Tomato necrosis and the 369 nucleotide Y satellite of cucumber mosaic virus: factors affecting satellite biological expression

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To determine which factors can affect biological expression of the Y satellite RNA of cucumber mosaic virus (CMV) in tomato, three laboratories collaboratively exchanged their natural satellite variants, the corresponding recombinant DNA clones and helper virus strains, as well as tomato varieties, on which different observations previously reported were based. The effects of these materials and the influence of temperature on symptom expression were systematically studied. The results show that in a standardized tomato bioassay at 24 °C, the Y satellite, when supported by either CMV-1 or CMV-Y, did not induce tomato necrosis in the Rutgers variety but elicited a slower necrotic response in the Best of All variety that was variably lethal, as compared to the faster inevitably lethal response induced by a prototype necrogenic D satellite variant in both tomato varieties. At higher temperatures (26.5 to 32 °C) an extremely fast-killing necrosis caused by CMV-Y itself was observed. The study demonstrates that in experiments on virus symptom modulation induced by CMV satellites, the nature of the helper virus, host plant varieties, as well as the environmental conditions should be precisely defined, and the effects of each parameter change determined separately.

Introduction

Lethal tomato necrosis, first described following a devastating cucumber mosaic virus (CMV) epidemic in the Alsace region of France (Putz et al., 1974; Marrou & Duteil, 1974), and later linked to a CMV satellite RNA that was designated CARNA 5 (for CMV-associated RNA 5) (Kaper & Waterworth, 1977), is a disease with a highly characteristic symptom progression which almost inevitably leads to death of the plant [for recent descriptions of symptomatology see Kaper et al. (1990b) and Montasser et al. (1991)].

Necrogenic CMV satellites have a highly conserved sequence profile, in contrast to the non-necrogenic variants which possess several domains of sequence variability (Kaper et al., 1988). Work from different laboratories has led to the identification of a tomato necrosis-determining conserved sequence element, of approximately 15 nucleotides in the 3' half of CMV satellites (Jacquemond & Lauquin, 1988; Masuta & Takanami, 1989; Devic et al., 1990; Sleat & Palukaitis, 1990). Although all necrogenic variants have been shown so far to possess this conserved 3' half sequence element, it is becoming apparent that other structural elements co-determine necrogenic expression of CMV satellites. A recent structural investigation of the Y satellite of CMV, the ability of which to elicit lethal necrosis in tomato having been in dispute (Takanami, 1981; Kaper et al., 1986), has shown that the presence of a massive insertion/deletion in its 5' half essentially prevents necrogenic expression of the 3' half necrogenic determinant. It was concluded that this 5' half insertion/deletion may have replaced an element that is a necessary part of a three-dimensional structure needed by CMV satellites to induce lethal tomato necrosis (Wu & Kaper, 1992).

In an attempt to establish which factors in the past contributed to the disputed Y satellite necrogenicity in tomato (Takanami, 1981; Kaper et al., 1986), a joint investigation was undertaken by three laboratories which have exchanged their natural satellite variants and the corresponding recombinant DNA clones, their helper virus strains, and tomato varieties.

Here we show that aside from structural element considerations, many other factors affect virus symptom modulation by CMV satellites. These include helper

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virus, host plant species, as well as environmental conditions. Each of these factors should be precisely defined, and the effects of any changes should be determined separately.

Methods

Virus strains, satellite RNAs and host plants. CMV-1 and CMV-Y are two CMV strains described previously by Kaper et al. (1981) and Takanami (1981), respectively. Y-CARNA 5 (Kaper et al., 1986) and Y-satRNA (Masuta et al., 1988a) both originate from the CMV-Y described by Takanami, but were subsequently maintained in different laboratories. D-CARNA 5 is the prototype necrogenic satellite RNA from CMV-D (Collmer & Kaper, 1986). Tobacco (Nicotiana tabacum L. cv. Xanthi nc) and two tomato varieties (Lycopersicon esculentum M. cv. Rutgers and cv. Best of All) were used in the study.

Nucleotide sequence determination, cloning of satellite RNA cDNA and in vitro transcription. The primary structure of gel-clut Y-CARNA 5 was determined using dideoxynucleotide chain termination sequencing with synthetic primer 5'GGGTCCCTG' (Kaper et al., 1986). Terminal sequences were determined by partial cleavage of end-labelled RNA as described by Kaper et al. (1988). Full-length cDNA of Y-CARNA 5 was cloned in pMM1 (Wu & Kaper, 1992). The Y-satRNA cDNA clone in pUT118GG-5 was previously constructed and sequenced by Kuwata et al. (1988). In vitro transcription of the pMM clones of D- and Y-CARNA 5 was carried out as described by Collmer & Kaper (1986). Y-satRNA was transcribed from its cDNA clone as described by Kuwata et al. (1988).

