% of tomato in New York is affected by CMV (13). In 1983, nearly 100% infection of squash with severe disease symptoms in New Jersey was associated with CMV (6). In California, CMV was found widespread in the coastal region, while aphid- and white fly-transmitted cucurbit viruses occurred in Imperial County (7). At this time, CMV is associated with a severe necrotic disease of tomato in Italy, resulting in dramatic crop losses (9,10). In addition, we know of problems in Spain, France, China, Japan, and Indonesia.

CMV is a small icosahedral virus composed of single-stranded RNA of positive polarity. This virus encapsidates a small satellite RNA together with its own tripartite genomic RNA and a fourth subgenomic RNA (19). This CMV satellite, which in our laboratory is designated CARNA 5 (for CMV-associated RNA 5 [21]), modulates the symptom expression of its helper CMV. The capability of certain CARNA 5 sequence variants to ameliorate symptoms of infection by CMV (3,26) has been hypothesized to result from an interference phenomenon based on the replicative competition of CMV and CARNA 5. With CARNA 5 overtaking and suppressing the synthesis of viral RNA in the beginning of infection (16,27), the synthesis of CARNA 5 also declines because of its one-way dependence on the viral RNA. This allows temporary resumption of viral RNA replication, but the cycle will repeat, and viral RNA synthesis is immediately outcompeted again by CARNA 5. The overall result is that alternating cycles of low-level viral and satellite replication continue as the plant grows. As a consequence, a virtually symptomless persistent infection spreads throughout the plant, particularly if the satellite RNA does not express any disease symptoms of its own (17). A new infection by a virus capable of supporting the satellite at any time during the plant's lifetime could trigger a resumption of CARNA 5 replication to higher levels, which could then outcompete the invading virus and stop the infection.

CMV frequently increases in incidence during the growing season, causing 100% infection in some late plantings. It is transmitted primarily by the green peach aphid (*Myzus persicae* (Sulzer)) and the melon aphid (*Aphis gossypii* (Glover)) in a nonpersistent manner (24). Attempts to control CMV by controlling the aphid vectors with insecticides have not been effective in reducing loss attributable to disease. The development and use of alternative methods of virus control, such as satellite-mediated protection, could reduce virus infection and disease loss and also reduce the use of insecticides.

The objective of the present work was to determine the efficacy with which a tomato crop can be protected from virulent CMV strains in the greenhouse and under simulated epidemic conditions in the field. Satellite-mediated protection and conventional cross-protection (by a satellite-free, mild strain of CMV) of tomato plants against challenge by a severe CMV strain were compared. Challenge-inoculation by mechanical or aphid transmission was also compared.

MATERIALS AND METHODS

Virus source and maintenance. The origins of CMV strains S and D were described previously (1,20). CMV-S contains S-CARNA 5, is symptomless in tomato (3), and is referred to as the "vaccine" in this study. Two severe strains were used. CMV-D contains D-CARNA 5 and causes lethal tomato necrosis (3). CMV-16 is a Japanese isolate from tomato (from H. Sayama, Kikko Foods Corporation) that contains no detectable satellite when maintained in tomato but causes severe stunting and fruit malformation. CMV-R76B is a satellite-free isolate from raspberry provided by M. Mayo of the Scottish Crop Research Institute. Like CMV-S, it causes only very mild chlorosis in tomato. All CMV isolates were maintained and propagated for purification

Part of this work was funded by the IPM/PT program, USDA-ARS, and was carried out at the Beltsville Agricultural Research Center under a cooperative agreement with the University of Maryland, College Park.

Mention of a commercial company or specific equipment does not constitute its endorsement by the U.S. Department of Agriculture over similar equipment or companies not named.

Accepted for publication 4 July 1990 (submitted for electronic processing).

in tomato (*Lycopersicon esculentum* Mill. 'Rutgers' or 'UC82B'), with the exception of CMV-D, which was maintained and propagated in tobacco (*Nicotiana tabacum* L. 'Xanthi-nc').

Tobacco mosaic virus (TMV), potato virus Y (PVY), potato virus X (PVX), tobacco etch virus (TEV), and pepper mottle virus (PMV) were provided by J. Hammond, USDA-ARS, and were maintained in *N. sylvestris* Speg. and *N. benthamiana* Domin. Tomato ringspot virus (TomRV) was a gift of E. Podleckis, USDA-ARS, and was maintained in cucumber (*Cucumis sativus* L.). Potato spindle tuber viroid (PSTV) was a gift of T. O. Diener of the University of Maryland and was maintained in tomato.

