Etiology

Tomato White Leaf: The Relation of an Apparent Satellite RNA and Cucumber Mosaic Virus

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ABSTRACT

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In 1970, an apparently new disease of tomato (*Lycopersicon esculentum*) was observed near Geneva, New York. Affected plants had a striking whitish green mottle on the leaf laminae, whereas petioles and stems were greenish white instead of the normal purplish green. Immature fruit were almost white, but eventually developed normal red color at maturity. Although affected plants were small, the disease was not lethal. Cucumber mosaic virus, designated CMV-WL, was consistently recovered from symptomatic plants. In sucrose density gradients, RNA extracted from CMV-WL sedimented as five components designated as RNAs 1+2, 3, 4, 4a, and 5 in order of decreasing sedimentation velocities, respectively. While only RNAs 1+2+3 were required for infectivity, tomatoes inoculated with these RNAs did not develop white leaf symptoms, but developed a downward leaf curling along the midvein and greatly reduced leaf lamina. However, white leaf symptoms developed on tomatoes inoculated with CMV-WL RNAs 1+2+3+5. Inoculum that contained CMV-WL RNA 5 and RNAs 1+2+3 of the CMV-C strain also produced white leaf on tomato, but symptoms were milder. *Nicotiana tabacum* 'Havana 423' often developed a brilliant chlorosis when inoculated with CMV-WL RNAs 1+2+3, but were symptomless when inoculated with CMV-WL RNAs 1+2+3+5.

Additional key words: CARNA 5, virus strains, host passage.

In 1970, an apparently new disease of tomato (*Lycopersicon esculentum* Mill.) was observed in experimental plots at the New York State Agricultural Experiment Station (NYSAES) at Geneva, New York. Cucumber mosaic virus (CMV) was consistently recovered from diseased plants, but the mosaic-fernleaf symptoms commonly associated with CMV infection of tomato were absent (3). Instead, leaf laminae had a prominent whitish-green mottle and the petioles and stems were greenish-white which contrasted to the purplish-green color of healthy plants. Affected plants were stunted, but the disease was not lethal. Fruits of affected plants were greenish-white when immature, but did develop the normal red color at maturity. In this report, this disease is called tomato white leaf.

Although most tomato plants which were inoculated with extracts from affected plants developed white leaf symptoms, some inoculated plants showed strikingly different symptoms. These

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plants retained the normal green color, but leaf laminae became narrow with a prominent downward curl. Infected plants were slightly stunted. Moreover, CMV was always recovered from plants showing leaf curl symptoms. Thus, it appeared that CMV was causing both the white leaf and leaf curl symptoms on tomato.

Recently, investigators (13,17,22–24) have shown that low-molecular-weight (~100,000 daltons) satellite RNAs, some of which are also referred to as CARNA 5 (13) or more recently (n)CARNA 5 (12), can often cause distinct symptom modifications in CMV-infected plants. For example, Kaper and Waterworth (13) showed that CARNA 5 in tandem with the CMV-S strain caused a lethal disease of tomato, whereas this same strain without detectable CARNA 5 incited mosaic and fern leaf symptoms. Waterworth et al (23) also showed that CARNA 5 attenuated symptoms caused by CMV in many other plants such as pepper and corn. Other workers have reported changes in symptoms caused by CMV in tobacco (22) and alleviation of symptoms in tomato (17) induced by satellite RNAs from other CMV isolates.

Since our observations with the white leaf disease of tomato indicated some similarities with the disease of tomato incited by (n) CARNA 5 (13), we sought to determine if the white leaf disease was caused by a satellite RNA associated with CMV. In this report, we show that a low-molecular-weight RNA associated with CMV caused the white leaf symptom of tomatoes whereas the genomic RNA of CMV, in the absence of detectable amounts of this RNA, caused the leaf curl symptom. A preliminary report was published (6).

