

Mapping Determinants Within Cucumber Mosaic Virus and Its Satellite RNA for the Induction of Necrosis in Tomato Plants

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A mutant of the WL47 satellite (WL47-sat) RNA of cucumber mosaic virus (CMV), constructed *in vitro*, induces lethal necrosis in tomato plants when associated with either of two subgroup II strains of CMV: LS- or WL-CMV (D. E. Sleat and P. Palukaitis, Proc. Natl. Acad. Sci. USA 87:2946-2950, 1990). The phenotype of this mutant (WLM2-sat RNA) has been examined on tomato plants, after co-inoculation with a wider range of CMV strains. Necrosis was observed when WLM2-sat RNA was associated with any of the subgroup II CMV strains tested. However, WLM2-sat RNA ameliorated the symptoms induced by subgroup I CMV strains without inducing necrosis. Inoculation with WLM2-sat RNA with pseudorecombinants formed between subgroup I and subgroup II CMV strains showed an association of the necrosis induction phenotype with RNA 2 of the subgroup II strains. In contrast to WLM2-sat RNA, infectious transcripts of the naturally necrogenic D4-sat RNA induced lethal necrosis in tomato with all CMV strains tested. Experiments involving the exchange of sequences between WLM2- and D4-sat RNAs indicated that nucleotide sequences in either of two separate regions could influence the necrosis phenotype. Thus, the subgroup-specific necrosis-inducing phenotype may be due to subtle alterations in secondary and/or tertiary structure in the satellite RNA, as well as the presence or absence of particular nucleotide sequences.

Additional keywords: cucumoviruses, pathogenicity.

Cucumber mosaic virus (CMV) strains can be divided into two subgroups, depending upon whether they differ only slightly (2–5%) in sequence from either Fny-CMV, a subgroup I strain, or Q-CMV, a subgroup II strain (Palukaitis *et al.* 1992). Although the RNAs of subgroup I and subgroup II strains differ by 26–29% in sequence, the gene products encoded by RNAs 1 and 2, which are involved in the replication of CMV and its satellite RNAs, are completely compatible in pseudorecombinants formed between the subgroups (Palu-

kaitis *et al.* 1992). Satellite RNAs of CMV have been extensively studied as model systems for viral pathogenesis in plants (research on this topic has been reviewed by Francki [1985] and Roossinck *et al.* [1992]). This is in part because CMV satellite RNAs can modify symptoms induced by their helper virus, CMV, in a number of different ways, despite their small size (330–391 nucleotides) and relatively high degree of sequence conservation (Garcia-Arenal *et al.* 1987; Kaper *et al.* 1988; Palukaitis 1988; Roossinck *et al.* 1992; Sayama *et al.* 1993).

The pathology of CMV alone on tomato plants is diverse, ranging from asymptomatic infection to severe stunting with leaf distortion, leaf curl, and general malformation. However, when associated with certain satellite RNAs, CMV can induce lethal necrosis, which usually develops 2–3 weeks after inoculation (Kaper and Waterworth 1977). Not all CMV satellite RNAs induce necrosis in tomato plants; in fact, most satellite RNAs attenuate the symptoms induced by CMV (Mossop and Francki 1979; Kaper *et al.* 1981; Gonsalves *et al.* 1982; Palukaitis 1988).

Analyses of chimeras constructed between necrogenic (i.e., necrosis-inducing) and nonnecrogenic CMV satellite RNAs have localized the sequences involved in the induction of necrosis to within the 3' half of the molecule (Devic *et al.* 1989; Kurath and Palukaitis 1989; Masuta and Takanami 1989), although sequences that affect necrosis induction by one satellite RNA also have been delimited to the 5' half of the

Table 1. Necrosis induction in tomato by WLM2-sat RNA and D4-sat RNA with various cucumber mosaic virus (CMV) strains