Virus purification and inoculum preparation. CMV-S was purified from tomato 7–14 days after inoculation using the method of Lot et al (25). RNA was isolated from the virus by SDS/phenol extraction (22). The presence of S-CARNA 5 and the absence of n-CARNA 5 (term used to generically denote a necrogenic satellite) were verified by semidenaturing polyacrylamide gel electrophoresis (PAGE) (12), which differentiates S-CARNA 5 from n-CARNA 5 (1), and by tomato necrosis bioassay. For preinoculations, CMV-S total RNA was used at 10 μg/ml in 0.03 M Na_2HPO_4.

CMV-R76B was purified from tomato using the same methods as with CMV-S. RNA was extracted as above, used at 10 μg/ml in inoculation buffer for cross-protection preinoculations, and was supplemented with 2.5 μg/ml S-CARNA 5 for satellite protection preinoculation.

Mechanical transmission. Inoculations with sap were made by grinding infected tissues in a suitable buffer (1 g/9 ml) for each virus tested and rubbing, with a cotton swab, the first true leaves of 8- to 10-day-old tomato plants that had been previously dusted with 600-mesh Carborundum. Immediately after inoculation, the leaves were rinsed with distilled water. Test plants were kept in 10-cm-diameter pots containing a mixture of soil, peat moss, and vermiculite (2:1:1, v/v) provided with a complete fertilizer in a greenhouse where the temperature ranged from 24 to 35 C.

Vaccinations and challenge-inoculations in greenhouse experiments. Tomato UC82B plants were preinoculated or vaccinated with CMV-S total RNA. Plants were then divided into four groups. The first group represented the protected control. The three remaining groups were each divided in two to form six subgroups. One, two, or three weeks later, two protected subgroups were challenge-inoculated with either CMV-D or CMV-16 in crude sap. Two unprotected groups of tomato plants were inoculated as above 2 wk after vaccination of the test plants and served as challenge controls. An additional group of tomato plants was left to serve as untreated controls after mock inoculation with inoculation buffer.

Mixed infections. Six commonly occurring viruses (TMV, PVY, PVX, TEV, PMV, and TRSV) and a viroid (PSTV) were mechanically inoculated singly as well as in mixed infection with CMV-S total RNA on young tomato seedlings. CMV RNA was in 0.03 M Na_2HPO_4; TMV-, TEV-, TRSV-, PMV-, PVY-, and PVX-infected tissue was triturated in 0.01 M K_2SO_4 solution; and PSTV was in 0.01 M potassium phosphate buffer, pH 7.

Mixed infections were carried out by inoculating one-half of the available leaves with the CMV-S total RNA and the other half with one of the other viruses or viroid at the same time. Symptoms were monitored and compared to noninoculated plants, protected plants, and plants inoculated singly with each of the six viruses or viroid. The presence of viruses was determined by indirect enzyme-linked immunosorbent assay (ELISA) and by bioassay.

Aphid culture. Green peach aphids provided by J. Hammond, USDA-ARS, were raised from a single aphid on a healthy turnip (*Brassica rapa* L.) plant. Aphids were fed on turnip plants and kept at the larvae stage (unwinged) through weekly passages on fresh turnips. After the culture was well established, some aphids were transferred onto tobacco (*Nicotiana tabacum* 'Xanthi-nc') plants for adaptation, where they were allowed to grow and multiply to large numbers before use for virus transmission.

Manual application of aphids. Aphids were collected from culture on tobacco plants, placed in petri dishes, and kept fasting for at least 1 hr before being placed on CMV-D- or CMV-16-infected tobacco plants where they were allowed to feed for about 20 min. These two virus sources were kept separate from each other and from other healthy plants in separate small greenhouses to prevent any cross-contamination of the test plants. After the 20-min acquisition period, 10 viruliferous aphids were transferred to each test plant with camel's-hair brushes and small disks of filter paper. They were allowed to feed overnight (inoculation feeding) to ensure transmission of enough inoculum into plant tissues, then killed by spraying with insecticide (malathion). Test plants were indexed for virus by ELISA 2 wk after the inoculation feeding.

Design of field trial. Tomato seedlings, sown in 2-wk intervals, were simultaneously inoculated with CMV-S total RNA 11, 25, and 41 days from the cotyledon stage. These inoculated seedlings were kept in the greenhouse for 3 wk to allow the virus and S-CARNA 5 to become established. Plants were then challenge-inoculated with CMV strains D or 16 in the greenhouse using mechanical or aphid transmission, or in the field with natural aphid transmission. The following controls, sown at the same time intervals, were included in the experimental design: untreated plants (H); plants preinoculated with CMV-S only (S); and control plants mechanically inoculated with CMV-D (D), CMV-16 (16), or aphid inoculated with CMV-D (H/D) or CMV-16 (H/16). The plants were then moved to a cold frame for hardening and 1 wk later were transplanted to the field. A total of 1,763 tomato UC82B seedlings were tested in three separate fields. In one field where mechanical transmission was employed for challenge-inoculation, the tests for CMV-D and CMV-16 were combined; where aphid transmission was employed, the tests for CMV-D and CMV-16 were in separate fields. Tobacco plants infected with each of the two severe strains of CMV and infested with aphids were transplanted to the field and were randomly distributed among the tomato test plants to allow aphid spread, thus simulating a natural challenge-inoculation. Four tobacco plants were used in each test plot of 20 tomato plants.