MATERIALS AND METHODS

Virus isolates. The CMV isolate (CMV-WL), which was used in most experiments, originated from a tobacco mosaic virus-resistant tomato line (80-18-1) showing white leaf symptoms. The plant was from an experimental field at the NYSAES, Geneva, New York. The CMV-C isolate (19), which causes fernleaf and mosaic symptoms on tomato, was also used for comparative studies in some experiments. CMV isolates were maintained in tomato line 80-18-1 which was also used for routine infectivity and purification experiments involving tomato.

Virus and RNA purification. Virus was purified from tomato and zucchini squash (*Cucurbita pepo* 'President') by the method of Lot et al (15) except that the virus was finally resuspended in PEN buffer (0.01 M sodium phosphate + 0.001 M EDTA + 0.001 M NaN_3, pH 7.0).

RNA was extracted from purified virus preparations by a modification (7) of the method described by Brakke and Van Pelt (1). All viral RNAs were suspended in sterile PEN buffer. RNA was separated into components by three centrifugation cycles in 7.5–30% (w/v) linear sucrose gradients (5). Centrifugation was at 35,000 RPM for 18 hr at 6 C in a Beckman SW 40 Ti rotor. Sucrose was dissolved in PEN buffer.

Additional methods. Serological tests in medium containing 0.75% Ionagar and 0.1% NaN_3 were done by using antisera prepared to CMV-B (19) and CMV-C. Tissue extracts used as inoculum were prepared in 0.01 M sodium phosphate, pH 7.0. Preparations of RNA containing ~250 μg of fractionated bentonite (4) were inoculated to plants by rubbing their leaves with ground glass spatulas that had been dipped in the inoculum. Inoculations with virus and RNA were performed throughout the year and plants were routinely kept in insect-free greenhouses maintained at 21–30 C. Fluorescent lights were used to ensure a daily illumination of at least 12 hr.

