Competition Between Cucumber Mosaic Virus Satellite RNAs in Tomato Seedlings and Protoplasts: A Model for Satellite-Mediated Control of Tomato Necrosis

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ABSTRACT

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The competition between two satellite RNAs of cucumber mosaic virus (CMV) in inoculated tomato seedlings or in electroporated tomato protoplasts was examined using semidenaturing polyacrylamide gel electrophoresis and northern hybridization. Analysis of total nucleic acid extracts revealed that in infections with two simultaneously inoculated satellite RNAs, or in infections where the more virulent satellite was inoculated at a later time, the amount of the two satellite RNAs that accumulated differed. The amount of accumulation depended on the concentration of each satellite RNA in the inoculum and the length of time between inoculations. On the basis of symptomatological and molecular analyses, inoculation with CMV containing a mild satellite RNA prior to challenge by a severe satellite RNA with or without CMV interfered with the replication and symptom expression of the severe strain. These results support the feasibility of the use of mild virus-satellite combinations in the biocontrol of CMV.

CARNA 5, the designation for RNA 5 associated with cucumber mosaic virus (CMV), comprises a group of sequence-related viral satellites of varying lengths (334–386 nucleotides) found in association with different strains of CMV (5, 10,13). Certain CARNA 5 sequence variants drastically alter the symptom expression of CMV. On tomato seedlings, D-CARNA 5 associated with CMV strain D converts virus-induced chlorosis and leaf deformation into a lethal necrosis, and S-CARNA 5 associated with CMV strain S ameliorates the viral symptoms. A natural variant of D-CARNA 5 was recently identified as the causal agent of lethal tomato necrosis in Italy (8), and ameliorative CMV-satellites such as S-CARNA 5 are being used in China as a vaccine on an extensive scale (more than 10,000 ha) for the prevention of CMV disease in a number of crops (17).

In nature many factors can decisively alter the biological outcome of a virus infection. One specific example is where a plant is infected with a CMV strain that supports two or more different CARNA 5 variants that were introduced simultaneously or sequentially. The CARNA 5 variants could ameliorate or aggravate the symptom expression of the virus or induce a disease syndrome of their own. Biological experiments showing competition between CARNA 5s were first reported in 1982 (6), and molecular evidence for competition in the CARNA 5 replication during serial passage of the helper virus was reported in 1983 (7). In both these cases, it was noted that if an undetectable amount of a more virulent, quicker replicating CARNA 5 was initially present in a CMV preparation containing a less virulent CARNA 5, the more virulent CARNA 5 would manifest itself in a bioassay.

We report here the results of experiments where two CARNA 5 sequence variants, one ameliorative and one necrogenic, assisted by a given helper CMV, were used to infect tomato. Symptomatological and molecular analyses were used to determine the competition occurring between two CARNA 5s inoculated simultaneously or sequentially and the relationship of the competition to the degree of tomato necrosis observed.

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MATERIALS AND METHODS

CMV purification and RNA isolation. The source, methods of propagation, and purification of CMV strains S, 1, and D and isolation of viral and satellite RNAs have been previously described (1,11,12).

Preparation of satellite-free CMV RNA. CMV-1 RNA was fractionated by sucrose density gradient centrifugation, and fractions containing genomic RNAs 1, 2, and 3 were pooled and ethanol-precipitated. After centrifugation, the RNA was resuspended in water, heated at 65 C for 5 min, and then quick-cooled. The solution was adjusted to 4 M urea, 1% SDS, prior to electrophoresis in a 1% low melting temperature (LMT, Seakem) agarose gel for 1 hr at 100 V. The position of the RNAs in the gel was determined by UV-imaging (2), then RNAs 1–3 were excised together and melted at 45 C. Bromophenol blue was added to the melted agarose prior to layering it on a 6% polyacrylamide gel prepared at an acrylamide:bisacrylamide ratio of 39:1 in TAE (40 mM Tris, 20 mM sodium acetate, and 2 mM Na EDTA, adjusted to pH 7.8 with acetic acid). Electrophoresis was conducted at 250 V, which maintained the temperature of the gel at approximately 45–50 C. The RNAs were again visualized by UV-imaging, excised, and eluted from the gel matrix (14) in a buffer without Mg++, ethanol-precipitated, and resuspended in water. The RNA was then inoculated to *Chenopodium quinoa* Willd. at a concentration of 100 μg/ml in 0.03 M Na_2HPO (inoculation solution). Four days later, a single lesion was triturated in the same solution and inoculated to *C. quinoa*. After 4 days, several lesions were excised, triturated together, and inoculated to tomato (*Lycopersicon esculentum* Mill. 'Rutgers') seedlings. Virus purified from the second passage in tomato was determined to remain satellite-free after subsequent inoculations to tobacco (*Nicotiana tabacum* L. 'Xanthi-nc') or tomato by total nucleic acid extraction of infected tobacco or tomato tissues and analysis of such extracts by gel electrophoresis and northern blot hybridization as described below.

