A Variant of Cowpea Chlorotic Mottle Virus Obtained by Passage Through Beans

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ABSTRACT

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After continued propagation of the type strain (T) of cowpea chlorotic mottle virus (CCMV) in California Blackeye cowpeas for several months, the intensity of the chlorotic symptoms diminished. When Bountiful beans were inoculated with sap extracts from such plants, a new variant of CCMV, designated M, was derived. M caused very mild symptoms on cowpeas rather than the bright chlorosis of strain T. There was a close relationship between T and M in serology, replication, specific infectivity, host range, and several biophysical properties. However, isolate T differed from M in symptoms produced on cowpeas, replication in resistant cowpea lines, ability to compete in cowpeas and beans, and in the nature of RNA species 3. Results of studies of pseudorecombination of the RNAs of isolates T and M suggested that RNA-3 of CCMV has two genes, one controlling systemic symptoms and the other controlling coat protein.

For several years, we have noted that the intensity and extent of chlorosis caused by the type strain of cowpea chlorotic mottle virus (CCMV-T) declined after continuous propagation in cowpeas, *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye.' This suggested that a CCMV variant had developed in susceptible cowpeas and had tended to become dominant or at least competitive with the type strain. Our objective was to isolate and identify such a variant and to compare its biological and physicochemical properties with those of CCMV-T.

MATERIALS AND METHODS

CCMV-T has been maintained in California Blackeye cowpeas or in desiccated cowpea tissue (reactivated every 3–4 yr) since 1961. Unless otherwise specified, inoculations were made with leaf tissue ground in 0.01 M neutral potassium phosphate buffer containing 1% Celite (Johns-Manville Corp., Denver, CO 82017). Most studies were performed in the greenhouse with summer temperatures of 25–35 C and winter temperatures of 21–30 C. Symptomatology and replication studies of the virus in California Blackeye cowpeas and Bountiful beans (*Phaseolus vulgaris* L.) were conducted in growth chambers programmed for a 16-hr photoperiod, illumination ~10,000 lx, and specific temperatures between 16 and 32 C.

Several procedures used in this study were described previously (5): virus culture, spectrophotometric analysis to determine virus yield, virus purification, infectivity assays on soybeans (*Glycine max* [L.] Merr. 'Bragg'), and density gradient analyses. Electrophoretic mobility comparisons were made with a Perkin-Elmer Model 238 electrophoresis apparatus equipped with a Tiselius cell (neutral sodium phosphate buffer containing sodium chloride; ionic strength = 0.1) and by electrophoresis of whole virus in 2.7% acrylamide gels (acrylamide/bisacrylamide, 20:1) with 0.1 M Na acetate (pH 5) electrolyte. Additional studies were conducted in gels to determine the electrophoretic mobilities of viral RNA (10) and sodium dodecyl sulfate (SDS)-disrupted coat protein (11). Tryptic peptide map analyses of coat proteins were made by the procedure described by Bancroft et al (3). Antisera were prepared by injecting white rabbits intravenously (1 mg of purified virus in 1 ml per injection) and intramuscularly (10 mg of purified virus in 1 ml per injection). Both injections were made weekly for 3 wk, and the animals were bled during the 4th wk.

With a few modifications, pseudorecombinant tests with RNA species from CCMV isolates were conducted by the procedure described by Bancroft and Lane (2). After isolation of the viral RNA and separation of RNA species 1, 2, and 3 by gel electrophoresis (15), various mixtures of inoculum were prepared by adjusting the concentration of each species to 0.4 μg/ml. Three hours after inoculation of the primary leaves of Bragg soybeans, the leaves were excised and incubated in petri dishes, with moist filter paper, in the dark at 30 C. Three days later, single lesions were excised and used to inoculate three California Blackeye plants.

RESULTS

Loss of chlorosis. When 8 to 10 day old California Blackeye cowpea plants were inoculated with CCMV-T, chlorosis was intense on the first two trifoliolate leaves and 80–100% of the leaf area was affected. On subsequent growth, both the intensity and area affected by the chlorosis were diminished by 50% or more. Sap extracted from any portion of the plant caused a similar intense chlorosis on new cowpea seedlings. However, if sap inoculations were made from cowpea to cowpea every 1–2 wk for a period of 3–6 mo, mild chlorosis was observed on the first trifoliolates and only 10–25% of the leaf area was affected. It is important to distinguish between chlorosis that did not develop in individual plants and decline in intensity of chlorosis associated with continuous propagation of the virus in cowpeas.

Selection of mild isolate. In an attempt to isolate a variant of CCMV, strain T was serially transferred through five single lesions on Bragg soybeans. The virus was then propagated continuously in California Blackeye cowpeas, with weekly transfers, for 6 mo. At this stage, the chlorosis was much less extensive than that in control cowpea plants that were inoculated each week with virus from single lesions. Nine plant species were inoculated with the virus that had been continually propagated in cowpeas. Sap from infected Bountiful beans caused mild mottle symptoms only on California Blackeye cowpeas (Fig. 1); sap from the other plant species caused various degrees of bright chlorosis. The virus isolated from beans, designated M, has been maintained in both cowpeas and beans since 1973, and the mild symptoms on California Blackeye cowpeas have remained essentially the same.