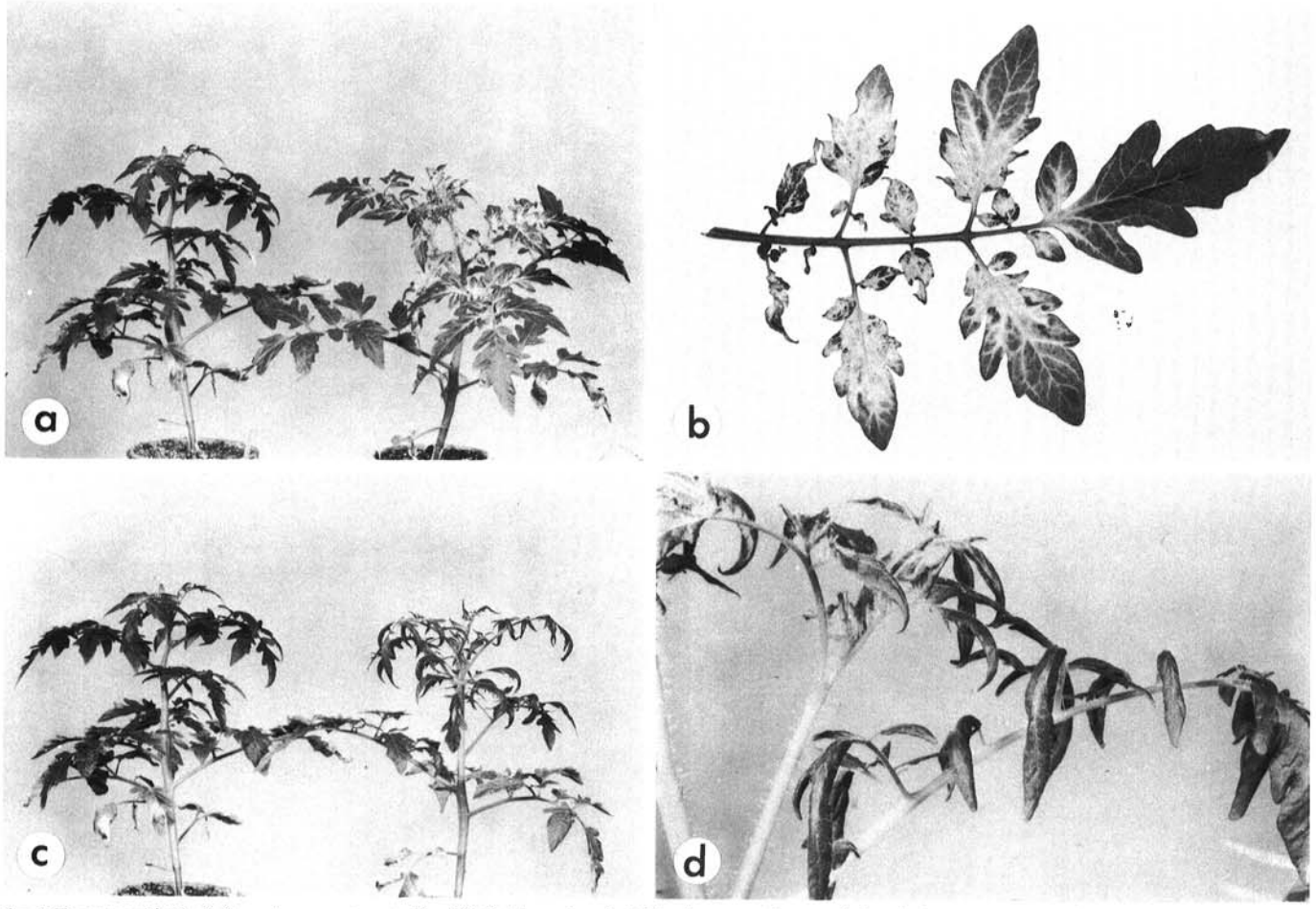

Fig. 1. Tomato white leaf. Symptoms on tomato line 80-18-1 inoculated with leaf extracts from an infected plant. **A and B,** White leaf symptoms. **C and D,** Leaf curl symptoms. Plants on the left in **A and C** are healthy.

RESULTS

Field observations of tomato white leaf disease. Since it was first seen in 1970, the tomato white leaf disease has occurred every year in the same field at the NYSAES in Geneva, New York. Disease incidence has been only 1–2% even though 50–80% of plants in the field often showed the mosaic-fernleaf symptoms typical for CMV-C type infection. None of the plants have shown both mosaic-fernleaf and white leaf symptoms. The white leaf disease has not been seen either in other areas around Geneva or elsewhere.

Association of CMV with tomato white leaf. White leaf symptoms developed 7–14 days postinoculation on most tomato plants inoculated with leaf extracts from field-collected tomato affected by white leaf (Fig. 1). However, a few plants occasionally developed leaf curl symptoms (Fig. 1). In subsequent inoculations with extracts from the tomato plants showing white leaf in the greenhouse, the white leaf condition often occurred on all inoculated plants (Table 1). However, if the inocula were prepared from plants that had been infected for several months, some inoculated plants would show either leaf curl or no symptoms (Table 1). Tomato inoculated with leaf extracts from tomato plants with leaf curl symptoms always produced leaf curl symptoms. White leaf symptoms were reproduced on a number of tomato lines or cultivars including Veemore, New Yorker, Nova, Rutgers, C-1327, and H-1350, and wild species such as *L. peruvianum*, *L. hirsutum*, and *L. pimpinellifolium*.

Cucumber mosaic virus was always recovered from tomato plants with white leaf or leaf curl symptoms. Inocula from these affected plants produced mosaic symptoms on squash and cucumber (*Cucumis sativus* 'Marketmore') and local lesions on *Chenopodium quinoa*. Antisera to CMV-C (Fig. 2) and CMV-B isolates reacted strongly with CMV-WL. Electron microscopy of purified CMV-WL showed particles of similar size and morphology to CMV-C and CMV-B (*unpublished*).

Induction of tomato white leaf disease by CMV-RNA 5 in association with CMV-WL RNAs 1+2+3. In the initial phase of this work, we observed that tomato plants developed only leaf curl symptoms when inoculated with crude extracts from cucumber or squash that had been inoculated with crude extracts from tomato with white leaf symptoms. However, virus purified from leaves of these cucumber or squash plants caused white leaf symptoms on tomato, indicating that the concentration of the causal agent(s) for white leaf was too low in crude extracts of infected squash or cucumber to incite white leaf in tomato plants.