Inoculation of tomato seedlings. Tomato seedlings were inoculated at the cotyledon stage with CMV-1 genomic RNA at 10 μg/ml as above. S-CARNA 5 was added to the viral inoculum to give a final concentration of 2.5 μg/ml. D-CARNA 5 was added to the CMV-1 + S-CARNA 5 inoculum to provide an exponential dilution series with final concentrations ranging from 2.5 μg/ml to 2.5×10^{-5} μg/ml (3). Plants were grown in growth chambers as described previously (8) for periods of up to 21 days. Samples for total nucleic acid extraction were taken at intervals throughout the course of the experiment. The variables of D-CARNA 5 inoculum concentration and the time delay for mechanical challenge inoculation were chosen arbitrarily, as no factual knowledge with regard to the influence of either factor was available.

Protoplast isolation, electroporation, and incubation. Protoplasts were isolated from mature mesophyll leaf tissue

of tomato plants grown in a greenhouse or under the controlled conditions of a growth chamber as above. Electroporation was performed essentially as described by Smith et al (16). The satellite-free control was prepared by inoculating aliquots of 2.5×10^5 protoplasts in 0.78 M mannitol, pH 7.0, with CMV-1 genomic RNA at 10 μg/ml. S-CARNA 5 was added to a concentration of 2.5 μg/ml for the CMV + CARNA 5 inoculum. D-CARNA 5 was added in exponential dilutions to a final concentration of 2.5×10^{-5} μg/ml as above. Samples were incubated for periods of up to 72 hr and extracted for total nucleic acid analysis as described (16).

Total nucleic acid isolation and replication footprint profile (RFP) analysis. Total nucleic acids were extracted from infected plant tissues and protoplasts as described (16,18) and analyzed by 9% polyacrylamide gel electrophoresis (PAGE) in order to differentiate the single-stranded CARNA 5 species (1). The gels were treated and transferred to Biotrans nylon membranes (ICN, Irvine, CA) for northern hybridization. With these methods, replication footprint analysis visualizes the relative proportions of viral RNA and single-stranded and double-stranded CARNA 5 that exist at a given time in infected plant tissue. It represents the sum total of past replicative, degradative, and processing events. A comparative analysis over several time intervals has been termed replication footprint profile (RFP) analysis (9,16).

RESULTS AND DISCUSSION

Symptom development in tomato plants infected with CMV-1 alone and with S-CARNA 5 or D-CARNA 5. If two CARNA 5 sequence variants are present in plant tissue and supported by the same CMV strain, the questions that immediately arise are whether a competition for replication will occur between the CARNA 5s and, more important, how their competition will affect symptom expression if the two variants are on the opposite ends of the disease-inducing spectrum. If a competition ensues, both CARNA 5s are expected to accumulate to an extent that may depend on the virulence of the variants and their proportional presence in the inoculum. The symptomatology expressed in such an infection might be directly related to the respective rates of accumulation of the two CARNA 5s in the plant tissues.

The symptoms caused by CMV in the absence or presence of S-CARNA 5 or D-CARNA 5 are presented in Figure 1 (3). CMV-1 alone caused chlorosis, stunting, and mild fern leaf. Plants inoculated with CMV-1 and S-CARNA 5 appeared virtually symptomless when compared with the mock-inoculated plants. CMV-1 and D-CARNA 5 induced leaf necrosis, epinasty, and death of the plant.

