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Satellite RNAs of Cucumber Mosaic Virus: Recombinants Constructed *in Vitro* Reveal Independent Functional Domains for Chlorosis and Necrosis in Tomato

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The biological activity of RNA transcribed from cDNA clones of three cucumber mosaic virus satellite RNAs was assessed in plants and found to correlate with the phenotype of the satellite RNA from which each clone was derived. Thus, transcript RNA of the D-sat, B-sat, and WL_1-sat clones all replicated well in tobacco, and induced necrosis, chlorosis, and amelioration, respectively, in tomato. To localize the satellite RNA domains responsible for symptom induction in tomato, we constructed six recombinant satellite RNA genomes *in vitro* from the infectious cDNA clones. Each recombinant contained a 185 nucleotide 5′ region and a 150 nucleotide 3′ region from different parental satellite RNA clones. Transcript RNA from each recombinant clone was infectious in tobacco, and replication in tomato induced symptoms that clearly demonstrated that the domain for chlorosis is in the 5′ 185 nucleotides, and the domain for necrosis is in the 3′ 150 nucleotides of the satellite RNA sequence. Analyses of amelioration in tomato indicated that this domain is more complex and will require further investigation. Characterization of the progeny satellite RNAs by RNA protection assays indicated that the recombinant genomes did not undergo major sequence alterations in the host species examined.

Additional keywords: chimeric satellite RNAs, pathogenicity domains.

The satellite RNAs of cucumber mosaic virus (CMV) are small RNA species found in association with many strains of CMV in nature (Francki 1985). Satellite RNAs are not part of the viral genome and have essentially no sequence homology with CMV, but they are completely dependent on the presence of CMV for their replication and encapsidation (Kaper *et al.* 1976; Mossop and Francki 1978; Takanami 1981; Gonsalves *et al.* 1982). The three CMV satellite RNAs used in this study are all approximately 335 nucleotides (nt) long and share greater than 85% sequence homology (García-Arenal *et al.* 1987), but they express different biological properties in certain host plant species. D-sat (previously called [D]CARNA 5 or n-CARNA 5), B-sat, and WL_1-sat RNAs have been reported to induce necrosis (Kaper and Waterworth 1977), chlorosis, and amelioration (García-Arenal *et al.* 1987), respectively, on tomato in the presence of some helper strains of CMV.

To investigate the molecular basis for this difference in pathogenicity, we have generated cDNA clones of these satellite RNAs (Kurath and Palukaitis 1987, unpublished). The biological activity of RNA transcribed *in vitro* from the D-sat clone has already been described (Kurath and Palukaitis 1987). This paper describes the biological activity of transcripts of the B-sat and WL_1-sat clones and the construction of six recombinant satellite RNA sequences from these three parental clones. The biological activity of transcripts of the recombinant clones localized separate domains responsible for chlorosis and necrosis in tomato to the 5′ and 3′ regions of the satellite RNA molecule, respectively. The results regarding amelioration in tomato indicated that this domain may be more complex and will require further investigation.

MATERIALS AND METHODS

Parental and recombinant CMV satellite RNA clones. The construction of the three parental cDNA clones used in this study has been described elsewhere, along with the nucleotide sequence of each clone (Kurath and Palukaitis 1987, manuscript submitted elsewhere). The prototype D-sat clone, pDsat4, has the D-sat sequence inserted as a *Bam*HI-*Sma*I fragment in the transcription vector pIBI76. The B-sat and WL_1-sat prototype clones, pBsat5 and pWLsat47, respectively, contain satellite sequences inserted as *Eco*RI-*Sma*I fragments in the transcription vector pBS-. Each parental clone has a *Nhe*I site at position 185 of the 335-nt satellite sequence.

The six recombinant clones were constructed by using the common *Nhe*I site (Table 1), and the identity of each clone was confirmed by restriction mapping and RNA protection assays with probes for the parental clone sequences used to make each recombinant (see below). Each recombinant clone thus consisted of a 185-nt 5′ region and a 150-nt 3′ region from different parental satellite cDNA clones. All recombinant satellite sequences were made in the pBS- vector in an orientation identical to the parental pBsat5 and pWLsat47 clones.

