CARNA 5, the Small Cucumber Mosaic Virus–Dependent Replicating RNA, Regulates Disease Expression

Abstract. *CARNA 5, the small cucumber mosaic virus-dependent replicating RNA which is the causal agent of lethal tomato necrosis disease, causes a drastic reduction of disease symptoms in at least two other plant species. Satellite-like RNA's associated with plant viruses have a disease-regulating function.*

Cucumber mosaic virus (CMV) was first described as the cause of a plant disease in 1916 (*1*). It is now known to be the causal agent of disease in nearly 100 crops throughout the world (*2*). Scarcely a single major crop, including forages, fruit trees, tropical crops, and ornamentals, is immune to CMV. This virus exists in nature as many different strains, is readily transmitted by some 60 species of aphids, and causes losses of up to 100 percent in some crops. It is known in various parts of the world and in various crops by dozens of other names and synonyms (*2*).

Recently we reported the association, for several strains of CMV, of large quantities of a satellite-like, replicating, low-molecular-weight RNA, which we designated CARNA 5 (for CMV-associated RNA 5) (*3*). It is encapsidated with CMV protein and is therefore isolated along with the CMV-RNA's (*4*). Previously we had found CARNA 5 (associated with the S strain of CMV) to be responsible for a lethal necrotic disease of tomato when it infected the plants along with CMV, its helper virus (*5*). This experimentally produced disease is identical to the disease that devastated the tomato crop in the Alsace region of France in 1972 (*6*). In the absence of CARNA 5, CMV in tomato usually causes the familiar fernleaf syndrome often accompanied by mosaic (*2*).

Whereas experiments with tomato demonstrated that CARNA 5 can dramatically increase disease severity caused by CMV (*5*), we report here that with WT isolate of CMV (*7*), CARNA 5 has an opposite effect on disease in certain other crops. The WT and S strains behave alike in tomato plants.

When CMV-WT isolate is introduced directly into tomato, the fernleaf mosaic syndrome results and plants do not develop necrosis (Fig. 1a). However, if the same inoculum is first placed in tobacco (*Nicotiana tabacum* L. Xanthi nc) and the resulting virus is then passed to tomato (*Lycopersicon esculentum* Mill cv. Rutgers), it causes necrosis and ultimate death of the plants (Fig. 1b). Electrophoretic patterns of the virus RNA extracted from these two types of diseased tomato plants show a correlation between the absence of CARNA 5 (Fig. 2a) and the occurrence of fernleaf mosaic disease (Fig. 1a) as with other strains (*5, 8*), and the presence of CARNA 5 (Fig. 2b) and the necrotic condition (Fig. 1b). Apparently, small, physically undetectable quantities of CARNA 5 present in CMV-WT, or incited in tobacco by CMV-WT, are rapidly built up in this host (*3, 7, 8*) and then multiplied further in tomato plants, resulting in their death. In the experiments described here, we used fernleaf-affected tomato plants as a source of inoculum without CARNA 5 and necrotic tomato plants as a source of inoculum with CARNA 5.

Table 1. Hybridization of 0.097 μg of ^{3}H-labeled CARNA 5 (2363 count/min) and 0.194 μg of double-stranded CARNA 5 from CMV-S in the presence of 100-fold quantities of competing CARNA 5 from CMV-WT produced in different host plants.

Competitor	Radioactivity of duplex (count/min)	f*
None	780	0
S-CARNA 5 from tobacco	80	0.90
WT-CARNA 5 from tomato	77	0.90
WT-CARNA 5 from pepper	68	0.91
WT-CARNA 5 from corn	61	0.92

*Fraction of ^{3}H-labeled CARNA 5 prevented from annealing to (−) strands of double-stranded CARNA 5. Because of the large excess of competitor over the labeled CARNA 5, this value also approximates the degree of nucleotide sequence homology with the labeled CARNA 5.

Table 2. Bioassay of CARNA 5 preparations isolated from CMV infections of tomato, pepper, or corn plants.

CARNA 5 in inoculum* (μg/ml)	Number of tomato seedlings that died with necrosis/ number of diseased plants for CARNA 5 obtained from		
	Tomato	Pepper	Corn
2.5	38/38	35/35	36/36
2.5×10^{-1}	37/37	37/38	36/37
2.5×10^{-2}	35/39	36/37	32/35
2.5×10^{-3}	27/42	22/39	18/34
2.5×10^{-4}	5/42	7/32	4/37
2.5×10^{-5}	5/42	4/37	2/34
2.5×10^{-6}	3/39	0/40	1/35
None	2/39	2/40	1/41

*The inoculum also contained 10 μg of the genomic RNA's 1, 2, and 3 of the helper virus per milliliter.