At each site, the different treatments and controls were in three replicate blocks. Each block contained one plot of each treatment or control randomly distributed (complete random block design), and each plot contained two rows of 20 plants with 48-cm spacing between plants and 120 cm between rows. Natural aphid-transmission test plots were separated from manual aphid-transmission plots by a plot of control, untreated plants. Insecticides were used on all but the natural aphid-transmission plots. The experimental plants at each site were surrounded by a guard row of uninoculated plants at the same spacing.

Symptom scoring, yield assessment, and statistical analysis. Plants were scored for symptom development and disease intensity by the same individual 4, 6, and 8 wk after the challenge-inoculation according to the following scale: grade 0 = no visible symptoms on leaf or stems; grade 1 = very mild mosaic (or mottle) symptoms; grade 2 = leaf distortion or malformation; grade 3 = slight stunting and/or partial necrosis on leaves; grade 4 = stunting and/or necrosis on leaves and streaks on stems; and grade 5 = severe stunting and/or lethal necrosis. Symptom scores, weights, and fruit number scores of all tested plants provided the data from which the yield values or "yield rate percent" for each treatment was calculated with the mean yield from the healthy replicates of untreated plants as a baseline. Healthy plants were determined to be healthy by ELISA. Data collected at the final reading for each

plant were used for statistical analysis using Student's *t* test and the general linear models procedure (GLM analysis).

Serological detection of viruses. To ensure the establishment of the vaccine throughout the plant, plants were tested for CMV-S with ELISA (2,5) before being transplanted to the field. Sap from 0.1–0.5 g of tissue was mixed with 10× (w/v) coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6, containing 2% polyvinyl pyrrolidone [PVP]) and either filtered through cheesecloth or centrifuged at 6,000 *g* for 2 min. One hundred microliters of the clarified extract was pipetted into wells of polystyrene microtiter plates. Polyclonal antiserum against CMV-D (American Type Culture Collections, Rockville, MD) was appropriately diluted in PBS (phosphate buffered saline: 0.02 M phosphate, 0.15 M NaCl, pH 7.4, containing 0.05% Tween 20, 2% PVP, and 0.2% ovalbumin) (5). Substrate (disodium *p*-nitrophenyl phosphate [Sigma Chemical Co., St. Louis, MO]) was used at a concentration of 1.0 mg/ml in substrate buffer (10% diethanolamine, pH 9.8). All buffers contained 0.02% sodium azide as a preservative. Absorbance at 405 nm of the hydrolyzed substrate was measured using a Biotek (Burlington, VT) Model EL307 or a Dynatech MR700 ELISA reader. Readings were made 0.25–1.0 hr after addition of the substrate and repeated at 1.5–4.0 hr in some instances. Values that exceeded twice that of the healthy and/or buffer controls were considered positive. Noninfected plants were discarded.

Polyacrylamide gel electrophoresis and northern hybridization. Purified viral RNA preparations and total nucleic acid extracts (TNA), prepared from random leaf samples taken four times during the season from different treatments, were analyzed by electrophoresis on 6% polyacrylamide (39:1 acrylamide: bisacrylamide containing 7 M urea and 1× TBE [90 mM Tris-borate, pH 8.3, 2.5 mM EDTA) (33). After staining the gels with ethidium bromide and UV-photography, the RNA was electrotransferred to nylon membranes, which were later probed for the presence of CARNA 5 as described (33).

RESULTS

Efficacy of satellite-containing CMV-S in protecting tomato against disease induced by severe strains of CMV. Vaccinated plants were challenge-inoculated with severe strain CMV-D or CMV-16 1, 2, or 3 wk after inoculation with CMV-S to determine the optimal length of time for the vaccine to become established in the test plants. CMV-S-inoculated plants were only partially protected when challenged 1 wk later with the severe strains; however, the disease prevention percentage increased sharply when plants were challenged after 2 wk and almost complete protection was obtained 3 wk after vaccination. In Figure 1, one plant from each of the above treatments is shown to illustrate the effect of challenge-inoculation on each treatment. The age of the plant at the time of inoculation with CMV-S had no effect on the efficacy of the vaccination (*data not shown*).

