A Severe Strain of Cucumber Mosaic Virus from China and Its Associated Satellite RNA

CHRISTOPHER M. Kearney, Graduate Research Assistant, DENNIS GONSALVES, Professor, and ROSARIO PROVVIDENTI, Professor, Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva 14456

ABSTRACT

Fields of tomato (Lycopersicon esculentum) in the Fujian province of southern China were afflicted with a severe disease characterized by purpling, yellowing, necrotic lesions, and severe stunting. By using electron microscopy, host indexing, serology, and RNA analysis, we have determined that a new strain of cucumber mosaic virus (CMV-Chi) is the causal agent. A satellite RNA (sat-Chi) was found in the culture. CMV-genomic RNA purified free of the satellite caused the same severe symptoms in tomatoes as seen in the field, excluding the necrotic lesions. The addition of purified satellite to the inoculum caused necrosis and death of tomatoes within 6 wk. CMV-Chi free of satellite induced severe symptoms in several hosts. This CMV strain also displayed a longevity in partially dried tissue greater than two other CMV strains, suggesting an especially stable virion.

Cucumber mosaic virus (CMV) infects a wide variety of plants (18). It is most commonly a problem in peppers (Capsicum annuum L.), cucurbits, tomatoes (Lycopersicon esculentum Mill.), and bananas (Musa L. spp.; 18,22). Symptoms usually consist of a mild to strong mosaic and stunting, with or without leaf deformation. On tomatoes, the most severe symptoms are "fernleaf" leaf deformation, mosaic, and stunting (5), though milder symptoms can occur (7).

CMV is anicosahedral virus with a genome divided among three single-stranded, positive-sense RNAs, called RNAs 1, 2, and 3 (20,25). A subgenomic RNA, RNA 4, is a duplication of the 3' half of RNA 3 (10). RNA 4 serves as the messenger RNA for coat protein production (29) but is not necessary for infection (20,25). A satellite RNA, usually composed of 334-342 nucleotides (16), is often associated with CMV cultures in the greenhouse and sometimes appears in the field (7,17,30; Kearney, Zitter, and Gonsalves, unpublished data). This molecule has little homology to the genomic RNAs of CMV (9,28) yet is dependent on them for its replication (15) and is encapsidated in CMV virons (19). As a group, CMV satellites are highly homogeneous in sequence (6,16).

CMV satellites can greatly affect symptom development by the CMV genomic RNAs. The tomato necrosis epidemic of 1972 in Alsace, France, was caused by the presence of a necrosis-inducing satellite (17). When added to CMV RNA 1, 2, and 3, which normally cause tomato fernleaf symptoms, the necrotic satellite can induce a quick necrotic death in tomatoes (12). Other CMV satellites can cause severe white leaf symptoms in tomato (7), brilliant chlorosis in tobacco (30), or act as protective agents, eliminating or reducing normal CMV-infected symptoms in tobacco or tomato (7,24).

Recently, a serious disease of tomatoes has appeared in the Fujian province in southern China. The severity of symptoms is atypical of those caused by CMV, suggesting the involvement of a satellite. In this study, we have determined that the disease is caused by an extremely severe strain of CMV and that a satellite is present in the CMV culture but that most of the severity seen in the field can be attributed to the genomic RNAs. The satellite was shown to induce a complete necrosis in tomato seedlings, and so it may add necrotic lesions or blotches to the already severe symptom complex of the field tomatoes.