Twenty-five plant species known to be susceptible to CMV were inoculated with sap from the two types of diseased tomato plants. They represented these genera or crops: ageratum, barley, beets, catnip, *Chenopodium quinoa* Willd., chickweed, cowpeas, *Capsella*, gomphrena, jimsonweed, lettuce, nightshade, oats, pepper varieties California Wonder, Cayenne, and Tabasco, petunia, *Plantago*, rye, salpiglossis, sesame, Caserta squash, sweet corn, tithonia, Xanthi tobacco, and zinnias.

The presence of CARNA 5 in the CMV inoculum resulted in conspicuously reduced disease symptoms in several species. Among them were *C. quinoa*, Caserta squash (*Cucurbita maxima* Duch.), Tabasco pepper (*Capsicum frutescens* L.), and Bantam sweet corn (*Zea mays* L.). The latter two important crops were selected for detailed study. During the ensuing year, 110 pepper and 380 corn plants were inoculated with sap from CMV-infected tomato plants.

In six experiments, 80 to 100 percent of the pepper plants inoculated with sap from fernleaf-affected tomato tissue (that is, without CARNA 5) developed severe mosaic, stunting, and necrosis of leaves, petioles, and stem (Fig. 1c). This symptom was described as early as 1957 (*9*). Plants are rendered useless. Little or no CARNA 5 was detected in virus isolated from four of these batches of tissues (Fig. 2c). On the other hand, plants inoculated with sap from necrotic tomato tissue developed only a mild chlorosis in some trials and no symptoms beyond the inoculated leaves in others (Fig. 1d). No virus could be extracted from the symptomless leaves. Plants that did develop mild chlorosis yielded virus with an RNA component composition in which CARNA 5 dominated, while some of the genomic CMV-RNA components were barely discernible (Fig. 2d).

Sweet corn, like Tabasco pepper, is more severely diseased when CARNA 5 is absent. When sap from fernleaf-affected tomato tissue was used as inoculum in six experiments, 90 to 98 percent of the infected sweet corn plants developed severe necrosis and stunting, and death occurred within 3 weeks after inoculation (Fig. 1e). Plants in three experiments were analyzed, and although CMV was present in the corn, no CARNA 5 could be detected from plants with incipient necrosis (Fig. 2e). On the other hand, when sap from necrotic tomato tissue was used as inoculum, sweet corn plants developed varying degrees of systemic chlorosis and stunting (Fig. 1f), but they did not die during a 3-month observation period. In three analyses, virus

0036-8075/79/0525-0845$00.50/0

from these plants contained a large proportion of CARNA 5 (Fig. 2f).

To ascertain that the CARNA 5 produced in necrotic tomato plants (Fig. 1b) was identical to that in the mildly diseased pepper and corn plants (Fig. 1, d and f), we isolated the CARNA 5 produced in these species and compared the nucleotide sequences for homology with competition hybridization and performed biological tests. To this end, large numbers of tomato, pepper, and corn plants were inoculated with purified CMV-WT (20 μg/ml) to which CARNA 5 was added (2.5 μg/ml). The plants produced symptoms like those shown in Fig. 1, b, d, and f, respectively. The RNA polyacrylamide electrophoresis patterns of the virus preparations from the three species resembled those shown in Fig. 2, b, d, and f. CARNA 5 was separated from the viral RNA's and purified by described methods (*10*). Nucleotide sequence homology was compared by allowing each of the three preparations to compete with the hybridization of ^{3}H-labeled CARNA 5 and double-stranded CARNA 5 from CMV-S, as described (*7*). It is evident from Table 1 that the CARNA 5 preparations from CMV-WT infections of tomato, pepper, and corn were as effective as CARNA 5 from CMV-S (homologous competitor) in preventing the incorporation of ^{3}H-labeled CARNA 5 into a ribonuclease-resistant duplex. The ability of the three CARNA 5 preparations to induce tomato necrosis was compared in tests in which the genomic RNA's 1, 2, and 3 of CMV-WT (10 μg/ml) were used to infect tomato seedlings in the presence of each of the CARNA 5 preparations at increasing dilutions. At a concentration of 2.5×10^{-3} μg/ml, all three CARNA 5 preparations caused necrosis and death of about half of the test plants (Table 2). These tests show that the CARNA 5's produced and supported by CMV-WT in three host species are chemically and biologically the same, if not identical, molecules. Moreover, they are also identical to CARNA 5 produced by CMV-S in tobacco (*5*).