Assay for tomato necrosis and replication footprint analysis. Transcription mixtures with cloned satellite transcripts adjusted to equal concentration after electrophoretic analysis, or 2.5 µg/ml of the natural satellites, were combined with helper CMV genomic RNA at a concentration of 10 µg/ml in 0.03 M-Na2HPO4. At Beltsville, 20 tomato seedlings at the cotyledon stage were inoculated with each combination and with CMV genomic RNA alone. Inoculated plants were kept in growth chambers with a 16 h light period (8000 to 12000 lux) at 24 °C, except in one experiment in which the temperature was raised to investigate its effect on symptom expression. In Yokohama and Madrid the temperature range of growth chambers was 25 to 26 °C.

Replication footprint analysis (Kaper et al., 1990b) showing the amounts of single-stranded and double-stranded satellite present was carried out with tissue sampled 14 days after inoculation or before the plant's death from necrosis. Replication footprint analysis was also performed with tomato protoplasts electroporated with appropriate CMV/CARNA 5 combinations (Smith et al., 1992). Total nucleic acid (TNA) extracts from the tissue samples (White & Kaper, 1989) or protoplasts were subjected to 9% semi-denaturing PAGE and the gel was blotted onto a Biotrans nylon membrane. The membrane was hybridized either with a riboprobe prepared from the cDNA clone of D-CARNA 5 or with a riboprobe prepared from a partial cDNA clone of CMV-D RNA 4 (White & Kaper, 1989).

Results and Discussion

Tomato necrosis in CMV-Y infections can be the result of a necrogenic satellite contamination

Gel-clut Y-CARNA 5 from the previous study in Beltsville (Kaper et al., 1986) was compared with Y-CARNA 5 obtained from an independent repetition of the series of tobacco passages initiated by the same lyophilized preparation of CMV-Y used in the 1986 investigation. These experiments showed that in the 1986 study the tobacco infection had become contaminated with a highly necrogenic satellite Yn-CARNA 5 (data not shown). This residual contamination probably accounted for the occurrence of some background tomato necrosis in the dilution endpoint bioassays of this work. From the sequence of Yn-CARNA 5 (Kaper et al., 1986) we suspect that the origin of the contamination was a CMV satellite previously isolated and characterized in the Beltsville laboratory (Collmer et al., 1983).

A single nucleotide difference in the sequences of Y-CARNA 5 and Y-satRNA

In the previous Beltsville work (Kaper et al., 1986) a partial nucleotide sequence of Y-CARNA 5 was determined to verify the identity of Y-CARNA 5 with the satellite of CMV-Y sequenced by Hidaka et al. (1984). Three differences were noted in the sequence, and confirmed by the Yokohama group (Masuta et al., 1988b); this group also found a fourth difference in the form of a C insertion at position 234, which was confirmed in the complete sequence analysis by the Beltsville group. However, one difference at position 252 remained. Here Y-CARNA 5 has a C residue (data not shown) and Y-satRNA a U residue (Masuta et al., 1988b).

Identical biological response of tomato to Y-CARNA 5 and Y-satRNA using different helper CMV strains

The bioassay at 24 °C with Y-CARNA 5 and Y-satRNA clone transcripts (or the two natural variants) in tomato using either CMV-1 or CMV-Y as the helper virus showed no difference in symptoms between the two satellites. Neither induced the characteristic lethal necrosis syndrome in the Rutgers tomato variety observed with the D-CARNA 5 transcript; rather, a mild chlorosis and distinct amelioration of viral symptoms were seen with the satellites of CMV-Y (Fig. 1). Similarly, no symptom differences between the two Y satellites were observed for the Best of All tomato variety (see below). This eliminated their single nucleotide difference as a source of uncertainty with regard to the ability of Y-CARNA 5 and Y-satRNA to induce tomato necrosis. Therefore other factors, e.g. helper virus, tomato variety or environmental conditions, would have to be considered and tested. However, since the two helper CMV strains (CMV-Y and CMV-1) used in this reinvestigation were the same as those which resulted in conflicting reports for the necrogenicity of CMV-Y satellites (Takanami, 1981; Kaper et al., 1986), the result shown in Fig. 1 also eliminated the helper virus strain as the
Tomato necrosis and the Y satellite of CMV

Fig. 1. Symptoms induced by D-, Y-CARNA 5 and Y-satRNA on plants of tomato variety Rutgers (Rt) using CMV-1 or CMV-Y as helper viruses. Tomato plants were inoculated with 10 μg/ml CMV RNA alone, or in the presence of \textit{in vitro} transcripts of the satellites, as indicated in the figure. The picture was taken 25 days after inoculation.

source of these conflicting results, at least for the tomato variety and the temperature (24°C) used in the present experiment.