To obtain more definitive data on the causal agent of white leaf, experiments were done with RNA extracted from virus that had been purified from infected squash. These plants had been inoculated with leaf extracts from tomato with white leaf symptoms, or with extracts from zucchini previously inoculated with extracts from tomato with white leaf symptoms. In sucrose gradients, CMV-WL RNA separated into five reproducible components designated, in order of decreasing sedimentation velocities, as RNAs (1+2), 3, 4, 4a, and 5, respectively (Fig. 3). Two other very slowly sedimenting zones (RNAs 6 and 7) were also often observed. RNA (1+2) separated into two species after electrophoresis in acrylamide gels (*unpublished*). RNAs (1+2), 3, 4, and 5 from sucrose gradients thus corresponded to CMV-RNAs 1, 2, 3, 4, and 5, respectively, which are observed after electrophoresis in polyacrylamide gels (14,18). RNA 4a has been reported by some workers (8,18). The RNA sedimentation profile of CMV-C (purified from squash) was similar to CMV-WL RNA except that RNAs 4a, 5, 6, and 7 were not observed (Fig. 3).

CMV-RNA components did not show detectable cross contamination after purification through three density gradient cycles (Fig. 3). Individual RNA components were not infectious, but combinations of RNAs 1+2+3 of CMV-WL and CMV-C were highly infectious. Tomato plants inoculated with mixtures of CMV-WL RNAs 1+2+3 alone or in combination with either 4, 4a, or 6 developed leaf curl symptoms (Table 2). Conversely, mixtures of RNAs 1+2+3+5 produced white leaf symptoms on tomato. In another experiment, all possible combinations of RNAs 1+2+3, 4, 4a, and 5 were each inoculated to two tomato plants. White leaf symptoms developed only in plants inoculated with RNA mixtures containing at least 1+2+3 and 5.

Symptoms produced on tomato inoculated with RNAs from CMV-C and CMV-WL. Others have shown that (n)CARNA 5 or CMV-satellite RNA 5, can cause lethal necrosis of tomato (13,22,24) or chlorosis of tobacco (22) even when associated with heterologous isolates of CMV. Consequently, tomato plants were inoculated with RNA mixtures containing CMV-C RNA 1+2+3 and CMV-WL RNA 5 (Table 3). Plants inoculated with CMV-C RNAs 1+2+3 developed typical mosaic-fernleaf symptoms, whereas those inoculated with CMV-C RNAs 1+2+3+CMV-WL RNA 5 developed white leaf symptoms. However, white leaf symptoms were milder on newer emerging leaves, and eventually were almost absent in new growth 4 wk after inoculation. When these plants were moved from 25 C to 16 C, the white leaf symptoms became more prominent but still were significantly milder than those of comparable plants inoculated with CMV-WL RNAs 1+2+3+5.

Effect of WL RNA 5 on virus yield in tomato and squash. Plants of zucchini squash cultivar President were routinely used to increase a number of CMV isolates for purification in our laboratory. Virus yield was fairly uniform between isolates except that the yield of CMV-B was lower. Yield of CMV-WL from squash averaged 200–300 mg per kilogram of tissue in seven purification trials. In tomato, however, average yield (five to seven experiments) of CMV-WL from plants showing white leaf symptoms was significantly less (4 mg/kg) than from those with

TABLE 1. Effect of cucumber mosaic virus-WL inoculum source on symptom production in tomato line 80-18-1

Exp.[a]	Tomato inoculum source[b]	Symptoms on tomato[c]		
		WL	LC	NS
A	WL	93	3	10
B	WL	20	0	0
C,D	WL	20	0	0
E	WL	12	3	15
F	LC	0	5	0
G	LC	0	15	0

[a] Inoculum source in Exp. B, C, D, F, and G from plants inoculated 3–6 wk earlier and A and E from plants inoculated 10–12 wk earlier.