The correlation between the presence

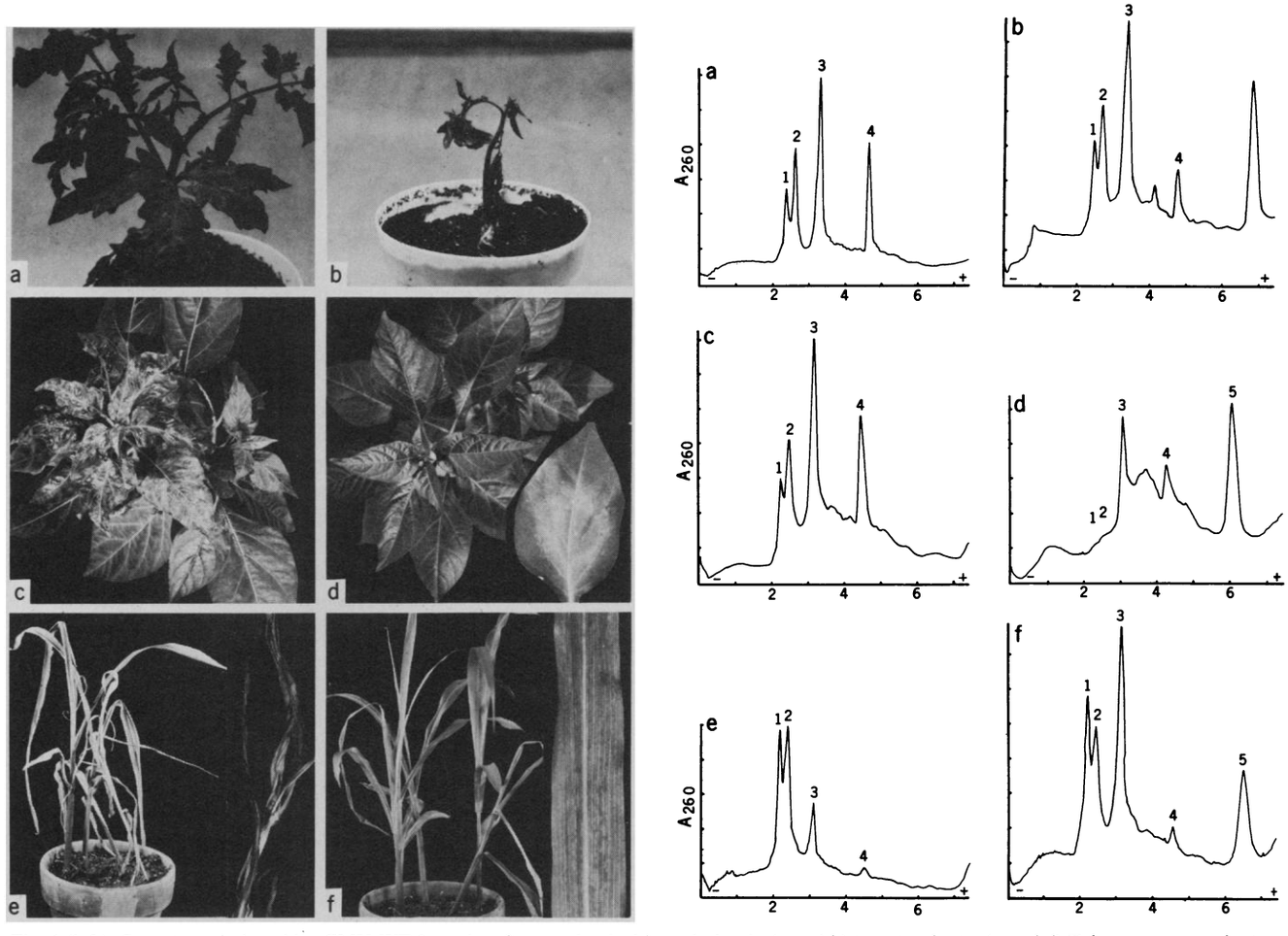

Fig. 1 (left). Symptoms induced by CMV-WT 3 weeks after mechanical inoculation in (a and b) tomato plants, (c and d) Tabasco pepper plants, and (e and f) sweet corn. Plants on the left were inoculated with virus free of CARNA 5 and those on the right with CMV that contained CARNA 5. Pepper plants in (d) show symptoms of virus infection only in some of the inoculated leaves (inset), and on analysis virus was often not systemic in these plants. Close-ups of the disease symptoms are shown in (e) and (f). The RNA composition of the virus in these six treatments is shown in the corresponding position in Fig. 2. Notice that the presence of CARNA 5 in the inoculum (right side) causes a more severe disease than the absence of CARNA 5 (left side) in tomato, but a less severe disease in Tabasco peppers and sweet corn. Inoculations were performed by triturating infected leaves in 0.025*M* (*p*H 7.8) phosphate buffer (1:5, weight to volume) and with Q-tips rubbing the liquid on the surface of abrasive (Carborundum)-dusted leaves of small test plants. Plants were held in a greenhouse at 19° to 23°C. Fig. 2 (right). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis profiles of CMV-WT RNA. Virus was purified by methods described previously (*3*) 3 weeks after mechanical inoculation. The virus (200 μg) was mixed with 30 to 50 μl of 0.01*M* sodium phosphate and 0.01*M* EDTA, *p*H 7, containing 10 percent sucrose and 1 percent SDS, and layered on 2.4 percent gels. Electrophoresis was performed at ambient temperatures for 3.25 hours at 4 mA per gel in buffer consisting of 0.04*M* tris, 0.02*M* sodium acetate, 0.002*M* EDTA, and 0.1 percent SDS, *p*H 7.8. The CMV was isolated from (a) tomato plants inoculated directly with the original CMV-WT isolate (*7*), (b) tomato plants inoculated with the CMV-WT isolate after one passage in tobacco, (c) Tabasco pepper plants inoculated with sap from fernleaf tomato tissue, (d) Tabasco pepper plants inoculated with sap from necrotic tomato tissue, (e) sweet corn plants inoculated as in (c), and (f) sweet corn plants inoculated as in (d).