Incidence of lethal necrosis in tomato plants infected with inocula containing different proportions of S- and D-CARNA 5. Disease symptoms were observed in larger groups of tomato plants 3 wk after infection with CMV-1 alone, CMV-1 + S-CARNA 5, and CMV-1 + D-CARNA 5 (all at standard concentrations of 10 μg/ml for viral RNA and 2.5 μg/ml for CARNA 5) and CMV-1 (10 μg/ml) + S-CARNA 5 (2.5 μg/ml) to which D-CARNA 5 was added in an exponential dilution series starting from the standard concentration of 2.5 μg/ml (Table 1). In the infections with mixtures of satellites, the symptoms typical of D-CARNA 5 predominated until the D-CARNA 5 in the inoculum was reduced to 10^{-3} times the concentration of S-CARNA 5. In earlier work, a similar dilution series of CMV-1 + D-CARNA 5 in the absence of S-CARNA 5 gave a necrotic response that was about one log higher (3).

CARNA 5 in tomato plants infected with inocula containing different proportions of S- and D-CARNA 5. Replication footprint analysis of total nucleic acid extracts from the tomato seedlings 10 days after they were inoculated are presented in Figure 2. D-CARNA 5 (lane 1) and S-CARNA 5 (lane 8) accumulated to similar amounts when inoculated at the standard concentration of 2.5 μg/ml, with CMV-1 at 10 μg/ml. Lanes 3–7 show the accumulation of the CARNA 5s inoculated in a series of mixtures in

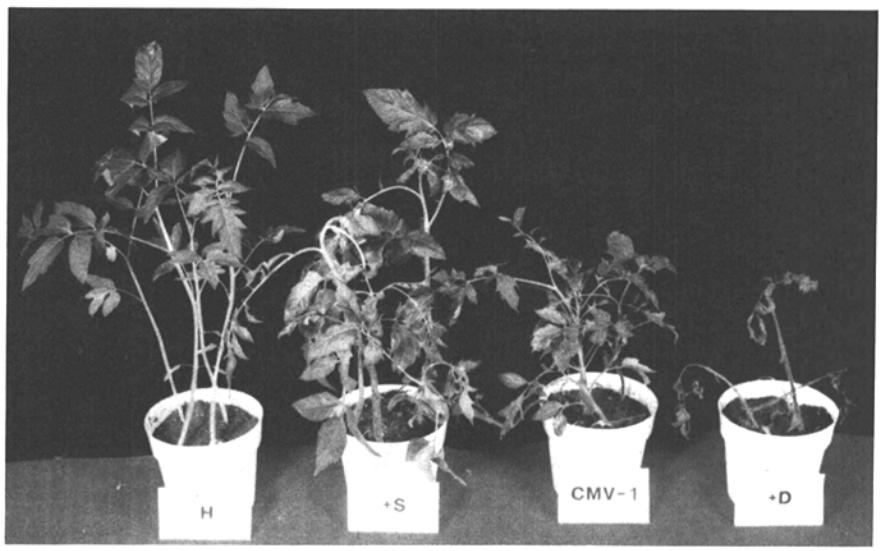

Fig. 1. Tomato plants 3 wk after inoculation (left to right): Mock-inoculated (controls) and inoculated with 10 μg/ml of CMV-1 + 2.5 μg/ml of S-CARNA 5, 10 μg/ml of CMV-1 alone, and 10 μg/ml of CMV-1 + 2.5 μg/ml of D-CARNA 5.