Helper virus strain	Virus alone ^a	Virus + D4-sat RNA	Virus + WLM2-sat RNA
Subgroup I			
Fny-CMV	– (0/0/5)	+ (5/5/5)	– (0/14/15)
Uh-CMV	– (0/0/5)	+ (5/5/5)	– (0/9/10)
K-CMV	– (0/0/5)	+ (5/5/5)	– (0/10/10)
Subgroup II			
LS-CMV	– (0/0/10)	+ (5/5/5)	+ (73/73/81)
SB-CMB	– (0/0/5)	+ (5/5/5)	+ (5/5/5)
U-CMV	– (0/0/5)	– (ND) ^b	+ (7/7/13)
WL-CMV	– (0/0/5)	+ (5/5/5)	+ (5/5/5)

^a + = Induction of necrosis. – = Satellite RNA either ameliorated or had no effect on the symptoms induced by the particular helper virus. The quantitative data are presented as (number of plants showing necrosis)/(number of plants containing satellite)/(number of plants inoculated), determined 3 weeks postinoculation.

^b ND = not determined. In two experiments, eight or 10 tomato seedlings inoculated with U-CMV containing D4-sat RNA showed no replication of the U-CMV.

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molecule (Wu and Kaper 1992). Furthermore, site-directed mutagenesis of cDNA clones of various satellite RNAs has shown that as few as one to three nucleotide changes can alter the satellite RNA phenotype on tomato from ameliorative to necrogenic, and vice versa (Masuta and Takanami 1989; Sleat and Palukaitis 1990a; Devic *et al.* 1990). In this report, we compare a necrogenic mutant of this type, WLM2 satellite (WLM2-sat) RNA (Sleat and Palukaitis 1990a), with the naturally necrogenic D4-sat RNA (Kurath and Palukaitis 1987) and demonstrate a significant difference in the phenotypes of these two satellite RNAs on tomato plants. This difference has been used to map additional sequences in the satellite RNA and the helper virus involved in the induction of necrosis in tomato.

RESULTS

Subgroup specificity of necrosis induction in tomato plants by WLM2-sat RNA.

The site-directed mutant satellite RNA, WLM2-sat RNA, has been shown to induce necrosis in tomato plants with the subgroup II strains LS- and WL-CMV (Sleat and Palukaitis 1990a). To examine the specificity of necrosis induction with respect to CMV helper strains, WLM2-sat RNA was gel-purified from either LS- or Fny-CMV, which had been passaged through tobacco plants, and tomato plants were then inoculated with the purified WLM2-sat RNA and a range of CMV strains of both subgroups (Table 1). D4-sat RNA, which is derived from a naturally necrogenic satellite RNA (Kurath and Palukaitis 1987), was also passaged through tobacco with LS-CMV and gel-purified, and tomato plants were then inoculated with the purified D4-sat RNA and different CMV strains (Table 1). Both WLM2-sat RNA and D4-sat RNA attenuate symptoms induced in tobacco plants by CMV strains from either subgroup (data not shown).

D4-sat RNA induced lethal necrosis in tomato plants with all CMV strains tested, within 2–3 weeks after inoculation (Tables 1 and 2). D4-sat RNA was previously shown to induce necrosis in tomato plants with the subgroup I strain Fny-CMV (Kurath and Palukaitis 1987) and the subgroup II

strains LS- and WL-CMV (Kurath and Palukaitis 1989). WLM2-sat RNA, however, only induced necrosis in tomato when associated with subgroup II CMV strains (Table 1). Necrosis induced by WLM2-sat RNA with subgroup II CMV strains was phenotypically identical to that induced by D4-sat RNA and was induced in a similar time, 2–3 weeks post-inoculation (p.i.). With subgroup I CMV strains, WLM2-sat RNA caused a mild amelioration of the symptoms induced by the virus alone. Plants infected with WLM2-sat RNA and either of the subgroup I strains Fny- and Uh-CMV were maintained up to 6 weeks without the induction of necrosis.

Induction of necrosis by WLM2-sat RNA with pseudorecombinant CMV strains.

Tomato plants were inoculated with gel-purified WLM2-sat RNA together with a number of pseudorecombinants formed between subgroup I and subgroup II CMV strains, to determine which RNA or RNAs of the subgroup II strains, in association with WLM2-sat RNA, induce necrosis (Table 2). With pseudorecombinants formed between U- and K-CMV, WLM2-sat RNA induced necrosis in tomato plants only when associated with the pseudorecombinant containing RNA 2 of the subgroup II strain (U-CMV), although it was replicated by the other two pseudorecombinants. This was also the case with pseudorecombinants involving Fny- and LS-CMV (i.e., necrosis was induced only after replication of WLM2-sat RNA by F₁L₂L₃, F₁L₂F₃, or L₁L₂F₃) (Table 2), although the satellite RNA was also replicated by the other three pseudorecombinants.