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Infrequently, however, a few small (3 mm in diameter) bright chlorotic spots occurred on cowpea plants infected with M. Careful isolation of 12 spots with a razor blade established that isolate T predominated in the brightly colored areas. Except for the infrequent occurrence of these chlorotic spots, there has been no evidence of reversion of isolate M to T in cowpeas. When approximately 50 single lesions of each isolate, T and M, were transferred from Bragg soybeans to California Blackeye cowpeas, symptoms produced on cowpeas were like those produced by the parent isolate.

During February, March, and April of a 3-yr period, culture of isolate T, which caused bright chlorosis, in Bountiful beans, frequently resulted in a selection of isolate M. After local lesion cloning of T, similar trans-species transfers made in other months failed to produce mild symptoms. Attempts to establish an artificial growth chamber environment to induce a change from T to M were unsuccessful.

Most single lesions formed on Bragg soybeans by virus propagated continuously in cowpeas caused bright chlorosis on cowpeas. Infrequently, however, isolates from a few lesions caused mild mottle symptoms. This isolate selection did not depend on the time of year.

Symptomatology and host range. In the greenhouse, symptoms on California Blackeye cowpeas were mild for isolate M and bright for T throughout the year, but slightly brighter symptoms were produced by both isolates during the winter and spring months. When cowpea plants were maintained at 16, 21, 27, and 32 C, isolate M caused mild symptoms at the three lower temperatures and none at 32 C. Isolate T caused bright chlorosis at all temperatures, but the coloration was most distinct at 27 C.

Symptoms on other known hosts of CCMV-T (7) were similar for both isolates M and T, and no new host species were found for isolate M. However, two cowpea plant introductions (147562 and 186465), known to be resistant (slow rate of replication and localized infection) to CCMV-T (13), were more resistant to M than to T; plants were more difficult to infect and less virus (only 10–25% as much) was produced in them. Virus quantities were measured by local lesion infectivity assays and spectrophotometric analyses of purified samples.

Mixed inoculum. California Blackeye cowpeas and Bountiful beans were inoculated with mixtures of purified T and M. Subsequent subinoculations to the test host, California Blackeye cowpeas, indicated a strong tendency for isolate M to become dominant in the mixed infection, particularly in beans (Table 1). The bright symptoms caused by isolate T were observed only when the mixed inoculum had at least 100 μg/ml of T and a concentration of T that was 10–100 times greater than M.

Serology. Isolates T and M were either identical or very similar

TABLE 1. Effect of mixed inoculum of cowpea chlorotic mottle virus type strain (T) and mild strain (M) isolates on symptom type in cowpeas and beans[a]

Original inoculum (μg/ml)		Symptom type on cowpeas[b] (source plants are indicated)	
M	T	Cowpeas	Beans
10	0	Mi	Mi
0	10	B	Mi
1	1	Mi	Mi
10	10	Mi	Mi
100	100	B	Mi
100	1	Mi	Mi
100	10	I	Mi
10	100	B	I
1	100	B	B

[a]California Blackeye cowpea or Bountiful bean plants were inoculated with mixed inoculum of isolates T and M. Then 10 days later, sap from these source plants were used to inoculate California Blackeye cowpeas.
[b]Abbreviations for chlorosis symptom types: B = bright, Mi = mild, I = intermediate.

Fig. 1. Symptoms of cowpea chlorotic mottle virus isolates T (left) and M (right) on California Blackeye cowpeas.

serologically. With either T or M antiserum, precipitin lines fused and no spurs developed in double diffusion tests with agar prepared in 0.1 M acetate buffer, pH 5. Furthermore, the homologous and heterologous reactions were the same in ring precipitin tests; the titer of each of the four reactions was between 1/1,024 and 1/2,048.

Isolate M was unstable in agar prepared in water or 0.01 M neutral potassium phosphate buffer. The virus wells became cloudy and precipitin lines did not develop with either T or M antiserum. Strain T was much more stable; it diffused through the agar and formed precipitin lines under these conditions.

Two serologically distinct strains of CCMV, A and BYS (6), were compared with isolates T, M, and the soybean strain S (9) in agar double diffusion tests at pH 5. With isolate T or M antiserum in a center well and virus in the outer wells, distinct spurs occurred between isolates T and A, T and BYS, M and A, M and BYS, and A and BYS; complete fusion and no spurs occurred between T and M, T and S, and M and S.

Virus characteristics. The results of several tests indicated that isolates T and M were similar in many aspects. Virus yield and specific infectivity studies were conducted in two hosts, California Blackeye cowpeas and Bountiful beans, and at three environmental conditions: greenhouse, 20 C, and 35 C. Although the virus yield varied with the host and the condition, there were no significant differences between isolates T and M. Furthermore, the infectious quality (specific infectivity) of the two isolates was similar at 5–10 days after inoculation for each comparison. The loss of infectivity in vivo (8) was rapid and similar for both isolates during a 30-day infection period.