TNA extracts from randomly sampled tissue of the plants represented in Fig. 1 after replication footprint analysis showed that the amounts and the proportions of satellite ss- and dsRNA were very similar in the infections with D-CARNA 5, Y-CARNA 5 and Y-satRNA (Fig. 2). Identical results were obtained in tomato protoplasts 16 and 40 h after infection with the same combinations of helper virus and satellites (data not shown). Thus differences in satellite titre in the infected tissues, as well as differences in initial replication rate, could be eliminated as possible reasons for the entirely different biological response of Rutgers tomato to D-CARNA 5 on the one hand, and Y-CARNA 5 and Y-satRNA on the other with either helper virus (Fig. 1). It was therefore concluded that in Rutgers tomato CMV-Y satellite replication does not elicit the lethal necrotic response that results from replication of the prototype necrogenic satellite of CMV-D (Collmer & Kaper, 1986).

Different biological response to Y-CARNA 5 by two different tomato varieties

In previous work from the Beltsville group 96 different varieties of tomato (\textit{L. esculentum}) were reported to develop tomato necrosis in response to infection with CMV-D containing the highly necrogenic D-CARNA 5, although certain exotic species of the genus \textit{Lycopersicon}}
showed only mosaic or chlorotic symptoms (White & Kaper, 1987). However, since the Yokohama group had reported tomato variety Best of All to give a higher incidence of necrotic response to infection with CMV-Y + Y-satRNA than tomato variety Rutgers (Masuta et al., 1988a), this comparison was repeated at Beltsville with natural Y-CARNA 5, using CMV-1 as helper virus and natural D-CARNA 5 as the prototype necrogenic control under the standardized bioassay conditions (24 °C). Both tomato varieties responded with fast lethal tomato necrosis to D-CARNA 5 (Fig. 1 and 3), but Best of All responded to Y-CARNA 5 with a slower form of necrosis from which some of the test plants recovered by sprouting new leaves (Fig. 3). The response of Rutgers tomato to Y-CARNA 5 was that of viral symptom attenuation (Fig. 1). Necrosis has also been reported by Devic et al. (1990) on tomato variety Ailsa Craig when it was infected with the Y satellite using a different helper virus strain (CMV-KIN). This might also reflect a more sensitive response on the part of that particular tomato variety or might be related to the use of the different CMV strain (see below). Replication footprint analysis of tissue from the plants of Fig. 3 again confirmed the presence of ss- and dsRNA of CARNA 5 of the D- and Y-satellites in amounts that were comparable to those shown in Fig. 2 (data not shown). This indicated that the differences in biological response to D- and Y-CARNA 5 in the two tomato varieties are not related to differences in satellite replication and/or movement in the plant.

![Fig. 3. Symptoms on tomato variety Best of All (BA) induced by D- and Y-CARNA 5 using CMV-1 as helper virus. Tomato plants were inoculated with CMV-1 genomic RNA (10 μg/ml) alone, or in the presence of 2.5 μg/ml of D- or Y-CARNA 5, as indicated in the figure. The picture was taken 28 days after inoculation.](image)

Different biological response of tomato to Y satellite in different laboratories

When experiments similar to those described above were performed in Yokohama or Madrid where growth chamber temperature ranged between 25 and 26 °C, both tomato varieties responded with a variable and slow form of stem necrosis which was more severe in Best of All and ultimately resulted in the death of some plants only of this variety (Table 1). Although plants were inoculated at the cotyledon stage in all three laboratories,