D4-sat RNA was tested with only six pseudorecombinant CMV strains: K₁K₂U₃, U₁K₂K₃, K₁U₂U₃, F₁L₂F₃, L₁F₂L₃, and F₁F₂L₃ (Table 2). In all cases, necrosis was induced after 2–3 weeks. However, the necrosis induced by WLM2-sat RNA, when replicated by the CMV pseudorecombinants F₁L₂F₃, L₁L₂F₃, and F₁L₂L₃, did not begin until after 3 weeks had elapsed and did not always cause complete plant death, but was often restricted to parts of the plants by 5 weeks p.i. (see below). This was not the case for the CMV pseudorecombinant helper virus K₁U₂U₃, which caused complete, systemic necrosis in the usual 2–3 weeks.

Induction of necrosis by WLM2-sat RNA is not due to adaptation or selection.

Prior to the onset of necrosis, WLM2-sat RNA was isolated from tomato plants that had been inoculated with this satellite and LS-CMV. The satellite was gel-purified and used with Fny-CMV to inoculate tomato seedlings. In no instance did WLM2-sat RNA with Fny-CMV induce necrosis (Table 3), indicating the absence of necrogenic mutants arising via passage with LS-CMV (Sleat and Palukaitis 1990a). WLM2-sat RNA passaged with either Fny- or Uh-CMV helper was purified 4 weeks after inoculation from tomato plants which did not show any necrosis. As a control, these two preparations of WLM2-sat RNA were gel-purified and then used to reinoculate tomato together with either LS-CMV, Uh-CMV, or Fny-CMV. As observed previously, both passaged WLM2-sat RNA preparations induced necrosis with LS-CMV but not with either Uh-CMV (Table 3) or Fny-CMV (data not shown). WLM2-sat RNA isolated from tomato plants co-inoculated with either Fny-, Uh-, or LS-CMV (first helper virus) was sequenced directly by dideoxynucleotide chain ter-

Table 2. Necrosis induction in tomato by WLM2-sat RNA and D4-sat RNA with pseudorecombinant viruses involving cucumber mosaic virus (CMV) strains in subgroups I and II

Helper virus strain ^a	Virus alone ^b	Virus + D4-sat RNA	Virus + WLM2-sat RNA
U ₁ K ₂ K ₃	–	+ (5/5/5)	– (0/6/15)
K ₁ U ₂ U ₃	–	+ (5/5/5)	+ (20/20/20)
K ₁ K ₂ U ₃	–	+ (3/3/5)	– (0/10/10)
F ₁ F ₂ L ₃	–	+ (4/4/5)	– (0/12/13)
L ₁ F ₂ L ₃	–	+ (3/3/5)	– (0/17/18)
F ₁ L ₂ L ₃	–	NT ^c	+ (11/11/16)
F ₁ L ₂ F ₃	–	+ (5/5/5)	+ (6/6/8)
L ₁ L ₂ F ₃	–	NT	+ (14/14/16)
L ₁ F ₂ F ₃	–	NT	– (0/13/16)

^a Pseudorecombinants between U-CMV (U) and K-CMV (K) or between LS-CMV (L) and Fny-CMV (F). The subscript numbers refer to the corresponding CMV RNA; e.g., U₁K₂K₃ contains RNA 1 from U-CMV and RNAs 2 and 3 from K-CMV.

^b + = Induction of necrosis. – = Absence of necrosis. The quantitative data are presented as (number of plants showing necrosis)/(number of plants containing satellite)/(number of plants inoculated).

^c Not tested.

mination, and the identity of the progeny satellite RNA was confirmed in each case. The identity of WLM2-sat RNA isolated from tomato plants in combination with LS- or Fny-CMV (second helper virus) was also confirmed by a ribonuclease protection assay (data not shown). The differential induction of necrosis by WLM2-sat RNA cannot therefore be attributed to contamination by a foreign satellite RNA or to a modification in sequence or structure resulting from passage with a particular helper CMV strain.