of CARNA 5 with the severe disease incited in tomato plants and the mild symptoms incited in Tabasco pepper and sweet corn plants contrasts strikingly with the absence of CARNA 5 and the resulting milder disease in tomato and severe diseases in pepper and sweet corn plants. In tobacco plants where high CARNA 5 results in reduced virus multiplication (*10*), a similar reduction in symptoms should be expected. However, with this host, the biological effects are hardly visible because the disease evoked by CMV is mild in either case.

It seems clear, therefore, that in natural hosts of CMV, which support multiplication of CARNA 5, the presence of CARNA 5 is expressed either as a new disease syndrome [shown to be lethal in at least one case (*5, 6, 8*)] or (and more often) as an attenuation of the viral infection with a resulting decrease in symptoms. We previously indicated how, during pathogenesis, the competition between CARNA 5 and the viral RNA's or the messenger capability of CARNA 5, or both, could decisively affect symptom expression (*3, 11*).

Huang and Baltimore (*12*) proposed that the competitive relationship of defective interfering (DI) particles and their helper viruses could play a major role in the evolution of viral disease. However, DI particles whose nucleic acid is directly related to the genome of their helper viruses (*12*) have not yet been described for plant viruses. On the other hand, viral satellites, equally dependent on their virus helpers for replication, are being found with increasing frequency in plant virus infections. Their genetic content has little if any relationship to the helper's genome (*13*). However, we have some evidence suggesting that they could have originated from the host (unpublished work). We therefore propose that small, satellite-like replicating RNA's such as CARNA 5 play a regulatory role in viral pathogenesis and ultimate disease expression. This report illustrates such regulatory effects by CARNA 5 in infections of CMV in three of its natural hosts, which are also important agricultural crops.

H. E. WATERWORTH
Plant Introduction Station, Agricultural Research, Science and Education Administration, Department of Agriculture, Glenn Dale, Maryland 20769

J. M. KAPER, M. E. TOUSIGNANT
Plant Virology Laboratory, Plant Protection Institute, Agricultural Research, Science and Education Administration, Department of Agriculture, Beltsville, Maryland 20705