Disease development and fruit quality. Preinoculation of tomato with CMV-S total RNA resulted in plants that were essentially symptomless for the length of the test (approximately 3 mo). When challenged by CMV-D or CMV-16, most of the vaccinated plants retained their healthy appearance. Plants inoculated with CMV-D or CMV-16 alone were also normal in appearance during the first 3 wk, but after about 4 wk, the characteristic symptoms of each virus strain developed. For CMV-D, foliage symptoms on the inoculated leaves started with chlorosis and veinal necrosis. The necrosis then spread from leaflets through the midrib into petioles toward the stem, then downward to cause black or dark brown streaks and constriction around the lower part of the stem. Ten days later, younger leaves and growing points started to show veinal necrosis and downward bending. By the time of blooming and fruit setting, the veinal necrosis reached the blossom, causing severe damage and death. The whole plant eventually collapsed and died.

CMV-16, on the other hand, caused mild mosaic symptoms with some leaf puckering initially. However, leaf size was severely reduced, and uneven marginal leaf growth caused twisting and clumping. The stem internodes were severely shortened so that the whole plant showed severe stunting.

The yield and quality of fruits from preinoculated plants were similar to that of healthy controls. Fruits from the surviving blossoms of plants infected with CMV-D showed dark brown blotches or narrow-banded concentric markings with a smooth shiny surface and diffused margins on the outer side of the skin. The browning appeared in

Fig. 1. Satellite-mediated protection of 13-wk-old tomato plants against challenge-inoculation with severe CMV strains (**A**) D and (**B**) 16: (left to right) mock-inoculated control plant, control plant vaccinated with CMV-S total RNA, challenge-inoculated plant 2 wk after vaccination, challenge-inoculated control plant.

the epicarp and extended through the mesocarp and placenta of tomato fruits. In other cases, severe veinal necrosis spread from the base of fruits into the main and small veins and then throughout the entire tomato, causing mummification and death even before ripening. On the other hand, the fruits obtained from plants infected with CMV-16 alone showed no symptoms except that they were very much smaller and ripened earlier than those from healthy controls or vaccinated tomato plants.

Comparison of conventional cross-protection and satellite-mediated protection of tomato. To determine how much CARNA 5 contributes to the protective effect in tomato following preinoculation with satellite-containing CMV, a comparison with conventional cross-protection was carried out using a satellite-free CMV strain R76B as the protecting virus. For the conventional cross-protection experiment, young tomato seedlings were preinoculated with CMV-R76B RNA, then challenged with the severe strain of CMV-16 after 1, 2, and 3 wk. For satellite-mediated protection, S-CARNA 5 was added to the same CMV-R76B RNA for preinoculation of tomato seedlings, which were then also challenged with CMV-16 after 1, 2, and 3 wk. Controls consisted of inoculations with CMV-R76B RNA, CMV-R76B RNA + S-CARNA-5, and CMV-16 alone, as well as mock-inoculated tomato plants.

Table 1 shows the results of this experiment with measurements of plant heights, fruit yield, and weight of shoot system. While both methods confer a certain degree of protection against the effects of CMV-16, satellite protection appears to be more complete with the use of the above parameters. In addition, the protection effects are established 1–2 wk sooner after vaccination with satellite than in conventional cross-protection.

Virus and CARNA 5 detection in vaccinated and subsequently challenged plants. Plants were indexed for CMV-S biologically on *Chenopodium quinoa* Willd. and California Blackeye cowpeas (*Vigna unguiculata* (L.) Walp.) and serologically by ELISA to verify the multiplication of CMV-S in protected plants before they were challenge-inoculated. Five percent of the plants (3/60) did not become infected upon vaccination. PAGE was used to detect single-stranded (ss) and double-stranded (ds) CARNA 5 and to monitor their accumulation in each part (root, stem, leaf, or fruit) of either young or old plants.