Six weeks p.i., tomato plants infected with WLM2-sat RNA and the pseudorecombinant U₁K₂K₃-CMV did not show lethal necrosis. The virus was isolated from these plants, and

WLM2-sat RNA was isolated by gel purification. This WLM2-sat RNA again induced necrosis in tomato plants when reinoculated with LS-CMV (Table 3) but not with Fny-CMV (data not shown). The WLM2-sat RNA isolated from tomato plants co-inoculated with U₁K₂K₃-CMV was partially sequenced with a primer complementary to the seven 3'-terminal nucleotides. The identity of the progeny was confirmed as WLM2-sat RNA (data not shown), again eliminating the possibility that the differential effect of WLM2-sat RNA was due to contamination, mutation, selection, or recombination.

To eliminate the possibility that the reproducible, delayed necrosis observed with the Fny-CMV/LS-CMV pseudorecombinants (Table 2) was due to a mutation in a subpopulation of either WLM2-sat RNA or the helper virus genome, the following experiment was done. Tobacco plants were inoculated with sap from partially necrotic tomato plants which had been inoculated 4 weeks earlier with F₁L₂L₃-CMV and WLM2-sat RNA. Two weeks later, the virus was purified from the infected tobacco plants and used to inoculate 16 tomato plants. Satellite RNA replicated in 11 of these plants, all of which developed partial to complete necrosis 3.5–5 weeks later. Thus, the delayed necrosis was the actual phenotype of this combination of pseudorecombinant and satellite RNA and was not due to mutation and selection to a threshold level for necrosis induction.

Delimitation of satellite RNA sequences regulating strain-specific necrosis induction.

WLM2- and D4-sat RNAs clearly differed in their ability to induce necrosis in tomato plants with different CMV

Table 3. Effect of passaging WLM2-sat RNA on the induction of necrosis in tomato^a

First helper virus strain	Second helper virus strain	Necrosis induction ^b
LS-CMV	Fny-CMV	– (0/8/10)
Uh-CMV	Uh-CMV	– (0/3/5)
Uh-CMV	LS-CMV	+ (8/8/10)
Fny-CMV	LS-CMV	+ (9/9/10)
U ₁ K ₂ K ₃ -CMV ^c	LS-CMV	+ (10/10/10)

^a WLM2-sat RNA was passaged in tomato with the first helper virus strain, cucumber mosaic virus (CMV) was isolated, viral RNA was extracted, and the WLM2-sat RNA was purified by polyacrylamide gel electrophoresis and elution. The recovered WLM2-sat RNA was then propagated in tomato with the second helper virus.

^b + = Induction of necrosis with the second helper virus. – = Absence of necrosis with the second helper virus. The quantitative data are presented as (number of plants showing necrosis)/(number of plants containing satellite RNA)/(number of plants inoculated).

^c U₁K₂K₃-CMV is a pseudorecombinant, containing RNA 1 from U-CMV and RNAs 2 and 3 from K-CMV.

PHENOTYPE ON TOMATO


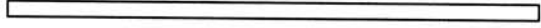




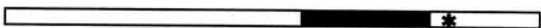

Plasmid	LS-CMV	Fny-CMV
 pDsat4	necrotic (13/13/15)	necrotic (5/5/5)
 pWLSat47	ameliorative (0/14/25)	ameliorative
 pWLSatM2	necrotic (73/73/81)	ameliorative (0/14/15)
 pDWsat191	ameliorative	ameliorative (0/23/24)
 pWDSat191	necrotic (8/8/10)	necrotic (21/21/24)
 pDW*sat191	necrotic (10/10/10)	ameliorative (0/23/24)
 pWDW*sat191/279	necrotic (23/23/23)	quasi-necrotic (38/38/53)
 pWDSat279	necrotic (22/22/23)	necrotic (25/25/38)

Fig. 1. Parental and chimeric satellite RNAs and phenotypes of satellite RNA transcripts of the corresponding plasmids when supported in tomato by cucumber mosaic virus (CMV) strain LS-CMV or strain Fny-CMV. The numerical data are presented as (number of plants showing necrosis)/(number of plants positive for satellite RNA by dot blot hybridization)/(number of plants inoculated). Phenotypes without quantitative data were quantified previously (Kurath and Palukaitis 1989).

strains. To map sequences within the satellite RNA that might dictate this specificity, a number of chimeras were constructed between cDNA clones of WL47-sat, WLM2-sat (W* in the following chimeras), and D4-sat RNAs, using their common *NheI* and *HgaI* sites at positions 191 and 279, respectively (Fig. 1).