MATERIALS AND METHODS
Propagation and inoculation of virus. Routine transfers of virus culture from host to host were done by grinding fresh infected tissue in 10 mM potassium phosphate buffer (pH 7.0) at 1:20 (wt:vol) and rubbing leaves with a pestle using Carborundum as an abrasive. Inoculations with purified viroids were performed the same way in a PEN buffer (10 mM NaH2PO4, 1 mM EDTA, 1 mM Na2SO4, pH 7.0) with 10-200 µg/ml of viroids, the concentration depending on the age and specific infectivity of the preparation. RNA was inoculated in the PEN buffer at a concentration of 30 µg/ml for genomic RNA (RNA 1, 2, and 3) and 5 µg/ml for satellite RNA and including 20 µl/ml of 2% fractionated bentonite (4) to protect against RNAases. Approximately 40 µl and 80 µl of RNA inoculum per plant was used for tomato (line 80-2-1) and tobacco (Nicotiana tabacum L. 'Havana 423'), respectively, with a ground glass spatula and Carborundum used for abrasion. For all types of inoculum, tomato plants were inoculated when the first secondary leaf was 1-2 cm long, tobacco and lettuce when the longest leaf was 5-8 cm long, pepper and bean at the stage of primary leaves and unexpanded secondary leaves, and cucurbits at the cotyledonary leaf stage.

Purification of viroids and viral RNA. Viroids were purified from infected zucchini squash (Cucurbita pepo L. 'Seneca') for satellite-free preparations or tobacco for satellite-containing preparations by the method of Lot et al (21). Cucurbitis poorly support the replication of most CMV satellites, while tobacco enhances replication (11,14). RNA was phenol-extracted from the viroids (8). Satellite RNA was separated from genomic RNA by three cycles of sucrose density gradient centrifugation (3).

Elimination of satellite from virus culture. To purify CMV-Chi inoculum free of satellites, RNAs 1, 2, and 3 were taken through three cycles of sucrose density gradient centrifugation (3) and then inoculated to quinoa (Chenopodium quinoa Willd.). Local lesions were excised with a new razor blade, ground in a chlorox-disinfested mortar and pestle with phosphate buffer, and inoculated to C. quinoa. Extracts from new local lesions were then inoculated onto tobacco. The cultures were examined for satellite by double-stranded RNA analysis (see explanatory section) and then inoculated onto zucchini squash for propagation. Virus purified at this stage formed the first satellite-free inoculum used in this study. To add an extra measure of certainty that sat-Chi was removed, this culture was then taken through three more C. quinoa local lesion passages and inoculated directly to zucchini squash after the last passage. After propagation in zucchini squash, the virus was purified and constituted the
Results

Field symptoms and initial identification. Leaf samples of infected tomato plants were taken in 1985 from tomato fields at Fuzhou in the Fujian Province of southern China. The symptoms were severe, with various plants showing one or more of the following: purple coloration, leaf curling, stunting, yellowing, and necrosis. These symptoms were observed in extensive patches of CMV infection in individual fields and in a large number of fields in the region. Leaf samples were dried and stored at room temperature with no vacuum or desiccant. A month later, they were ground in 10 mM phosphate buffer (pH 7.0) and inoculated onto tobacco. Severe leaf deformation, suggestive of a severe strain of CMV, developed. The culture was then transferred to cucumber (*Cucumis sativus* L. 'Marketer'), causing a mosaic; to zucchini squash, causing a mosaic and necrosis; to cowpea (*Vigna unguiculata* (L.) Walp.) and *C. quinoa*, producing large local lesions; and to tobacco, giving the symptoms seen in the field, excluding the necrotic lesions. Except for the atypical reaction in tomatoes, all of these symptoms were similar to those of other CMV strains, though more severe than usual. Initial immunodiffusion tests indicated the presence of CMV in the infected plants. The virus culture was then maintained in tobacco.

Confirmation by electron microscopy and RNA analysis. A virion preparation was made from infected tobacco according to the protocols used for CMV.

Fig. 3. Northern blot of virion RNA of Chinese (Chi) and other isolates of cucumber mosaic virus (CMV) using a probe for CMV satellite. In lanes from left to right are CMV-WL, CMV-L1, CMV-L3, CMV-Chi harboring satellite, CMV-Chi free of satellite by local lesion transfers with *Chenopodium quinoa*, and CMV-Chi satellite purified by sucrose density gradient centrifugation. One μg of glyoxylated CMV RNA was run in each lane, blotted onto a nylon membrane, and probed with a 3P-labeled in vitro transcript of cloned CMV-WL satellite.