The presence of unstable variants or unencapsidated CCMV-RNA was checked by extracting RNA (4) with phenol, 8-hydroxyquinoline, and sodium naphthalene disulfonate from infected plants maintained at moderate (20 C) and high (35 C) temperatures. Local lesion assays demonstrated that the ratio of infectivity for T and M RNA extracts was similar to the ratio for sap extracts. Furthermore, the specific infectivity of phenol-SDS-extacted RNA from purified preparations of each of the two isolates was similar.

Most biophysical properties of isolates T and M were identical or similar: sedimentation rate in sucrose density gradients, ultraviolet spectrum, electrophoretic mobility of viral nucleoprotein in moving boundary experiments and in polyacrylamide gels; RNA and SDS-protein profiles after electrophoresis in polyacrylamide gels; and location of tryptic peptides on two-dimensional electrophoresis chromatograms.

In addition to differences in stability of isolates T and M in agar at pH 7 (serology tests), M was found to convert from 88S to 78S (1) more easily than T. The conversion of M occurred at a lower molarity (0.01 M, rather than 0.1 M) of potassium phosphate buffer (pH 7.5) and in a shorter time. Furthermore, M particles released about twice as much RNA as T when purified preparations were dialyzed against 1.0 M sodium chloride at pH 5.

Pseudorecombination studies. With fractionated viral RNAs, a complete inoculum mixture containing RNA species 1, 2, and 3 induced 10–40 lesions per leaf. The number of lesions obtained was similar regardless of the source of each RNA species. When either RNA-1, RNA-2, or RNA-3 was omitted from the inoculum, either no lesions or occasionally one to two per leaf were induced.

In a study of the progeny of all the possible combinations of RNA species 1, 2, and 3 from T and M, it was obvious that the type of systemic symptoms on California Blackeye cowpeas was controlled by RNA 3 (Table 2). In 74 of 77 progeny tested, the symptoms were characteristic of the isolate from which the specific RNA-3 in the inoculum had been isolated; the remaining three resulted in an intermediate degree of chlorosis that we classified as a mild reaction.

TABLE 2. Effect of combinations of RNA species 1, 2, and 3 inocula of cowpea chlorotic mottle virus isolates T and M on symptoms on California Blackeye cowpeas

Inoculum[a]			Symptom type on cowpeas[b]			
RNA 1	RNA 2	RNA 3	Experiment 1		Experiment 2	
			Bright	Mild	Bright	Mild
T virions			8	0	8	0
M virions			0	8	0	8
T	T	T	8	0	7	0
M	M	M	0	8	0	7
M	T	T	7	0	4	1[c]
T	M	T	6	2[c]	6	0
T	T	M	0	8	0	5
T	M	M	0	7	0	6
M	T	M	0	8	0	5
M	M	T	8	0	4	0

[a] Bragg soybeans were first inoculated with the RNA mixtures. Then single lesions were used to inoculate one pot (three plants) of cowpeas.
[b] Numbers refer to the number of local lesions used as inoculum on cowpeas (ie, the number of trials).
[c] Intermediate degree of chlorosis.

DISCUSSION

Variants of viruses develop as a result of mutation and selection. With CCMV-T, it appears that a mutation occurs frequently when the virus is propagated in susceptible cowpeas. The new variant M coexists with T and gradually becomes dominant under certain conditions. With subsequent passage through susceptible beans, variant M was selected and T probably was excluded. Although variant M was relatively easy to maintain as a distinct isolate in either beans or cowpeas, it was necessary to pass T through single lesions in soybeans to maintain its characteristics.

Five naturally occurring isolates of CCMV have been reported: the type strain in cowpeas (7), a mild isolate in soybeans (9), a mild isolate in *Desmodium laevigatum* (14), strain A in beans (6), and bean yellow stipple in beans (6,17). Three serologic groups can be distinguished (6). Strain T and the soybean and *Desmodium* isolates are serologically identical or very similar (9,14); strains BYS and A are serologically distinct, and both are distinct from the strain T group (6). Isolate M is a variant that belongs to the strain T group.

Although strain M shares many biological and physicochemical properties with strain T, it should be recognized as a distinct strain of CCMV. Susceptible cowpeas react with milder symptoms to M than to T, and resistant cowpeas are more resistant to M than to T, based on rate of virus replication and virus content (16). Strain M developed spontaneously under greenhouse conditions, and it tends to become dominant over T in a mixed infection. Most important, genetic information on RNA-3 differs for the two isolates.

Previous RNA pseudorecombination studies (2) with nitrous acid mutants of CCMV demonstrated that both coat protein and systemic symptoms on cowpeas are genetically directed by RNA-3. Bancroft and Lane (2) suggested that the two phenomena are genetically linked in such a manner that they may be invariably coupled on the small RNA-3 (mol wt = 0.85×10^6) of CCMV. Robinson (12) reported that the smallest RNA (mol wt = 0.7×10^6) of bipartite tobacco rattle virus has two genes, one controlling symptoms and the other coat protein. The results for our pseudorecombination studies with CCMV are similar to those for tobacco rattle virus. Although the coat proteins of T and M appear to be identical (based on serology and tryptic peptide analyses), the RNA-3 of the two strains causes different systemic symptoms.

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