The chimeric satellite RNAs were passaged through tobacco with Fny-CMV and then used to inoculate tomato plants. The chimeras were also gel-purified from the Fny-CMV RNAs and were used with LS-CMV to inoculate tomato plants. The respective phenotypes of these chimeric satellite RNAs on tomato are indicated in Figures 1 and 2.

The bioassay results from RNA transcripts of the *NheI* chimeras pWdsat191, pDWsat191 (Kurath and Palukaitis 1989), and pDW*sat191 indicated that the determinant for strain-specific necrosis induction is located within the 3' half of the satellite RNA molecule (Fig. 1). The necrosis domain itself has been localized within this region (Kurath and Palukaitis 1989; Devic *et al.* 1989; Masuta and Takamami 1989; Wu and Kaper 1992).

The *NheI/HgaI* chimeras pWdsat279 and pWDW*sat191/279 further divided the 3' half of the satellite RNA. Satellite RNA derived from pWdsat279 induced necrosis in tomato with Fny-CMV and LS-CMV in a qualitatively and temporally similar manner (Figs. 1 and 2), indicating that the sequences which define the CMV specificity of necrosis induction map between the *HgaI* site and the 3' terminus (Fig. 1). WLM2- and D4-sat RNA differ at only two nucleotide positions within this region (positions 330 and 340). However, when Fny-CMV was the helper virus, satellite RNA derived from the chimera pWDW*sat191/279 (Fig. 1) induced a partial necrosis (Fig. 2B), the onset of which was delayed, occurring between 3 and 4 weeks p.i. This result suggests that sequences between positions 191 and 279 can also influence the necrogenic phenotype. There are 10 nucleotide differences between D4-sat and WLM2-sat RNA in this region. The time course and overall pattern of necrosis induced by the satellite RNA derived from pWDW*sat191/279 when supported by Fny-CMV (Fig. 2B) was identical to that observed when WLM2-sat RNA was supported by Fny-CMV/LS-CMV pseudorecombinant viruses that contained RNA 2 of LS-CMV (Table 2 and text).

All chimeric satellite RNAs were isolated from tomato plants before complete necrosis and characterized by di-deoxynucleotide sequencing, using primers complementary to the seven 3'-terminal nucleotides and to nucleotides 272–284. The identities of chimeric WD-, DW-, and DW*-sat RNAs were also confirmed by a ribonuclease protection analysis (data not shown).

All chimeric satellite RNAs were replicated by Fny-CMV to similar levels (data not shown), indicating that differences in phenotype (Figs. 1 and 2) were not a consequence of differences in the concentration of the various chimeras or mutants. In general, Fny-CMV supports satellite RNAs to higher levels than LS-CMV does (unpublished data), making inefficient satellite support an unlikely basis for the lack of necrosis induction.

DISCUSSION

We have shown that WLM2-sat RNA is not equivalent to D4-sat RNA with respect to its ability to induce necrosis in tomato. D4-sat RNA induced tomato necrosis when supported by CMV strains in either subgroup I or subgroup II, while WLM2-sat RNA induced tomato necrosis only in the presence of subgroup II CMV strains. The time course and severity of necrosis induction by WLM2-sat RNA were identical to those observed with D4-sat RNA, when supported by related subgroup II CMV strains, but not when supported by selected pseudorecombinants formed between CMV subgroups I and II.