Figure 2 compares different TNA extracts from protected and subsequently challenged tomato plants after 6% PAGE analysis and ethidium bromide staining or hybridization with CARNA 5-specific probes (33). It shows that 6% PAGE allows the differentiation of the dsCARNA 5 in CMV-S inoculated and CMV-D infected plants (lanes 2 and 3). In plants inoculated with CMV-S and subse-

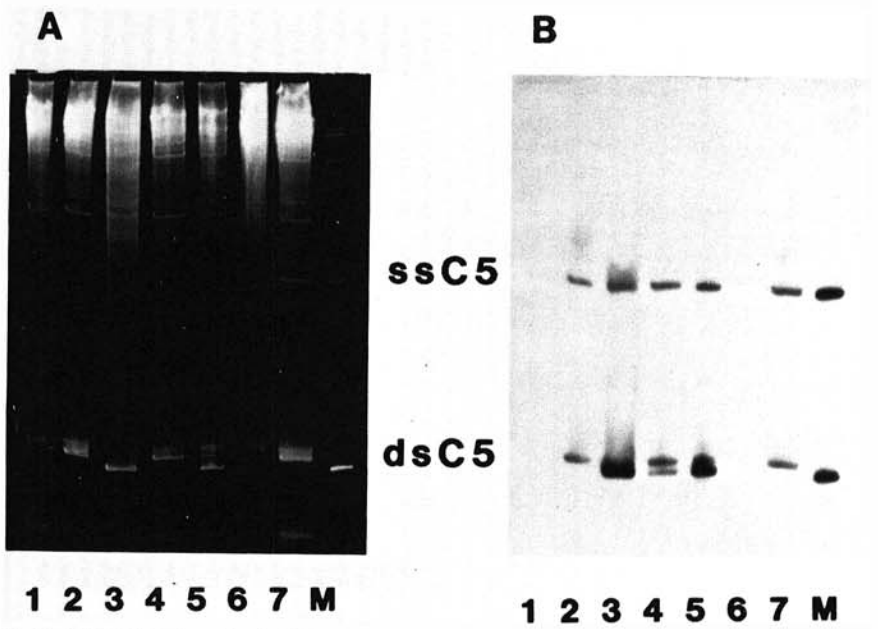

Fig. 2. CARNA 5 detection in TNA extracts of tomato preinoculated with CMV-S total RNA before and after challenge-inoculations with satellite-containing CMV-D or satellite-free CMV-16. Panels represent 6% PAGE patterns after (**A**) ethidium bromide staining and (**B**) northern transfer and hybridization with D-CARNA 5 specific probe. Approximate positions where ss and dsCARNA 5 migrate are indicated. Lane 1, mock-inoculated plant; lane 2, control plant preinoculated with CMV-S (vaccinated); lane 3, control plant challenged with CMV-D; lane 4, vaccinated plant challenged with CMV-D that remained asymptomatic; lane 5, vaccinated plant challenged with CMV-D showing partial necrosis; lane 6, control plant challenged with CMV-16; lane 7, vaccinated plant challenged with CMV-16; lane 8, CMV-D total RNA + D-dsCARNA 5.

Table 1. Comparison of the effects of cross-protection and satellite-mediated protection against infection by a severe satellite-free CMV strain

Treatment[x]	Duration (wk)	No. of plants	Average stem length (cm)	Average fruit yield		Average weight shoot system		Disease prevention[y] (%)
				Weight (g/plant)	Fruit/plant (no.)	Fresh (g/plant)	Dry (g/plant)	
Cross-protection								
R76B		20	28.5 a[z]	18.8 e	2.6 h	25.0 k	3.4 n	...
R76B/16	1	16	11.3 c	10.7 f	0.8 i	20.9 l	2.3 o	0
	2	16	10.8 c	20.1 e	1.75 i	23.3 l	2.6 o	30
	3	16	21.5 b	21.9 e	1.9 i	34.4 k	3.9 n	65
Satellite protection								
R76B + C5		20	27.3 a	21.1 e	3.2 g	42.2 j	5.7 m	...
R76B + C5/16	1	16	20.3 b	19.2 e	2.6 h	31.5 k	3.8 n	45
	2	16	27.8 a	26.4 d	2.1 h	32.9 k	3.4 n	95
	3	16	26.5 a	27.5 d	2.5 h	37.1 j	4.9 m	99
Controls								
CMV-16	2	16	14.3 c	9.6 f	1.0 i	19.2 l	1.8 o	...
Healthy UC82B tomato		20	27.5 a	23.2 d	3.6 g	41.4 j	5.8 m	...

[x]Data collected 3 mo after preinoculation.
[y]Calculated using $100[(C - V)/C]$, where C = disease intensity of plants inoculated with CMV-16 and V = disease intensity of vaccinated plants challenged with CMV-16.
[z]Values followed by the same letter do not differ significantly from each other at the 0.05 level according to Duncan's multiple range test.

quently challenged with CMV-D, a mixture of both dsCARNA 5 species can be seen (panels A and B, lanes 4 and 5). In plants that successfully resisted challenge, S-dsCARNA 5 predominated (panel A, lane 4); in plants where protection was not well established, for instance, with challenge-inoculation after 1 wk, D-dsCARNA 5 predominated (panel A, lane 5). The proportions of the two dsCARNA 5 species on the autoradiograph (panel B) deviate somewhat from the corresponding one stained with ethidium bromide (panel A) in favor of D-dsCARNA 5, because they were hybridized with a probe derived from cloned D-CARNA 5 (cDNA) (33). In TNA extracts from plants infected with CMV-16, no CARNA 5 was found (panels A and B, lane 6), as expected; those protected and then challenged with CMV-16 showed only the presence of ss and S-dsCARNA 5 (panels A and B, lane 7).