### Table 1. Observation of symptoms on tomato varieties induced by satellite RNAs in Yokohama*

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Tomato variety</th>
<th>Helper transcript†</th>
<th>Transcript of satRNA‡</th>
<th>No. of plants inoculated</th>
<th>Symptom§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rutgers</td>
<td>CMV-1</td>
<td>–</td>
<td>5</td>
<td>5 fern-leaf, mosaic</td>
</tr>
<tr>
<td>2</td>
<td>Rutgers</td>
<td>CMV-1</td>
<td>Y-CARNA 5</td>
<td>5</td>
<td>5 attenuation, partial stem necrosis</td>
</tr>
<tr>
<td>3</td>
<td>Rutgers</td>
<td>CMV-1</td>
<td>Y-satRNA</td>
<td>5</td>
<td>5 attenuation, partial stem necrosis</td>
</tr>
<tr>
<td>4</td>
<td>Rutgers</td>
<td>CMV-Y</td>
<td>–</td>
<td>5</td>
<td>5 severe mosaic</td>
</tr>
<tr>
<td>5</td>
<td>Rutgers</td>
<td>CMV-Y</td>
<td>Y-CARNA 5</td>
<td>5</td>
<td>5 severe mosaic</td>
</tr>
<tr>
<td>6</td>
<td>Rutgers</td>
<td>CMV-Y</td>
<td>Y-satRNA</td>
<td>5</td>
<td>5 attenuation, partial stem necrosis</td>
</tr>
<tr>
<td>7</td>
<td>Best of All</td>
<td>CMV-1</td>
<td>–</td>
<td>5</td>
<td>5 attenuation, partial stem necrosis</td>
</tr>
<tr>
<td>8</td>
<td>Best of All</td>
<td>CMV-1</td>
<td>Y-CARNA 5</td>
<td>5</td>
<td>5 severe stem necrosis</td>
</tr>
<tr>
<td>9</td>
<td>Best of All</td>
<td>CMV-1</td>
<td>Y-satRNA</td>
<td>5</td>
<td>5 severe stem necrosis</td>
</tr>
<tr>
<td>10</td>
<td>Best of All</td>
<td>CMV-Y</td>
<td>–</td>
<td>5</td>
<td>5 severe stem necrosis</td>
</tr>
<tr>
<td>11</td>
<td>Best of All</td>
<td>CMV-Y</td>
<td>Y-CARNA 5</td>
<td>5</td>
<td>5 severe stem necrosis</td>
</tr>
<tr>
<td>12</td>
<td>Best of All</td>
<td>CMV-Y</td>
<td>Y-satRNA</td>
<td>5</td>
<td>5 severe stem necrosis</td>
</tr>
</tbody>
</table>

* All plants were kept in a growth chamber with a 16 h light period (10000 lux) at 25 °C (±0.5 °C).
† Inoculum concentration was 10 μg/ml viral genomic RNA.
‡ Transcription mixtures with cloned satellite transcripts were adjusted to equal concentrations after gel electrophoresis.
§ Symptoms were scored 46 days for Expt. 1 to 7 and 43 days for Expt. 8 to 14 after inoculation.
the overall appearance of the plants at later stages of development was different, suggesting that environmental factors other than temperature (such as lighting conditions and soil nutrients) that might affect plant development also exerted an influence on the biological expression of the satellite.

The varying necrotic response of tomato to Y satellites of CMV therefore indicates that this satellite has a potential necrogenicity that can surface in the more sensitive Best of All tomato variety, but can be obscured in the less sensitive Rutgers variety. The notion of a potential necrogenicity of CMV-Y satellites which is structurally suppressed is corroborated by new evidence that shows that the absence of certain sequence elements in the 5' half of Y-CARNA 5 that are present in D-CARNA 5 and essential to necrogenicity prevent expression of the 3' half necrogenic determinant (Wu & Kaper, 1992).

Necrotic response of tomato to infection by CMV-Y alone and the effect of different temperatures

In the experiments shown in Fig. 1 it had been noticed that although no necrosis occurred with Y-CARNA 5 or Y-satRNA transcripts added to the CMV-Y inoculum, at 24 °C tomato seedlings responded to infection with CMV-Y alone with leaf necrosis from which the plants recovered after about 2 weeks (Fig. 1). This confirmed previous experiences of the Yokohama group, who prior to this investigation inoculated their tomato seedlings with the first true leaves emerged, in order to minimize necrosis damage by CMV-Y alone (unpublished results).

The influence of temperature on the lethal necrotic response of tomato to infection with CMV containing a necrogenic CARNA 5 was first reported by Kaper & Waterworth (1977), who noted a distinct restriction of systemic spread of the disease at higher temperatures. With the combination CMV-D/D-CARNA 5 we now know this to be a satellite replication-related effect (J. L. White et al., unpublished results). In contrast, Hanada & Tochihara (1980) reported that (satellite-containing) CMV-Y 'caused necrosis on tomato at 28 °C but not at 22 °C'.