The data from the incomplete set of K-CMV/U-CMV pseudorecombinants suggest that either RNA 2 or a combination of two RNAs from a subgroup II strain was sufficient for necrosis induction, while the results from the Fny-CMV/LS-CMV pseudorecombinants show that RNA 2 of the subgroup II CMV RNAs was sufficient for necrosis induction. However, the profile of necrosis induction with WLM2-sat RNA was altered, compared to that of D4-sat RNA. Taken together, these data indicate a complex association between satellite and CMV RNAs, which may vary depending on the particular strains used in the assessment. In this respect, the necrogenic phenotype associated with the WLM2-sat RNA is reminiscent of other pathogenic phenotypes mapped by pseudorecombi-



Fig. 2. Tomato plants infected with cucumber mosaic virus (CMV) and chimeric satellite RNAs. Tomato plants were inoculated with either LS-CMV (A and C) or Fny-CMV (B and D), each containing transcripts of either pWdsat279 (C and D) or pWDW*sat191/279 (A and B). The photo was taken 4 weeks postinoculation. Note that one of the four plants in D, inoculated with Fny-CMV and transcripts of pWdsat279, did not become infected. The other plants are either partly necrotic (B) or completely necrotic (A, C, and D).

nation between various CMV strains (Rao and Francki 1982).

WLM2-sat RNA is not unique with respect to its dependency upon the strain of its helper virus for necrosis induction. T-sat RNA does not induce necrosis in tomato when supported by Ix-CMV, although it is necrogenic when supported by two other CMV strains (Kaper *et al.* 1990). In most studies of the pathogenicity of CMV satellite RNAs, however, the helper virus is largely uncharacterized, despite the potential influence of the helper strain demonstrated here and in other reports (Masuta *et al.* 1988; Palukaitis 1988; Kurath and Palukaitis 1989; Kaper *et al.* 1990; Sleat and Palukaitis 1990b). Thus, a nonnecrogenic phenotype for WLM2-sat RNA would have been assigned if the initial experiments had used subgroup I strains as helper viruses instead of subgroup II strains. This raises the question of whether other satellites with the ambipathogenic properties of WLM2-sat RNA and T-sat RNA exist in nature.

Infectious transcripts of Y-sat RNA induce necrosis in tomato plants when supported by the subgroup II strain KIN-CMV (Devic *et al.* 1989) and by the subgroup I strain Y-CMV (Takanami 1981), although recent observations indicate that Y-CMV itself may be necrogenic (Wu *et al.* 1993). It is also unclear whether Y-sat RNA induces tomato necrosis with the subgroup I strain O-CMV; conflicting reports claim that Y-sat RNA did (Masuta and Takanami 1989) and did not (Masuta *et al.* 1988) induce tomato necrosis. Some of these contradictions appear to relate to differences in tomato cultivars and environmental conditions (Wu *et al.* 1993). It has also been shown that Y-sat RNA does not induce necrosis in tomato when supported by the subgroup I strain 1-CMV, and that sequences in the 5' half which are unique to Y-sat RNA,

as well as additional sequences outside the previously delineated necrosis domain, influence the necrogenicity of Y-sat RNA in both a qualitative and a quantitative manner (Wu and Kaper 1992).

Chimeric constructs made between cDNA clones of D4-sat RNA and WLM2-sat RNA were used to map two regions that affected tomato necrosis. Both sequences between the *NheI* site (position 191) and the *HgaI* site (position 279) and sequences between the *HgaI* site and the 3' end could affect necrosis induction in tomato (Fig. 2). While the latter sequence contains all of the elements for complete, systemic necrosis induction (pWDsat279), the former sequence (positions 191–279) could also impart a partial necrogenic phenotype to the chimera (pWDW*sat191/279). An examination of the sequences between the *HgaI* site (position 279) and the 3' end (Fig. 3) showed that WLM2-sat RNA differs from D4-sat RNA at two positions, one of which (position 340) is also an adenosine (A) in several necrogenic satellite RNAs (Kaper *et al.* 1988; Devic *et al.* 1990). By contrast, the A insert at position 330 for both WLM2-sat and Y-sat RNAs was absent from all other necrogenic satellite RNAs. Thus, incomplete necrosis observed for the RNA derived from pWDW*191/279 in this study and the mutant DY-mt (Wu and Kaper 1992) (Wu and Kaper changed the A at position 286 to G) may reflect an effect of the A insert at position 330, common to both chimeric satellite RNAs. Similarly, the sequence differences between WLM2-sat RNA and the D4-sat and Y-sat RNAs at positions 191 and 279 may indicate which nucleotides in this region also affect the necrogenic phenotype from ameliorative to partial necrosis in the presence of subgroup I helper virus strains. Of the two differences unique to WLM2-