[b] Leaf extracts ground in 0.01 M phosphate (Na^+), pH 7.0. Tomatoes showed white leaf (WL) or leaf curl (LC) symptoms.

[c] WL = white leaf, LC = leaf curl, NS = No symptoms. Numbers refer to number of inoculated plants in various symptom categories.

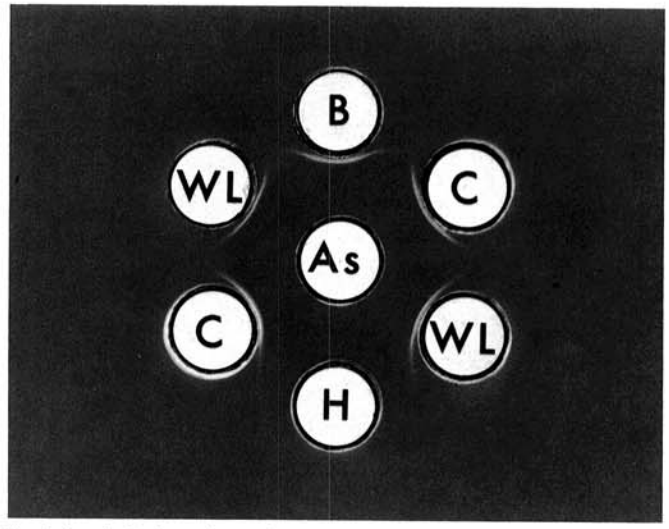

Fig. 2. Serological reactions of cucumber mosaic virus (CMV) isolates B, C, and WL in immunodiffusion tests. The center well contains antiserum to CMV-C. Outer wells contain 1 mg of virus per milliliter in suspensions purified from squash (designated B, C, and WL for corresponding CMV isolates). H = extract from healthy zucchini squash. Medium contains 0.75% Ionagar and 0.1% NaN_3.