Table 1. Symptomatology of tomato plants inoculated with cucumber mosaic virus strain 1 (CMV-1), S-CARNA 5, and D-CARNA 5[a]

Inoculum, μg/ml	Symptoms[b]			
	Necrosis	Leaf necrosis/ epinasty	Stunting/ chlorosis	Mild chlorosis/ healthy
CMV-1, 10	0	0	100	0
CMV-1, 10 + S-CARNA 5, 2.5	0	0	0	100
CMV-1, 10 + D-CARNA 5, 2.5	93	7	0	0
CMV-1, 10 + S-CARNA 5, 2.5 + D-CARNA 5, 2.5	100	0	0	0
+ D-CARNA 5, 2.5×10^{-1}	80	20	0	0
+ D-CARNA 5, 2.5×10^{-2}	73	10	7	10
+ D-CARNA 5, 2.5×10^{-3}	20	3	13	64
+ D-CARNA 5, 2.5×10^{-4}	10	0	7	83
+ D-CARNA 5, 2.5×10^{-5}	7	0	0	93

[a] Data combined from two experiments in which 20 seedlings per experiment were inoculated with each treatment.
[b] Percentage of seedlings showing symptoms 21 days after inoculation.

which S-CARNA 5 was kept at the standard concentration and D-CARNA 5 was exponentially diluted. Although S-CARNA 5 and D-CARNA 5 (single-stranded as well as double-stranded forms) are equally evident in the samples of tissue when introduced individually (lanes 8 and 1, respectively), when inoculated as a mixture, no S-CARNA 5 was detected in the tissues until the proportion of D-CARNA 5 in the inoculum was reduced to 10^{-2} times the standard concentration (lane 4). At a 10^{-3} dilution of D-CARNA 5 in the inoculum, the two satellites were present in about equal proportions (lane 5). At a dilution of 10^{-4}, D-CARNA 5 was only found in trace amounts in the plants (lane 6), whereas S-CARNA 5 was detected in amounts comparable to those when inoculated alone (lane 8).

CARNA 5s in tomato protoplasts infected with inocula containing different proportions of S- and D-CARNA 5. To verify whether interference of S-CARNA 5 accumulation by D-CARNA 5 could be related to events at the cellular level, infections were produced in tomato protoplasts using electroporation (16). At 43 hr after inoculation (Fig. 3), D-CARNA 5, when present at the standard inoculum concentration (2.5 μg/ml) in a mixture with S-CARNA 5, interfered with the accumulation of S-CARNA 5 (lane 1). This interference appeared less pronounced than in plants (compare with lane 2 in Figure 2). There was an equal accumulation of the two CARNA 5s when the concentration of D-CARNA 5 was 10^{-1} times that of S-CARNA 5 (which was kept at the standard concentration of 2.5 μg/ml) (lane 2). However, at a dilution of 10^{-3}, only accumulation of S-CARNA 5 was detectable (lane 4).

RFP analysis. In order to determine if the competition for replication between two CARNA 5s introduced at a specific concentration ratio was maintained over time, seedlings inoculated with CMV 1 + S-CARNA 5 at 2.5 μg/ml + D-CARNA 5 at 2.5×10^{-3} μg/ml were sampled at 7, 10, 14, 18, and 24 days after inoculation (Fig. 4). S-CARNA 5 was the only satellite detected by day 7 (lane 2). However, at days 10 and 14 (lanes 3 and 4), the amount of D-CARNA 5 in the sample was greater than that of the S-CARNA 5. At day 24 (lane 6), both CARNA 5s appear at a similar, low concentration.

Symptom development in tomato inoculated with CMV-1 + S-CARNA 5, then challenge-inoculated with D-CARNA 5 alone or CMV-D + D-CARNA 5. In an effort to model recent field tests for the protection of tomato from tomato necrosis disease (4,15), we inoculated tomato seedlings with a combination of helper CMV and ameliorative S-CARNA 5 and determined the rate of accumulation of necrogenic D-CARNA 5 introduced as a challenge