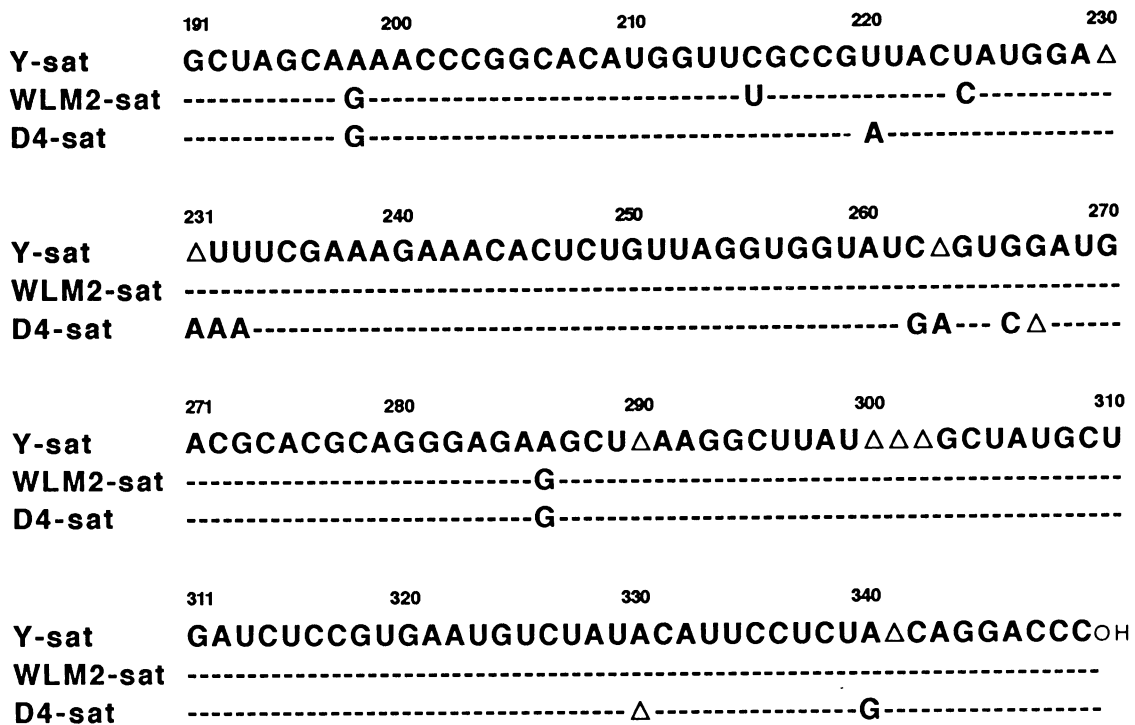


Fig. 3. Nucleotide sequence comparison of the 3' half of the tomato-necrogenic D4-sat RNA with similar regions of two tomato-quasi-necrogenic satellite RNAs (Y-sat and WLM2-sat). Sequences absent from a satellite RNA, with respect to the others, are represented by a triangle, to facilitate comparisons and maintain the published numbering system including other satellite RNAs (Garcia-Arenal *et al.* 1987; Sleat 1990).

sat RNA here (positions 215 and 224), the latter was present in other necrogenic satellite RNAs, while the former was always C in necrogenic satellite RNAs. Thus, if changes at positions 215, 286 (Wu and Kaper 1992), or 330 can affect necrosis induction independently of each other, then it seems more likely that such changes alter a secondary or tertiary structure essential for necrosis induction.

Models based on the secondary structure of such satellite RNA variants *per se* have not been instructive in determining what structures are important for necrosis induction, since necrosis is also dependent on interaction with the helper virus. It is apparent that determining the nature of the interaction with the helper virus as well as interaction with the host is going to be the key to determining the mechanism of tomato necrosis induction.

MATERIALS AND METHODS

Materials.

Restriction endonucleases, RNA polymerases, reverse transcriptases, and T4 DNA ligase were supplied by US Biochemicals. RNasin and RNase-free DNase were from Promega. T7 DNA polymerase and nonradioactive deoxynucleoside triphosphates were from Pharmacia. [α - 32 P]dATP and [α - 32 P]UTP were from Amersham.