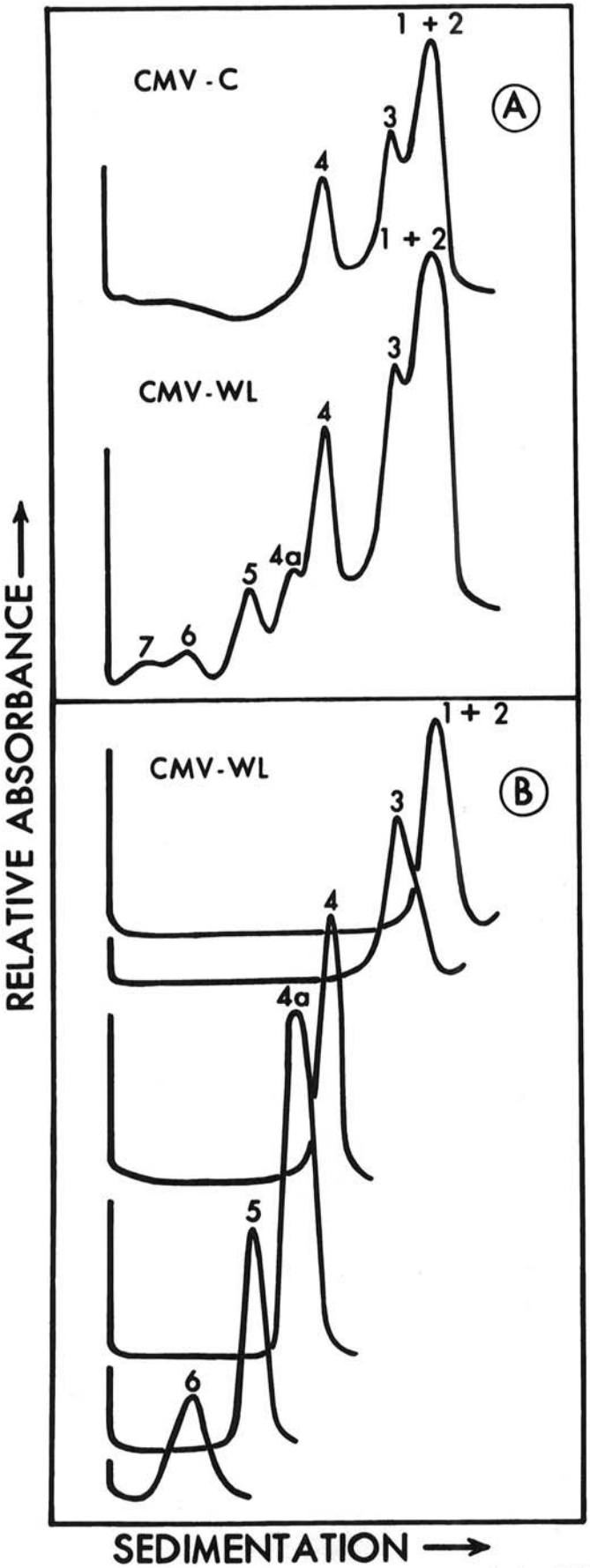

Fig. 3. Ultraviolet absorbance patterns of cucumber mosaic virus RNA preparations centrifuged in a 7.5–30% linear sucrose gradient. Unfractionated CMV-C and CMV-WL RNAs (top frame). CMV-WL RNAs separated into components by three sucrose density gradient cycles (bottom frame). Centrifugation was in a Beckman SW 40 Ti rotor at 350,000 ppm for 18 hr at 6 C.

leaf curl symptoms (28 mg/kg). In a single experiment, virus yield from tomato plants inoculated with CMV-RNAs 1+2+3 (leaf curl symptoms) was 260 mg/kg as compared to 20 mg/kg from tomato inoculated with CMV-RNAs 1+2+3+5 (white leaf symptoms).

Symptoms on *Nicotiana tabacum* inoculated with CMV-WL RNAs. Since Takanami (22) had shown that a CMV satellite RNA associated with CMV-Y produced a striking chlorosis on tobacco which was somewhat similar to white leaf symptoms on tomato, we were interested in determining if CMV-WL RNA 5 would have a similar effect on tobacco. Contrary to our expectations, all plants inoculated with RNAs 1+2+3+5 remained symptomless (Table 4, Fig. 4). However, in the first two experiments (A and B of Table 4) three of ten plants inoculated with RNAs 1+2+3 developed an intense chlorosis (Fig. 4). The other seven either developed a mild mottle accompanied by a slight narrowing of leaves or remained symptomless. Because WL-RNA 5 attenuated symptoms in tobacco, we suspected that residual contamination of RNA 5 in the RNA 1+2+3 inoculum prevented the development of intense chlorosis in these seven plants. Thus, RNA was extracted from an aliquot of the same virus preparation which supplied RNAs 1+2+3 in the first two experiments, fractionated and purified through three sucrose density gradient cycles, and subsequently tested on tobacco (experiment C of Table 4). Four of five plants inoculated with RNAs 1+2+3 developed intense chlorosis, while all five plants inoculated with RNAs 1+2+3+5 remained symptomless. Virus was recovered from all symptomless plants.

DISCUSSION

CMV-WL RNA 5 is probably a satellite RNA that is dependent on CMV for replication. Its properties are similar in many respects to those of satellite RNAs reported by others. Primarily, it alters or attenuates the symptoms in some plants, reduces virus yield, and can act in conjunction with other CMV isolates (10,11,17,22,23).

TABLE 2. Tomato white leaf. Symptoms on tomato line 80-18-1 inoculated with RNAs from cucumber mosaic virus-WL

Inoculum RNA 1+2+3 plus:	Symptoms on tomato[a]	
	Undiluted[b]	1/5[b]
...	LC (5/5)[c]	LC (5/5)
4	LC (5/5)	LC (5/5)
4a	LC (5/5)	LC (5/5)
5	WL (5/5)	WL (5/5)
6	LC (5/5)	LC (5/5)

[a] LC = leaf curl, WL = white leaf.
[b] RNA concentrations in undiluted preparations were from 0.4 to 1 μg RNA/ml for each RNA species. 1/5 indicates five-fold dilution of undiluted preparation.
[c] Number of plants showing symptoms divided by the number inoculated.