The effect of different higher temperatures (32 °C, 28 °C and 26.5 °C) on the necrotic response of tomato to CMV-Y and CMV-1 in the absence or presence of satellites was subsequently examined systematically in Beltsville by inoculating seedlings at the cotyledon stage with each virus strain alone and in the presence of clone transcripts of D-CARNA 5, Y-CARNA 5 and Y-satRNA. In each experiment, half the number of plants (12) from each inoculation were grown at the higher temperatures while the other half served as controls at 24 °C. In the experiment at 28 °C (Fig. 4), within 1 week all plants infected with CMV-Y as helper virus (regardless of satellite presence) had succumbed to lethal necrosis (rows 5 to 8), whereas all plants infected with CMV-1 (rows 1 to 4) and the uninfected plants (row 9) had survived. The symptoms of the infected plants in rows 1 to 4 after 3 weeks were similar to those kept at 24 °C where only the plants inoculated with D-CARNA 5 had died, as shown in Fig. 1. In view of these temperature-dependent differences in the biological response of tomato to infections with two different helper...
Fig. 5. Replication footprint analysis of TNA extracts from tomato plants infected with CMV-Y alone, and in the presence of D- or Y-CARNA 5. Tomato (Rutgers) plants grown at 24 °C (lanes 1, 3 and 5) and 32 °C (lanes 2, 4 and 6) were inoculated with CMV-Y RNA (10 µg/ml) alone, or CMV-Y + D- or Y-CARNA 5 (2.5 µg/ml). TNA extracts from the plants were obtained 4 (a) and 7 (b) days after inoculation, followed by 9% semi-denaturing PAGE and Northern blot hybridization to riboprobes prepared from cDNA clones of CMV-D RNA 4 [left panels of (a) and (b)] and CARNA 5 (right panels). Lanes 1 and 2, CMV-Y alone; lanes 3 and 4,
CMV strains, it seems possible that the reported necrogenic response of tomato to infection by Y satellites in the presence of helper viruses CMV-O (Masuta & Takanami, 1989) or CMV-KIN (Devic et al., 1990) might also have been influenced by unspecified variable temperature conditions during their tests.

To determine whether the effect of the higher temperatures on the necrotic response of tomato to CMV-Y is related to viral replication, experiments were carried out at 32 °C and 24 °C and TNA extracts were obtained from the infected plants at 4 and 7 days after inoculation. Fig. 5(a) shows a replication footprint analysis of 4 day TNA extracts using labelled specific probes for both CMV genomic RNA and CARNA 5. In addition to reduced amounts of CMV-Y genomic RNA, due presumably to the high level of D- or Y-CARNA 5 accumulation (lanes 3 to 6 compared to lanes 1 and 2), the results consistently showed a higher amount of CMV-Y genomic RNA in tissues from the plants at 32 °C than at 24 °C. This is indicated by the higher amount of CMV RNA 3 and at 32 °C (lanes 2, 4, and 6) than at 24 °C (lanes 1, 3, and 5) detected by the CMV genomic probe. TNA extracts collected after 7 days (Fig. 5b) simply show blank 32 °C lanes, since no tissue could be extracted from dead plantlets. An identical control experiment using CMV-1 did not show this difference in viral RNA presence in the infected plants at the two temperatures (data not shown). The correlation between the fast-developing necrosis and the larger amounts of viral RNA in tissues at higher temperature suggests that the necrotic response of tomato to CMV-Y is viral RNA replication-related. This explanation may be too simplistic, however, since in the 32 °C infections with Y- and D-CARNA 5 added, CMV-Y RNA reached levels only slightly higher than those at 24 °C with CMV-Y alone, where no death occurred. To complete the experimentation that appears to relate the fast-killing tomato necrosis to CMV-Y itself, and not to the effects of satellite, we have also infected tomato at 24 °C and 32 °C with CMV-Y to which the prototype non-necrogenic satellites S-CARNA 5 and 1-CARNA 5 were added. This yielded results identical to those with D-CARNA 5 and Y-CARNA 5 (data not shown).

Clearly, more insight into the relationship between the titres of viral RNAs and CARNA 5 in plant tissues, their threshold levels and the biochemical and physiological events leading to death from tomato necrosis is required. What is evident from these results, however, is that in addition to satellite and helper virus strains and plant varieties, environmental conditions are also critical factors in experiments on virus symptom modulation by CMV satellites. These should be precisely defined and the effects of each change determined separately.

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(Received 9 July 1992; Accepted 8 October 1992)