Plasmids pWLSat47 (Kurath and Palukaitis 1989), pWLSatM2 (Sleat and Palukaitis 1990a), and pDsat4 (Kurath and Palukaitis 1987) as well as parental and pseudorecombinant CMV strains either have been described previously (Owen and Palukaitis 1988; Sleat and Palukaitis 1990b) or will be described elsewhere (L. Zhang and P. Palukaitis, in preparation). Additional chimeras between these cDNA clones were constructed at the common *NheI* and *HgaI* sites (positions 191 and 279, respectively) by means of standard procedures (Maniatis *et al.* 1982) to produce plasmids pDW*sat191, pWDSat279, and pWDW*sat191/279, where W* is an abbreviation for sequences from pWLSatM2 rather than the parental pWLSat47.

Synthesis of satellite RNA transcripts.

Transcripts of cloned satellite RNAs were synthesized from 5 μ g of *SmaI*-linearized template plasmid in a 100- μ l reaction volume with T7 RNA polymerase, as described by Kurath and Palukaitis (1987). Reactions were incubated at 37° C for 1 hr. Template DNA was removed by the addition of 5 U of RNase-free DNase and further incubation at 37° C for 10 min. Reactions were extracted with phenol/chloroform and then with chloroform. The nucleic acids were precipitated with an equal volume of isopropanol and recovered by centrifugation. Transcripts were dissolved in sterile water and quantified by gel electrophoresis.

Infection of tobacco and tomato plants with satellite RNA transcripts.

Satellite RNA transcripts were initially passaged through tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) before bioassay on tomato plants (*Lycopersicon esculentum* cv. Rutgers), because of the poor infectivity of the transcripts on the latter host (Kurath and Palukaitis 1987). Tobacco plants at the four- to six-leaf stage were placed in the dark for 24 hr before inoculation with 50 mM Na₂HPO₄, pH 9.2, containing either

LS- or Fny-CMV RNA (200 μ g/ml) and satellite RNA transcripts (25 μ g/ml). Both tobacco and tomato plants were dusted with Carborundum immediately prior to inoculation. Samples from inoculated plants were tested 1–2 weeks later by hybridization with satellite RNA-specific 32 P-labeled cDNA (Kurath and Palukaitis 1987). Plants containing satellite RNA were harvested, and the virus was purified (Palukaitis *et al.* 1992). RNA was isolated from the virions by extraction with phenol/chloroform (Palukaitis and Zaitlin 1984) and recovered by ethanol precipitation and centrifugation. Satellite RNA was separated from the helper viral RNAs by electrophoresis in and elution from a 6% polyacrylamide gel (Palukaitis and Zaitlin 1984). Fully expanded cotyledons of tomato seedlings were inoculated with satellite-free viral RNA (250 μ g/ml) and gel-purified satellite RNA (5–10 μ g/ml) in 50 mM Na₂HPO₄, pH 9.2. The plants were kept in the dark for 48 hr prior to inoculation. The replication of satellite RNA in tomato plants was also confirmed by dot blot hybridization (Kurath and Palukaitis 1987). The plants were maintained at either 21 or 24° C in controlled environment chambers with a 16-hr photoperiod.

For the various strains and pseudorecombinants, each combination of virus and mutant or chimeric satellite RNA was tested on tomato plants in at least two and up to six separate bioassays. In some cases, the values in the tables represent the accumulated data from several experiments.

Characterization of progeny satellite RNAs.

The identities of progeny satellite RNAs were confirmed by either of two methods. First, total viral RNAs containing chimeric satellite RNAs from tomato plants were hybridized to 32 P-labeled antisense transcripts and subjected to a ribonuclease protection assay (Winter *et al.* 1985; Kurath and Palukaitis 1989). Second, some satellite RNAs were gel-purified from the viral RNAs isolated from tobacco or tomato and sequenced directly by the use of avian myeloblastosis virus reverse transcriptase and chain-terminating dideoxynucleotides (Zimmern and Kaesberg 1978). Primers complementary to the seven 3'-terminal conserved nucleotides of satellite RNAs and to nucleotides 272–284 were used for sequencing.

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