TABLE 3. Tomato white leaf. Symptoms on tomato line 80-18-1 inoculated with RNA mixtures from cucumber mosaic virus-C and -WL

	Symptoms on tomato[b]					
	Exp. 1			Exp. 2		
Inoculum[a]	LC	WL	LD	LC	WL	LD
CMV-C 1+2+3	0	0	4	0	0	5
CMV-C 1+2+3 + CMV-WL5	0	2[c]	2	0	5[c]	0
CMV-WL 1+2+3	4	0	0	5	0	0
CMV-WL 1+2+3+5	0	4	0	0	5	0
CMV-WL 5	0	0	0	...	...	...

[a] RNA concentrations were: ~0.7 and 0.5 μg/ml for CMV-C 1+2 and 3; 1.0 for both CMV-WL RNAs 1+2 and 3; and 0.2 for CMV-WL5.
[b] LC = leaf curl, WL = white leaf, and LD = leaf distortion and fern leaf symptoms. Numbers refer to number of inoculated plants in each symptom category. Four and five plants used per inoculum in Exp. 1 and 2, respectively.
[c] Plants initially showed WL, but WL symptoms became milder with time.

TABLE 4. Tomato white leaf. Symptoms on tobacco cultivar Havana 423 and tomato line 80-18-1 inoculated with cucumber mosaic virus-WL RNAs 1+2+3 and 1+2+3+5

		Symptoms[b]				
		Tobacco			Tomato	
Exp.	Inoculum[a]	BC	Mot.	NS	WL	LC
A	(1+2)+3	2	1	2	0	3
	(1+2)+3+5	0	0	5	3	0
B	(1+2)+3	1	1	3	0	3
	(1+2)+3+5	0	0	5	3	0
C	(1+2)+3	4	1	0	0	3
	(1+2)+3+5	0	0	5	3	0

[a] RNAs concentrated to approximately 0.49–1.56 μg/ml per species. RNAs 1+2+3 were isolated from a single CMV-WL preparation purified from squash. RNAs 1+2+3 components in Exp. A and B were purified in the same sucrose gradients, whereas RNAs 1+2+3 components from Exp. C were purified in other sucrose gradient cycles. RNA 5 was from CMV-WL purified from cucumber.

[b] BC = bright chlorosis on leaves, Mot = mottle, NS = no symptoms, WL = white leaf, LC = leaf curl. Five tobacco and three tomato plants inoculated per treatment. Numbers refer to number of inoculated plants in each category.