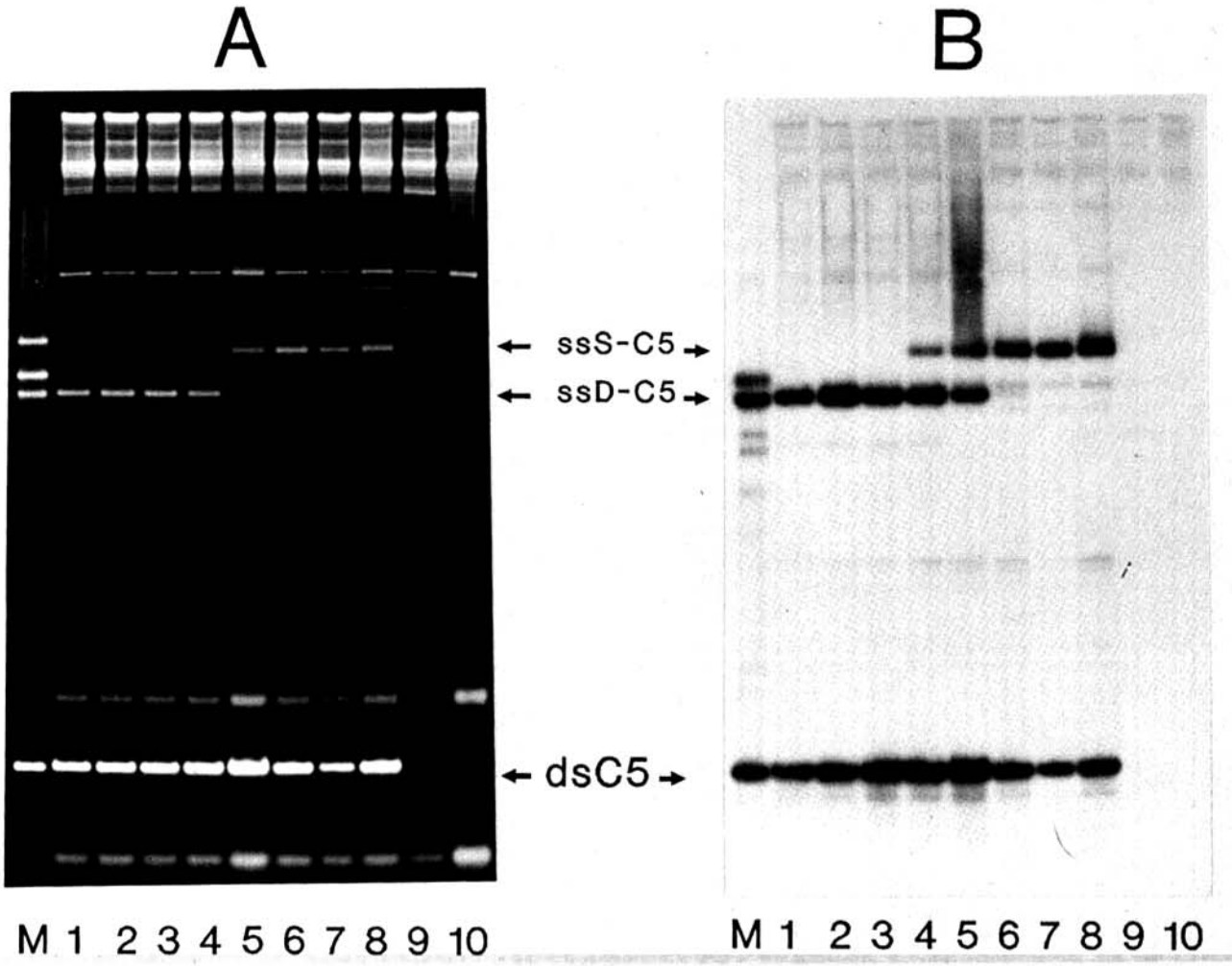

Fig. 2. Replication footprint analysis of total nucleic acid extracts of tomato plants sampled 10 days after inoculation with 10 μg/ml of CMV-1, 2.5 μg/ml of S-CARNA 5, and varying amounts of D-CARNA 5, then analyzed by 9% semidenaturing PAGE. Ethidium bromide **(A)** stained and UV-photographed and **(B)** northern-transferred and hybridized with a ^{32}P-labeled riboprobe to S- and D-CARNA 5s. M = marker RNAs containing, in descending order, PARNA 5, D- and I-CARNA 5, and dsD-CARNA 5. Total NAs from plants inoculated with (lane 1) CMV-1 + D-CARNA 5, (lane 2) CMV-1 + S-CARNA 5 at 2.5 μg/ml + D-CARNA 5 at 2.5 μg/ml, (lane 3) + D-CARNA 5 at 2.5 $\times 10^{-1}$ μg/ml, (lane 4) + D-CARNA 5 at 2.5 $\times 10^{-2}$ μg/ml, (lane 5) + D-CARNA 5 at 2.5 $\times 10^{-3}$ μg/ml, (lane 6) + D-CARNA 5 at 2.5 $\times 10^{-4}$ μg/ml, (lane 7) + D-CARNA 5 at 2.5 $\times 10^{-5}$ μg/ml, (lane 8) CMV-1 + S-CARNA 5, (lane 9) CMV-1, and (lane 10) mock-inoculated (uninfected tomato).