Fig. 2. The reaction of the Chinese isolate (CMV-Chi) with two cucumber mosaic virus (CMV) antisera and the placement of CMV-Chi into CMV serogroup WT. Dilutions of virions (CMV-Chi), -C,-WL, or -L2 were assayed by ELISA using antisera to (A) CMV-L2 (S serogroup) or (B) CMV-C (WT serogroup). CMV-WL is a member of the S serogroup.

Fig. 1. Polyacrylamide gel of RNA of cucumber mosaic virus (CMV) strains compared to the RNA of the Chinese isolate (CMV-Chi). The lanes contain, left to right, CMV-L1, CMV-L3, CMV-WL, CMV-Chi with satellite, CMV-Chi after removal of satellite by local lesion transfer using *Chenopodium quinoa*, and the satellite of CMV-Chi after purification by sucrose density gradient centrifugation. The genomic RNAs (RNA 1, 2, and 3), the subgenomic RNA (RNA 4), and the satellite RNA are indicated. The gel was composed of 5% acrylamide and 8.3 M urea, was run in TBE buffer (50 mM Tris, 50 mM borate acid, and 2 mM EDTA) at 100 V for 90 min, and was stained with ethidium bromide.

Second satellite-free inoculum of this study.

Serology and CMV strains. Enzyme-linked immunosorbent assay (ELISA) was performed according to Clark and Adams (1) with some modifications. A group of CMV strains used to define two serogroups of CMV (2) was reassayed and found to have retained serogroup fidelity and to give consistent group-specific reactions in several experiments. Antisera representing the two serogroups were used to type CMV-Chi: CMV-L2 antiserum of the S serogroup and CMV-C antiserum for the WT serogroup. The CMV strains used in this study were CMV-WL (white leaf; 7), CMV-L1 and L2 (lettuce strains 1 and 2; 27), CMV-C (2), and CMV-Mus, an isolate obtained from a mustard (*Brassica* L. sp.) sample collected in New York state.

Electrophoresis and northern analysis. The probe used for northern hybridization was a 32P-labeled in vitro transcript made from a CMV white leaf satellite cDNA cloned into an in vitro transcription vector (Kearney and Gonsalves, unpublished). Electrophoresis and hybridization procedures were carried out using glyoxylated RNA and DuPont company protocols for Gene Screen Plus (DuPont/NEN, Boston, MA), except that the SSC (sodium chloride-sodium citrate) concentration was reduced to 0.1 X for all washings and 250 μg/mL of yeast RNA replaced 100 μg/mL of salmon sperm DNA in the hybridization buffer to reduce nonhomologous binding by the RNA probe.

Polyacrylamide gels were made with TBE (50 mM Tris, 50 mM borate acid, 1 mM EDTA), 50% (8.3 M) urea, and 5% acrylamide/bis (20:1). RNA samples were preheated to 65°C in 3 M urea and quick-chilled on ice before loading.