Assessment of the biological activity of the clones. For all parental and recombinant clones, transcription from the T7 RNA polymerase promoter, using *Sma*I-linearized template DNA, produced a plus-sense satellite RNA transcript with a natural 3′ end and a vector-derived leader sequence of 57 nt (for pDsat4) or 10 nt (for pBsat5, pWLsat47, and recombinants) at the 5′ end. Transcript RNA was synthesized and was inoculated onto plants in the presence of helper virus RNA as described (Kurath and Palukaitis 1987). Each biological experiment included control plants, which were inoculated either with the helper virus alone or

with buffer alone. The helper virus CMVs used in this work were satellite-free preparations of either the L_2 or the WL strain of CMV as noted in the text. The host plant species tested were tobacco (*Nicotiana tabacum* 'Xanthi nc') and tomato (*Lycopersicon esculentum* 'Rutgers').

Tests of different clones were carried out in separate, enclosed chambers as described (Kurath and Palukaitis 1987). Approximately 2 wk after inoculation, individual plants were assayed for satellite RNA replication by dot blot hybridization with a satellite RNA-specific cDNA probe as described (Kurath and Palukaitis 1987). Symptoms on tomato were recorded at 2–4 wk postinoculation. All biological experiments were carried out a minimum of two independent times, and each involved 12–25 plants.

Characterization of progeny satellite RNAs. Virus was purified from plant tissue essentially as described by Lot *et al.* (1972), and RNA was extracted from purified virions as described (Palukaitis and Zaitlin 1984). Satellite RNA was visualized on denaturing 6% acrylamide gels containing 7 M urea (Sanger and Coulson 1978), and semidenaturing 7.5% acrylamide gels containing 8 M urea (Kaper *et al.* 1981).

The relationship of the progeny satellite RNA to the T7 transcript inoculum was assessed by RNA protection assays carried out by using a modification of the procedure of Winter *et al.* (1985) to be described elsewhere. Probes for these assays were ^{32}P-labeled, full-length minus-sense transcripts of the parental and recombinant satellite clones. Probes were synthesized as described (Kurath and Palukaitis 1987), using as template DNA *Bam*HI-cleaved pDsat4 and *Eco*RI-cleaved pBsat5, pWLsat47, pBWsat185, pWBsat185, pBDsat185, or pWDsat185 for probes designated D, B, W, BW, WB, BD, and WD, respectively. Due to the loss of the *Eco*RI site during construction of the pDBsat185 and pDWsat185 clones, DB and DW probes were synthesized from *Pvu*II-cleaved DNA templates. For each RNA protection assay, probes were hybridized with either 50 ng of RNA transcript or 500 ng of total progeny viral RNA, and the RNA species protected from RNase digestion were analyzed by electrophoresis on denaturing 6% acrylamide gels containing 7 M urea (Sanger and Coulson 1978) and autoradiography.

RESULTS

Phenotypes of parental D-sat, B-sat, and WL_1-sat clones. The construction and nucleotide sequences of D-sat, B-sat, and WL_1-sat cDNA clones have been described elsewhere (Kurath and Palukaitis 1987, manuscript submitted elsewhere). RNA transcribed *in vitro* from the prototype D-sat clone, pDsat4, has been shown to replicate in tobacco, and to induce necrosis in tomato, in the presence of satellite-free RNA of the Fny-CMV helper strain (Kurath and Palukaitis 1987). Thus, the initial stage of this study was to assess the biological activity of RNA transcribed from the prototype B-sat clone, pBsat5, and the prototype WL_1-sat clone, pWLsat47.