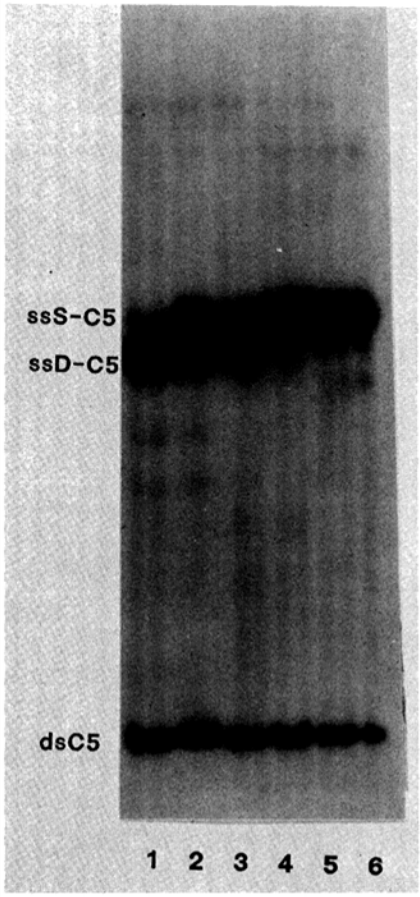

Fig. 3. Replication footprint analysis by 9% semidenaturing PAGE of total nucleic acid extracts of tomato protoplasts 43 hr after inoculation. After northern transfer, membrane was hybridized with a ^{32}P-labeled riboprobe to S- and D-CARNA 5s and autoradiographed. Protoplasts infected with CMV-1 at 10 μg/ml + S-CARNA 5 at 2.5 μg/ml + D-CARNA 5 at (lane 1) 2.5 μg/ml, (lane 2) 2.5×10^{-1} μg/ml, (lane 3) 2.5×10^{-2} μg/ml, (lane 4) 2.5×10^{-3} μg/ml, (lane 5) 2.5×10^{-4} μg/ml, and (lane 6) 2.5×10^{-5} μg/ml.

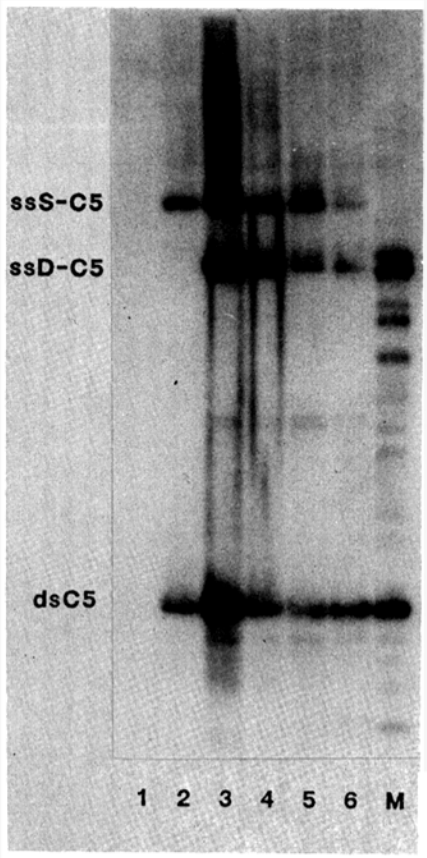

Fig. 4. Replication footprint profile of total nucleic acid extracts of 0.25 g of tomato leaf tissue harvested on different days after inoculation with 10 μg/ml of CMV-1 + S-CARNA 5 at 2.5 μg/ml + D-CARNA 5 at 2.5×10^{-3} μg/ml and analyzed by 9% semidenaturing PAGE. After northern transfer, membrane was hybridized with a ^{32}P-labeled riboprobe to S- and D-CARNA 5s. (Lane 1) uninfected plants; sample taken at (lane 2) 7 days, (lane 3) 10 days, (lane 4) 14 days, (lane 5) 18 days, and (lane 6) 24 days after inoculation. M = marker lane containing D-CARNA 5, 1-CARNA 5, and dsCARNA 5.