Mixed infection of CMV-S total RNA and other pathogens in tomato. To check for possible synergistic or antagonistic effects between CMV-S and certain commonly occurring viruses, tomato plants were inoculated with CMV-S total RNA and either TMV, PVY, PVX, TEV, PMV, or TRSV. Simple indirect ELISA was used to verify the presence of these viruses following these mixed infections. No synergistic effects were found. However, significant antagonistic effects were noticed in tomato plants inoculated with PSTV and CMV-S that showed significantly milder viroid symptoms than those infected with PSTV alone. In these dually inoculated plants, much less PSTV RNA was detected by PAGE analysis and northern hybridization than in samples collected from plants inoculated with PSTV alone (*results not shown*).

Field test with mechanical transmission of challenge viruses. Table 2 shows that, with few exceptions, disease intensity was much higher and the yield of fruits greatly reduced in control plants directly infected with CMV-D or CMV-16, as compared with plants preinoculated with CMV-S and then challenged. Disease prevention of up to 81% was observed for both virus strains. Figure 3 shows typical examples from the field test where essentially complete protection was achieved against either one of the challenge virus strains. Yield rates for fruit production were 94–95% for protected and challenged plants.

Field test with aphid transmission of challenge viruses. Because CMV is aphid-transmitted in a nonpersistent manner, viruliferous aphids carrying the virulent CMV-D or CMV-16 were used to infect untreated control plants as well as CMV-S-protected plants to simulate a CMV challenge under natural conditions such as might occur in a severe outbreak. Two methods of handling aphids were used to challenge two groups of tomato seedlings. For both methods of challenge, the mean fruit yield from all plants infected with CMV-D or CMV-16 was significantly less than from the protected/challenged plants. No significant differences were seen between results from the manual brush applica-