References and Notes

1. S. P. Doolittle, *Phytopathology* **6**, 145 (1916).
2. A. J. Gibbs and B. D. Harrison, *Commonw. Mycol. Inst. Descr. Plant Viruses No. 1* (1970); K. M. Smith, *A Textbook of Plant Virus Diseases* (Longman, London, ed. 3, 1972), p. 234; E. B. Martyn, Ed., *Plant Virus Names* (Commonwealth Mycological Institute, Kew, Surrey, England, 1968).
3. J. M. Kaper and M. E. Tousignant, *Virology* **80**, 186 (1977).
4. H. Lot and J. M. Kaper, *ibid.* **74**, 223 (1976).
5. J. M. Kaper and H. E. Waterworth, *Science* **196**, 429 (1977).
6. A. Vuittenez and C. Putz, *Alsace*, 20 and 22 August 1972; C. Putz, J. Kuszala, G. Spindler, *Ann. Phytopathol.* **6**, 1939 (1974).
7. J. M. Kaper and M. E. Tousignant, *Virology* **85**, 323 (1978).
8. H. E. Waterworth, M. E. Tousignant, J. M. Kaper, *Phytopathology* **58**, 561 (1978).
9. C. W. Anderson and M. K. Corbett, *Plant Dis. Rep.* **41**, 143 (1957).
10. J. M. Kaper, M. E. Tousignant, H. Lot, *Biochem. Biophys. Res. Commun.* **72**, 1237 (1976).
11. R. A. Owens and J. M. Kaper, *Virology* **80**, 196 (1977).
12. A. S. Huang and D. Baltimore, *Nature (London)* **226**, 325 (1970); in *Comprehensive Virology*, H. Fraenkel-Conrat and R. R. Wagner, Eds. (Plenum, New York, 1977), vol. 10, p. 73.
13. A. Shoulder, G. Darby, T. Minson, *Nature (London)* **251**, 733 (1974); J. R. Diaz-Ruiz and J. M. Kaper, *Virology* **80**, 214 (1977); J. M. Kaper *et al.*, *ibid.* **88**, 166 (1978).
14. We thank W. Povish, R. Holsinger, and F. Fan for technical assistance.

28 September 1978; revised 14 February 1979

Mortality of the Monarch Butterfly (*Danaus plexippus* L.): Avian Predation at Five Overwintering Sites in Mexico

Abstract. *Analyses of predated butterflies on the forest floor at five monarch overwintering sites in Mexico and observations of birds foraging in mixed flocks indicate that individual birds of several species have learned to penetrate the monarch's cardenolide-based chemical defense. Predation is inversely proportional to colony size and appears to be one evolutionary explanation of the dense aggregations.*

Occasional bird predation of overwintering monarch butterflies has been reported in California (*1, 2*) where small mammal predation was also inferred from wing caches and live, maimed butterflies falling from trees at night (*3*). In contrast to this minor predation in California, we report here extraordinarily heavy mortality at one previously described (*4*) and four newly discovered overwintering sites in Mexico (*5*).

Within these Mexican colonies and particularly beneath heavily laden trees (Fig. 1), accumulations of fallen butterflies increased throughout the 1977–1978 overwintering season, and by late February the forest floor was thickly carpeted in places (Figs. 2 and 3) with as many as 776 butterflies per square meter. These accumulations consisted mostly of dead butterflies. Some of the live ones were temporarily immobilized by the cold and flew off when warmed. Others were moribund, including intact individuals unable to fly, and still others, definitely predated, were missing one or more wings, abdomens, and/or heads (Figs. 4 to 11).

We collected random samples (*6*) (Fig. 3) totaling 1697 dead butterflies at site Delta on 26 March 1978, shortly after its abandonment. Undamaged butterflies constituted 11 percent of the total. The remaining 89 percent showed definite indications of predation. Of these, 35 percent lacked one or more wings, and 65 percent had all wings intact but were damaged in various other ways. A further breakdown of this last-named subgroup showed overlapping damage categories: 68 percent had damaged thoraces—from a small hole to a large eaten-out portion (Fig. 12)—and 53 percent had missing abdomens (Figs. 4, 8, 10, and 11). Moreover, of those with their abdomens still attached, 19 percent had the abdominal contents stripped, leaving only the outer cuticular shell. On three occasions, we observed a live female attached in copulo to a dead, stripped male (Fig. 13). The same categories of moribund and dead butterflies occurred at all sites.

Binocular and photographic observations at all sites revealed three species of birds preying extensively on the monarchs (*7, 8*). These were Scott's oriole (*Icterus parisorum* Bonaparte), the black-backed oriole (*Icterus abeillei* Lesson) (Figs. 14 and 15), and the black-headed grosbeak (*Pheucticus melanocephalus* Swainson) (Fig. 16). Both oriole species and the grosbeaks flew in mixed flocks of 25 to 30 birds, made repeated forays into the colonies, and attacked monarchs in the hanging clusters and, to a lesser extent, on the tree trunks. The orioles spent more time pecking the butterflies and frequently stripped the abdomens, whereas the grosbeaks often deftly snapped off and ate only the abdomens, dropping the rest of the butterfly.

In 37 percent of our direct observations (9 of 22 oriole attacks and 4 of 13 grosbeak attacks) the individual birds captured, apparently or actually damaged, and then released a butterfly without eating it. In addition, we repeatedly observed monarch wings and body parts with wings attached floating down from

0036-8075/79/0525-0847$00.50/0