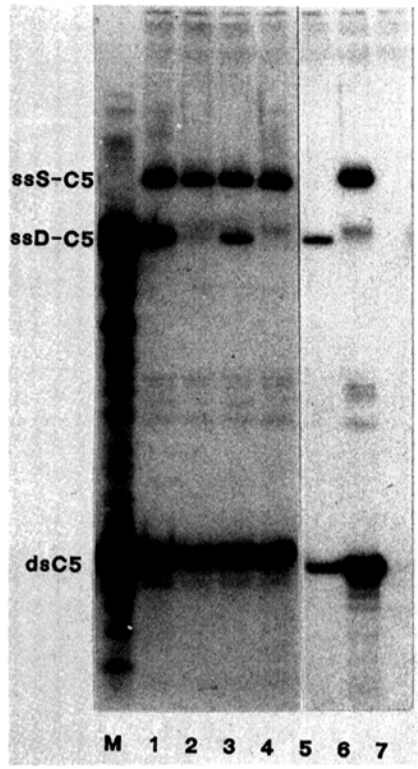

Fig. 5. Replication footprint analysis by 9% semidenaturing PAGE of total nucleic acids of samples taken 21 days after tomato seedlings were inoculated with 10 μg/ml of CMV-1 + 2.5 μg/ml of S-CARNA 5 and challenge-inoculated 3 days later with two concentrations of CMV-D + D-CARNA 5 or D-CARNA 5 alone. After northern transfer, membrane was hybridized with a ^{32}P-labeled riboprobe to S- and D-CARNA 5s. M = marker lane containing D-CARNA 5, 1-CARNA 5, and dsCARNA 5. (Lane 1) Challenge with CMV-D at 10 μg/ml + D-CARNA 5 at 2.5 μg/ml, (lane 2) challenge with CMV-D at 10 μg/ml + D-CARNA 5 at 2.5×10^{-2} μg/ml, (lane 3) challenge with D-CARNA 5 at 2.5 μg/ml, (lane 4) challenge with D-CARNA 5 at 2.5×10^{-2} μg/ml, (lane 5) uninfected plant inoculated with CMV-D + D-CARNA 5 at time of challenge inoculation, (lane 6) inoculated plant with no challenge, and (lane 7) plant inoculated with CMV-1 alone.

infection as early as 3 days after inoculation. The symptomatology expressed in an infection of this nature was found to depend on the concentration of the necrogenic CARNA 5 inoculum and the time of the challenge inoculation, two factors that in turn affect the rate of accumulation of the satellites competing for replication and expression. The symptoms that developed in groups of plants inoculated with CMV-1 + S-CARNA 5 at 2.5 μg/ml and then allowed to grow for 3 or 7 days prior to challenge inoculation with D-CARNA 5 alone or CMV-D + D-CARNA 5, at the concentrations indicated, are summarized in Table 2. Plants developed tomato necrosis or epinasty (usually a precursor of tomato necrosis) only when D-CARNA 5 was used at 2.5 μg/ml and was applied to the plants 3 days after their inoculation with CMV-1 + S-CARNA 5. If the challenge inoculation was delayed to 7 days after inoculation with CMV-1 and S-CARNA 5, or if the concentration of the necrogenic D-CARNA 5 was reduced, fewer plants showed necrosis caused by D-CARNA 5. These observations were generally consistent with those (Table 1) of the effects of the reduction of D-CARNA 5 concentration in the inoculum when applied simultaneously with S-CARNA 5.