Double-stranded RNA analysis. Infected plants were individually analyzed for the presence of satellite by double-stranded RNA analysis (13,23). Briefly, 0.15 g of frozen (−20°C) tissue was ground in a microcentrifuge tube with a plastic microfuge pestle (Kontes Scientific, Vineland, NJ) along with 400 μL of 2X STE buffer (2X is 0.2 M NaCl, 0.1 M tris, 2 mM EDTA, pH 6.8) containing 3% SDS (sodium dodecyl sulfate), 0.3% bentonite, and 0.5% (v/v) 2-mercaptoethanol. After phenol/chloroform extraction, the double-stranded RNA of the aqueous phase was bound to CF-11 cellulose in another microfuge tube and washed (vortexing followed by microcentrifugation [16,000 g] for 5 min) four times in 1X STE containing 16% ethanol. The purified double-stranded RNA was then released by a wash with STE alone.
purification (21). This preparation was examined by transmission electron microscopy. Both spheres and flexuous rods were present, giving the appearance of a mixed infection of CMV and a potyvirus. RNA was extracted from the virions with phenol and precipitated with ethanol. The resulting RNA showed a typical CMV pattern of four genomic bands and one satellite band (Fig. 1). No band indicative of the larger RNA of potyviruses was seen. This RNA preparation was then taken through three cycles of sucrose density gradient centrifugation, followed by two local lesion passages in C. quinoa. Upon inoculation onto tobacco, the typical symptoms of severe stunt and deformation again developed. A virion preparation produced from these tobacco samples was examined by electron microscopy, and only the spheres remained.

Placement of CMV-Chi in a CMV serogroup. The flexuous rod-free virion preparation just described was compared by ELISA against other CMV strains representing the two CMV serogroups WT (CMV-C) and S (CMV-L2 and -WL) as defined by Piazzolla et al (26) and applied to these strains by the method of Edwards and Gonsalves (2; Fig. 2). The four CMV isolates were tested against antisera from each serogroup. CMV-Chi reacted strongly with WT antiserum and weakly with S antiserum, placing it in the WT serogroup of CMV.

Identification of the satellite RNA and its effect on symptoms. Sat-Chi comigrated with other CMV satellite RNAs in polyacrylamide gel electrophoresis (Fig. 1). A northern blot was run with a [32P]-labeled in vitro transcript from a clone of sat-WL used as the radioactive probe. Sat-Chi clearly hybridized with the probe, showing that it is homologous to other CMV satellite RNAs (Fig. 3). When the genomic RNA of CMV-Chi was purified free of sat-Chi and inoculated onto tomatoes (Geneva line 80-2-1), symptoms were produced that were typical of those seen in the greenhouse and field originally (Fig. 4, middle). Two weeks after inoculation, the plants were severely stunted and the leaves were curled back and tinted purple. After 6 wk, these plants remained severely stunted, the new leaves were narrow and deformed, and the remaining expanded leaves sometimes turned yellow. A severe bushy stunt developed after a prolonged period. With the addition of the satellite, however, the plants did not reach the bushy stunt stage, dying after only 3-4 wk from a complete necrosis (Fig. 4, left). These results were seen in tomato line 80-2-1 in six trials with a total of 122 plants and in a separate trial with four different tomato cvs. (80-2-1, Heinz 1350, Campbell 1327, and Nova). Genomic CMV-Chi RNA caused a severe stunt and leaf deformation on tobacco with or without sat-Chi.

The two satellite-free CMV-Chi virus preparations used in this study were tested to verify their lack of satellite. Each preparation was inoculated onto eight tomato and eight tobacco plants, and the plants were checked for symptoms and the production of satellite. All tomato plants produced the symptom syndrome of the satellite-free plant in Figure 4, while the tobacco plants developed the typical severe stunt. In no case did satellite appear, as assayed by double-stranded RNA analysis (Fig. 5).

Reassortment studies using sat-Chi and other CMV strains. To determine if CMV-Chi genomic RNA was necessary for the induction of tomato necrosis by sat-Chi, Sat-Chi was tested with genomic RNA from three different CMV strains. Sat-Chi in tomatoes caused complete necrosis with CMV-WL as well as with CMV-Chi genomic RNA. Sat-Chi induced yellowing and necrosis more quickly with CMV-WL genomic RNA than with CMV-Chi genomic RNA. With CMV-L1 genomic RNA, sat-Chi induced mild yellowing and necrosis but not the death of the plant. CMV-WL and CMV-L1 genomic RNAs alone cause very mild symptoms in tomatoes.