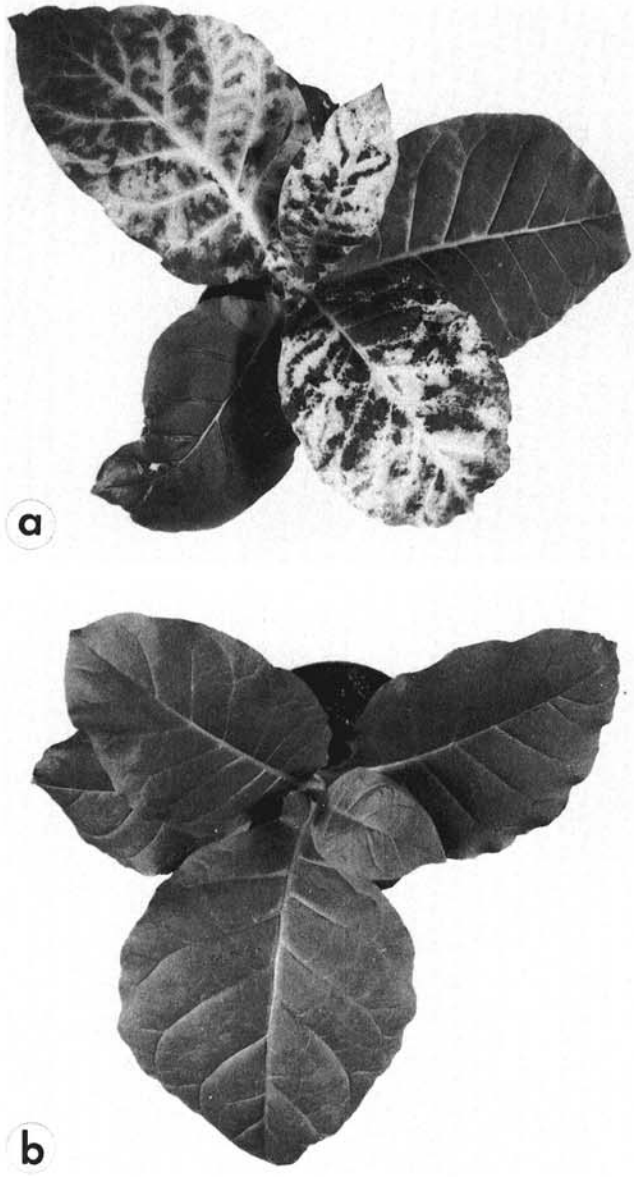

Fig. 4. Symptoms on inoculated *Nicotiana tabacum* 'H423.' **A,** CMV-WL RNAs 1+2+3 and **B,** CMV-WL RNAs 1+2+3+5. Healthy tobacco plants appear similar to B.

However, until it is demonstrated that CMV-WL RNA 5 does not replicate and that it is not a subgenomic RNA of CMV (2,8,11), the evidence that RNA 5 is a satellite RNA is mainly circumstantial. Thus, we will tentatively designate it as CMV-WL RNA 5.

The (n)CARNA 5 (13) and a CMV satellite RNA from Japan (22) both cause necrosis in tomato. In tobacco, however, the former attenuates symptoms while the latter causes a brilliant chlorosis along with some symptom attenuation. CMV-WL RNA 5 can be distinguished from these in that it causes white-chlorosis in tomato. It also differs from other CMV satellite RNAs that have no effect or attenuate symptoms in tomato (12,17).

The CMV isolate from which CMV-WL RNA 5 was originally obtained also incites unique symptoms on tomato and tobacco. The leaf curl symptom caused on tomato by CMV-WL RNAs 1+2+3 differs from the mosaic-fernleaf symptom caused by CMV-C, which predominates in tomatoes grown in New York State and elsewhere (3). The intense chlorosis seen on a number of tobacco plants inoculated with CMV-WL RNAs 1+2+3 is somewhat similar to that caused by CMV satellite RNA from Japan (22). Our results clearly show, however, that CMV-WL RNA 5 attenuates symptoms on tobacco. Thus, we feel that the absence of chlorosis on some tobacco plants inoculated with CMV-WL RNAs 1+2+3 (experiments A and B of Table 4) was due to residual contamination of these preparations with CMV-WL RNA 5. Contaminating RNA 5 or its double-stranded form (9), may have built up to sufficient levels to attenuate symptoms in tobacco. Others have shown that CMV satellite RNAs build up to high levels in tobacco (10,11,22,24). However, we have not completely excluded other possibilities such as the existence of another (or other) satellite RNA(s).

Very few reports of diseases caused by CMV satellite RNAs have appeared in the literature. The only reports are from France (16,20) on a necrosis of tomato in which (n)CARNA 5 has been implicated (13). Quiot et al (21) recently investigated the epidemiology of this disease and the "shoestring disease" of tomato which is caused by common strains of CMV. They found that epidemics of the necrosis occurred only when alate aphids were numerous, that these two diseases have independent epidemics, and that plants with the shoestring disease could also develop necrosis under field conditions. The consistent occurrence of the tomato white leaf disease since 1970 at only one location also makes it an ideal system to study the epidemiology of diseases caused by CMV satellite RNAs (assuming that WL-RNA 5 is a satellite). Since our data indicate that inoculum source influences the symptoms on tomato, this may be responsible for the low incidence of white leaf disease in the field. Also, the prevalence of other CMV isolates that cause mosaic-fernleaf symptoms, such as CMV-C, may mask symptoms or prevent establishment of the white leaf disease. We have shown that white leaf symptoms are milder when CMV-C is used as a helper virus for CMV-WL RNA 5.

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