CARNA 5s in plants inoculated with CMV-1 + S-CARNA 5 and challenge-inoculated with D-CARNA 5 alone or CMV-D + D-CARNA 5. Replication footprint analysis of tissue samples taken from the experiments of Table 2, where tomato plants inoculated with CMV-1 + S-CARNA 5 at 2.5 μg/ml were allowed to grow for 3 or 7 days prior to challenge inoculation with necrosis-inducing D-CARNA 5 in the presence or absence of helper virus CMV-D, are shown in Figure 5. Samples taken 21 days after the challenge inoculation showed that D-CARNA 5 accumulated when inoculated at 2.5 μg/ml in either the presence (lane 1) or absence (lane 3) of CMV-D. However, when the inoculum concentration of D-CARNA 5 was reduced 100-fold, D-CARNA 5 did not accumulate at all (lanes 2 and 4). Very similar results, but with lesser accumulation of D-CARNA 5, were obtained when the challenge inoculation occurred 7 days after inoculation of the plants with CMV-1 + S-CARNA 5 (*data not shown*). Apparently, late introduction of D-CARNA 5 did not affect the accumulation of S-CARNA 5, which was predominant in all samples.

The results obtained demonstrate that both a dilution end point bioassay (3)

Table 2. Symptomatology of tomato plants inoculated with cucumber mosaic virus strain 1 (CMV-1) at 10 μg/ml plus S-CARNA 5 at 2.5 μg/ml and challenge-inoculated 3 or 7 days later with CMV-D at 10 μg/ml and/or D-CARNA 5 at 2.5 or 0.025 μg/ml[a]

Challenge inoculation	Symptoms[b]			
	Necrosis	Epinasty	Stunting	Mild chlorosis
At 3 days				
CMV-D + D-CARNA 5, 2.5 μg/ml	7	11	0	2
CMV-D + D-CARNA 5, 0.025 μg/ml	1	3	0	16
D-CARNA 5, 2.5 μg/ml	1	12	0	7
D-CARNA 5, 0.025 μg/ml	0	3	0	17
At 7 days				
CMV-D + D-CARNA 5, 2.5 μg/ml	1	4	13	2
CMV-D + D-CARNA 5, 0.025 μg/ml	0	0	0	20
D-CARNA 5, 2.5 μg/ml	1	2	17	0
D-CARNA 5, 0.025 μg/ml	1	2	17	0
Nonchallenged controls				
CMV-1	0	0	17	3
CMV-1 + S-CARNA 5	0	0	17[c]	3
CMV-D + D-CARNA 5, 2.5 μg/ml	16	1	0	3
CMV-D + D-CARNA 5, 0.025 μg/ml	10	7	0	3

[a]Twenty plants were inoculated with each treatment.
[b]Number of plants showing symptoms 21 days after challenge inoculation.
[c]Very mild.

combined with an RFP (Fig. 4) are required to show a cause-effect relationship of a given CMV satellite to a given biological symptom, in this case, tomato necrosis. Figure 2 shows that significant accumulation of D-CARNA 5 can occur even when inoculated in the presence of much larger amounts of S-CARNA 5 with CMV-1 as helper virus. The symptoms that develop (Table 1) suggest that only small amounts of D-CARNA 5 in the inoculum may result in expression of necrosis. This type of analysis is needed to help detect possible contaminating necrogenic satellites (10) and to maintain quality control in cases where the ameliorative S-CARNA 5 is to be used for protection.

Developing a model for CMV/CARNA 5 infections may be of practical value when the relative protective capability of selected CMV satellites vs. that of other sequence variants (either naturally occurring or genetically modified) is to be assessed. Analysis of the competition between selected CARNA 5s may be accelerated by using protoplasts. Selected combinations of CMV/CARNA 5 can be inoculated and then the plants challenged to test effectiveness against severe, locally prevailing CMV strains or CMV/satellite combinations. One could also attempt to more closely mimic natural conditions by using aphids for the challenge inoculations. The present work demonstrates that it may be experimentally feasible to monitor mixed satellite infections in the field by replication footprint analysis and thereby correlate CARNA 5 presence in the plant tissues to the observed biological symptoms.

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