Symptoms on other hosts. With peppers, CMV-Chi genomic RNA caused a severe stunt in addition to the

---

**Fig. 4.** Symptoms induced by the Chinese strain of cucumber mosaic virus (CMV-Chi) and its satellite (sat-Chi) in tomato. **Left**, inoculated with CMV-Chi genomic RNA plus sat-Chi; **middle**, inoculated with genomic RNA alone; **right**, uninoculated. Inoculation was done 5 wk previous; all plants were the same age.

**Fig. 5.** Double-stranded (ds) RNA analysis used to verify the absence of satellite from plants inoculated with a satellite-free preparation of CMV-Chi. Tomato plants were inoculated with the virus preparation to allow the replication of any satellite residual in the inoculum. DsRNA was extracted from individual plants, run on a polyacrylamide gel, and stained with ethidium bromide. Lanes 1-3 represent three plants inoculated with satellite-free CMV-Chi; lane 4, tomato inoculated with CMV-Chi plus satellite-Chi; lane 5, 100 ng of a dsRNA standard, consisting of CMV-WL dsRNA purified from tobacco by LiCl fractionation; lane 6, tomato inoculated with CMV-WL plus in vitro transcript made from cloned satellite-WL cDNA.
typical CMV fine-grained mosaic and leaf distortion (Fig. 6). In lettuce (Lactuca sativa L. 'Ithaca'), the genomic RNAs caused a mild mosaic with necrotic flecks generally dispersed on the leaves. The lower and middle leaves were chlorotic, and the plant was stunted. The addition of satellite RNA reduced the intensity of the mosaic and limited the necrotic spots to the base of the leaves. In zucchini squash (Cucurbita pepo L.), necrotic lesions were observed on the leaves, with some plants eventually recovering from the infection. In cantaloupe (Cucumis melo L. cv. Pride of Wisconsin, Hale’s Best, and Rocky Ford), a mosaic and stunt occurred, but the plants eventually recovered from the stunt. No symptoms developed following inoculation of common bean (Phaseolus vulgaris L. cv. Red Kidney and Black Turtle 2).

**Longevity in partially dried tissue.** The original tissue samples from the field were not dried and stored with desiccant, a procedure that normally eliminates the infectivity of CMV. However, these samples retained high infectivity, suggesting a property of exceptional stability for CMV-Chi virions. Therefore, we compared the virion stability of CMV-Chi to that of three other CMV strains in dried tomato tissue. To develop infected tissue, eight tomato plants per strain were inoculated with purified virion preparations, all of which lacked satellite. The infectivity of the virion inoculum for each strain was assayed on C. quinoa: 20 lesions per leaf for CMV-Chi, 40 for CMV-Mus, 50 for CMV-L1, and >150 for CMV-WL. After 3 wk, the symptoms were a typical severe stunt with purple-tinted leaves for CMV-Chi plants, a fairly severe to moderate stunt plus leaf distortion for CMV-Mus, a mild leaf narrowing and slight stunt for CMV-WL, and virtually no symptoms with CMV-L1.

Three weeks after inoculation, extracts of fresh samples from the infected tomatoes were inoculated onto C. quinoa to determine the initial infectivity level for each strain. The tomato tissue was then placed between towels and left to dry, with checks for infectivity at 1 wk and 7 wk after harvest. Table 1 shows that although CMV-Chi had the highest infectivity level in fresh tomato tissue, the other strains were not much lower. After 1 wk of drying, however, the titers of CMV-WL and CMV-Mus fell precipitously relative to the titers of CMV-Chi and CMV-L1. By the seventh week, the titer of CMV-L1 had dropped slightly relative to CMV-Chi.