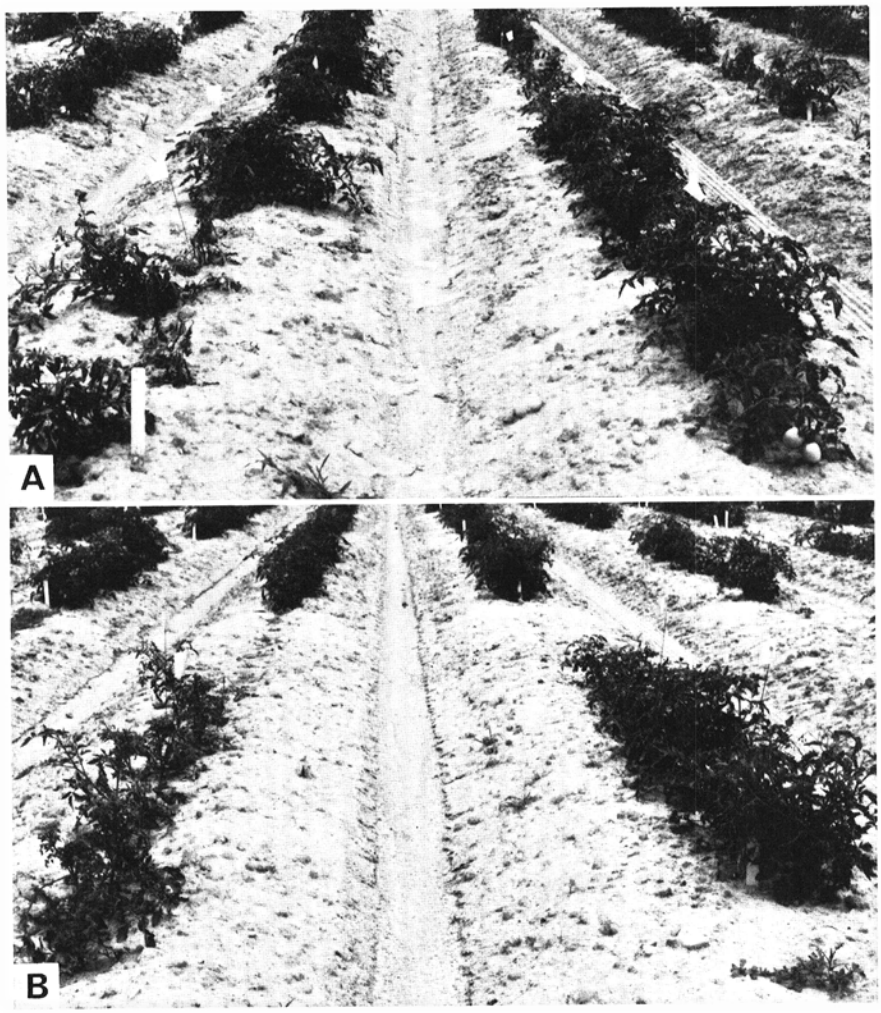

Fig. 3. Satellite-mediated protection of approximately 10-wk-old tomato plants against challenge-inoculation with severe CMV strains (**A**) D and (**B**) 16. Plants in left foreground were challenge-inoculated controls and plants in right foreground were vaccinated before challenge-inoculation. Left row in **A** shows spaces where plants have died and in **B**, severely stunted plants.

Table 2. Effect of satellite-mediated protection on disease and fruit yield in field-grown tomato after mechanical challenge-inoculation with two severe strains of cucumber mosaic virus

Treatment[v]	Disease intensity[w] (%)	Infected plants/ plants tested	Disease prevention[x] (%)	Average yield/plant (kg)	Average fruit/plant (no.)	Yield rate[y] (%)
H	2.7	2/180	···	6.7 a[z]	61.8 a	100.0
S	2.7	4/173	···	5.4 b	45.6 b	80.6
S/D	13.0	19/180	81	6.3 a	49.6 b	94.0
D	67.3	142/180	···	1.8 c	11.6 d	26.9
S/16	11.3	12/180	80	6.4 a	43.2 b	95.5
16	57.7	138/180	···	2.6 c	31.8 c	38.8

[v] H = healthy tomato (UC 82B) control, S = CMV-S inoculated, S/D = CMV-S inoculated plants challenge-inoculated with CMV-D, D = CMV-D inoculated, S/16 = CMV-S inoculated plants challenged with CMV-16, 16 = CMV-16 inoculated plants.

[w] Calculated using $100(\Sigma\, sn/SN)$, where s = disease index score, n = number of plants with the same score, S = highest score, and N = total number of plants tested. Disease was rated on a scale of 0–5, where 0 = no symptoms and 5 = severe symptoms.

[x] Calculated using $100[(C - V)/C]$, where C = disease intensity of plants inoculated with CMV-D or CMV-16 and V = disease intensity of CMV-S inoculated plants challenged with CMV-D or CMV-16.

[y] Ratio of average fruit yield from treated plants to average fruit yield from healthy plants.

[z] Means followed by the same letter are not significantly different according to Student's t test. LSD for yield = 0.85 and for number of fruit = 4.7.

tion method or the natural movement of aphids from the colony plants. In challenge-inoculations with either CMV-D or CMV-16, disease prevention of 79–86% and fruit yield rates of over 90% were obtained (Table 3). GLM analysis for the tomato yield of this experiment showed insignificant differences between the two methods of aphid transmission used in this study (*data not shown*) and thus the data of the two methods were combined for presentation in Table 3.

DISCUSSION

The results of this study demonstrate that introduction of nonnecrogenic CMV satellites, such as S-CARNA 5 (via preinoculation or vaccination in the presence of a helper virus), into tomato plants will prevent severe disease following later infection by a severe strain of CMV. This outcome was expected in view of the presumed replicative competition between satellite and viral RNA and the proposed interference mechanism (16) with which we have previously rationalized CARNA 5-induced attenuation of disease symptoms in CMV infections. Evidence for stable replication and spreading of CARNA 5 in tomato plants during long-term CMV infections was provided recently in preliminary experiments (33) preceding the work described here and in the following paper (11). Satellite-mediated protection of tomato (15,35) and pepper (34) against infection by CMV has been tested; however, molecular analysis (in the case of tomato), a search for possible synergistic effects, and a systematic comparison of satellite protection with conventional cross-protection of tomato were not undertaken.

Wu et al (34) detected the presence of the protective and challenge strains of CMV by protein A sandwich ELISA (PAS-ELISA) and dot-blot hybridization. In the present work, ELISA was used to detect the presence of the protective strain (CMV-S) before transplanting. However, specific monoclonal antibodies to the challenge strains were not available. The question of possible synergism and the comparison of conventional cross-protection with satellite-mediated cross-protection, problems that had not been addressed previously, were studied in greenhouse experiments before the field tests described here and in the following report (11).

An important factor in the commercial use of vaccination for the protection of tomato from the effects of CMV is its effect on the yield rate and the quality of the fruit. Yield rates for fruit production from protected and challenged plants reached 95% that of healthy, untreated plants, and the horticultural properties of tomato appeared not to be affected by the preinoculation. Much more extensive tests of fruit quality in similar field experiments in Japan have confirmed this observation (H. Sayama, Kikko Foods Corporation, *personal communication*).

There are two potential drawbacks in the use of satellite-mediated protection of tomato. The first, the possibility of synergistic effects occurring when plants preinoculated with CMV-S become infected with other viruses commonly found in tomato in the open field, can be negated by empirically screening for this eventuality through mixed infection tests, as was done in the work above. The second, the danger of a necrogenic CARNA 5 emerging or mutating from the vaccine components and its overtaking and eventually predominating in the infection, will be evident in tomato if it reaches the necessary threshold levels to incite tomato necrosis. However, this danger can be screened for and detected long before this point is reached with relatively rapid and sensitive biochemical techniques that involve TNA extraction and PAGE analysis combined with appropriate hybridization probes (33). As was shown in Figure 2, with 6% PAGE a necrogenic D-dsCARNA 5 (here introduced in challenge-inoculation) could easily be differentiated from the protecting S-dsCARNA 5, introduced by vaccination.

By using CMV-R76B, which can be maintained relatively free of satellite in tomato, it has been possible to compare systematically the effectiveness of satellite-mediated protection and conventional cross-protection of tomato against challenge-inoculation by the severe satellite-free CMV-16 strain. The results were clearly in favor of satellite protection (Table 1) and confirm a recently published comparison of satellite protection and cross-protection against CMV in pepper (34). Conventional cross-protection was found to be essentially ineffective against challenge by CMV containing a necrogenic CARNA 5 (15, *unpublished*). Apparently in that case the newly invading CARNA 5 is immediately supported by the cross-protecting helper virus and does not have to compete with a replicating protecting CARNA 5 introduced with the vaccination.

The demonstration of the effectiveness of vaccination against a natural challenge infection in the field induced by aphids (Table 3) further emphasizes the usefulness of the method of satellite-mediated biological control of CMV. The following report gives further data on satellite-mediated protection of a tomato crop under natural epidemic conditions in southern Italy.

Table 3. Effect of satellite-mediated protection on disease and fruit yield in field-grown tomato after challenge-inoculation via aphid transmission with two severe strains of cucumber mosaic virus

Treatment[v]	Disease intensity[w] (%)	Infected plants/ plants tested	Disease prevention[x] (%)	Average yield/plant (kg)	Average fruit/plant (no.)	Yield rate[y] (%)
H	9.3	13/140	···	4.8 a[z]	45.8 ab	100.0
S	6.7	7/110	···	4.6 a	44.2 b	95.8
S/D	15.0	18/120	79.1	4.4 a	33.0 c	91.7
H/D	71.7	86/120	···	2.1 b	22.2 d	43.8
D	81.7	49/60	···	1.4 c	7.6 e	29.2
S/16	13.4	8/60	86.3	4.5 a	49.4 a	93.8
H/16	97.5	39/40	···	0.04 d	0.7 f	0.8
16	75.0	45/60	···	0.2 d	6.3 e	4.2

[v] H = healthy tomato (UC 82B) control, S = CMV-S inoculated, S/D = CMV-S inoculated plants challenge-inoculated with CMV-D, H/D = healthy tomato challenge-inoculated with CMV-D via aphids at time of S/D challenge, D = plants mechanically inoculated with CMV-D at time of CMV-S inoculation, S/16 = CMV-S inoculated plants challenged with CMV-16, H/16 = healthy plants challenge-inoculated with CMV-16 via aphids at time of S/16 challenge, 16 = plants mechanically inoculated with CMV-16 at time of CMV-S inoculation.

[w] Calculated using $100(\Sigma\, sn/SN)$, where s = disease index score, n = number of plants with the same score, S = highest score, and N = total number of plants tested. Disease was rated on a scale of 0–5, where 0 = no symptoms and 5 = severe symptoms.

[x] Calculated using $100[(C - V)/C]$, where C = disease intensity of plants inoculated via aphids with CMV-D or CMV-16 and V = disease intensity of CMV-S inoculated plants challenge-inoculated with CMV-D or CMV-16.

[y] Ratio of average fruit yield from treated plants to average fruit yield from healthy plants.

[z] Means followed by the same letter are not significantly different according to Student's t test. LSD for yield = 0.2 and for number of fruit = 2.4.

ACKNOWLEDGMENTS

We thank Thomas H. Barksdale for facilitating the field activities. We also thank Peter McGarvey, Tomas Lattick, Carol Eichner, Bob Hoover, Deborah Block, Lynn Geletka, and Mike Blum for their participation in these studies.

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