**DISCUSSION**

In 1977, CMV satellites were first demonstrated to drastically affect symptom development (17). CMV satellites have been responsible for the severe symptoms of at least three outbreaks in the field. These are tomato necrosis (17), tomato white leaf (7), and tobacco chlorosis (30). In addition to these field observations, other studies have found that many isolates maintained in the greenhouse contain satellites that induce a variety of symptoms (12,28). Although these outbreaks of severity were induced by satellites, we have shown in this study that the severe stunting, deformation, and purpling of tomatoes seen in the fields of southern China can be duplicated in the greenhouse by the genomic RNA alone. Not only is the inoculum but also the inoculated, severely symptomatic plants were demonstrated to be free of satellite. CMV-Chi genomic RNA also severely affects zucchini squash, pepper, lettuce, and cantaloupe. In a separate study, CMV-Chi-free of satellite was shown to cause symptoms much more severe than six other CMV strains in 12 lines of pepper (Capsicum annuum L., Capsicum frutescens L., and Capsicum chinense Jacq.; Loaiza-Figueroa and Provvidenti, unpublished data). The wide range of hosts exhibiting severe symptoms argues against the involvement of sat-Chi in the severity, because most satellites incite severe symptoms in one host while being ameliorative or mild in another (7,17). Sat-Chi was shown to cause complete necrosis in tomato seedlings when coinoculated with genomic RNA of two different CMV strains. Sat-Chi may therefore be responsible for the necrotic symptoms seen in the field tomatoes, while the genomic RNA incites the severe stunt, purpling, and deformation. Alternatively, the necrosis may be caused by environmental influences on the disease. The necrosis induced in tomatoes by sat-Chi occurred at roughly the same time after inoculation as reported for another necrosis-inducing CMV satellite (17), though complete necrosis was occasionally not induced by sat-Chi, instead being replaced by a very severe stunt. It is difficult to say whether or not this satellite was present in appreciable amounts in the original field tomato sample. Propagation in the greenhouse, especially in tobacco, would be expected to alter the satellite population (16,24).

---

**Table 1.** Longevity of four CMV strains in infected tomato tissue dried at room temperature without desiccant or vacuum as assayed by local lesions on Chenopodium quinoa

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Strain</th>
<th>Fresh</th>
<th>Dried 1 wk</th>
<th>Dried 7 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:200</td>
<td>Chi</td>
<td>72 ± 11</td>
<td>6.5 ± 3.5</td>
<td>1.8 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>LI</td>
<td>25 ± 9.8</td>
<td>1.0 ± 1.1</td>
<td>0.75 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>Mus</td>
<td>19 ± 8.8</td>
<td>0.75 ± 0.50</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>WL</td>
<td>32 ± 21</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 1.0</td>
</tr>
<tr>
<td>1:20</td>
<td>Chi</td>
<td>182 ± 39</td>
<td>66 ± 23</td>
<td>43 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>LI</td>
<td>202 ± 30</td>
<td>22 ± 13</td>
<td>8.2 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>Mus</td>
<td>110 ± 12</td>
<td>1.8 ± 1.5</td>
<td>1.2 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>WL</td>
<td>175 ± 42</td>
<td>0.75 ± 0.50</td>
<td>0.50 ± 0.58</td>
</tr>
</tbody>
</table>

* Eight representative leaflets per strain were ground in 10 mM potassium phosphate buffer (pH 7.0) at the dilutions (tissue:buffer) indicated. The four strains were inoculated onto four leaves of four Chenopodium quinoa plants in a Latin square design.

* Average ± standard deviation of number of lesions per leaf (n = 4 leaves) appearing on C. quinoa 1 wk after inoculation with buffer extract of infected tomato sample.
CMV-Chi was first isolated from tomato tissue dried without desiccant, a procedure that is usually destructive to CMV infectivity. It was shown that CMV-Chi has greater longevity than CMV-WL and CMV-Mus in this dried tissue (Table 1). The infectivity decrease of CMV-LI, however, was of a similar degree, demonstrating that this longevity is not unique to CMV-Chi. Strains with differing stabilities, such as CMV-Chi and CMV-WL, might serve as models for coat protein structure comparisons.

LITERATURE CITED