Table 1. Symptoms induced in tomato by infectious RNAs transcribed from CMV satellite RNA clones

Construct	Clone	Modification of L_2-CMV symptoms [a]	Modification of WL-CMV symptoms [b]
5' □ 3'	pDsat4	necrosis	necrosis
5' ▨ 3'	pBsat5	chlorosis, mild amelioration	mild amelioration
5' ■ 3'	pWLsat47	mild amelioration	amelioration
5' ▨■ 3'	pBWsat185	chlorosis	amelioration
5' ■▨ 3'	pWBsat185	none	amelioration
5' ■□ 3'	pWDsat185	necrosis	necrosis
5' □■ 3'	pDWsat185	none	amelioration
5' ▨□ 3'	pBDsat185	chlorosis, necrosis	amelioration[c], necrosis
5' □▨ 3'	pDBsat185	none	amelioration

[a] L_2-CMV induces stunting and moderate fern-leaf on tomato in the absence of satellite RNA (Palukaitis 1988).

[b] WL-CMV induces stunting and severe fern-leaf on tomato in the absence of satellite RNA (Palukaitis 1988).

[c] Amelioration here was of the fern-leaf symptoms only, before onset of necrosis.

The pBsat5 clone has nucleotide sequence features of both the B_1-sat and B_3-sat RNAs (data not shown) that are closely related and were originally isolated from a B-sat RNA mixture (García-Arenal *et al.* 1987). Thus, it was necessary to determine whether the phenotype of the pBsat5 clone was like B_1-sat RNA or B_3-sat RNA. The biological distinction between these satellite RNAs involves the use of different helper strains of CMV (Palukaitis 1988). B_1-sat RNA induces chlorosis with either L_2-CMV or WL-CMV. B_3-sat RNA induces chlorosis with L_2-CMV, but not with WL-CMV. Despite the chlorosis, both satellites partially ameliorate the fern-leaf symptoms caused by the L_2-CMV and WL-CMV viruses in tomato.

Biological tests of the pBsat5 clone were carried out by using *in vitro* synthesized RNA transcripts that contained the satellite RNA sequence with a natural 3′ end and 10 nt of nonsatellite vector sequence at the 5′ end. This transcript RNA was initially inoculated onto tobacco plants in the presence of satellite-free L_2-CMV and WL-CMV RNAs. In both cases satellite RNA replication was detected in almost all the plants by dot blot hybridization analyses done 2 wk after inoculation (results not shown). Virus purified from these tobacco plants was used to inoculate tomatoes, due to the low efficiency of infection in tomato with naked RNA inocula (Kurath and Palukaitis 1987). Dot blot hybridization analyses of the tomatoes 2 wk after inoculation indicated that satellite RNA was replicating in the majority of the plants (results not shown). All the tomatoes that replicated satellite RNA after inoculation with L_2-CMV containing the pBsat5 transcript progeny also developed yellow, veinal chlorosis, whereas those replicating WL-CMV containing the pBsat5 transcript progeny remained green (Table 1). Both showed slight amelioration of the fern-leaf distortion caused by the helper virus. No satellite RNA replication or chlorosis was detected in control plants inoculated with the helper virus strains in the absence of satellite RNA. Thus, RNA transcripts of the pBsat5 clone are infectious in tobacco and tomato and have a phenotype in tomato identical to that of the B_3-sat RNA.

RNA transcripts of the pWLsat47 clone were tested in the same way and were found to replicate well in tobacco with either the L_2-CMV or the WL-CMV helper viruses. Replication of the progeny of the pWLsat47 transcript RNA in tomato resulted in partial amelioration of the fern-leaf symptoms caused by both L_2-CMV and WL-CMV (Table 1). The amelioration was more clear with WL-CMV, due to the greater severity of the fern-leaf distortion induced by that strain when no satellite RNA is present (Palukaitis 1988).

Because the helper strain used previously to test the pDsat4 clone was Fny-CMV (Kurath and Palukaitis 1987), RNA transcripts of pDsat4 were retested by using the L_2-CMV and WL-CMV helper viruses. In both cases the transcript RNA replicated well in tobacco and induced necrosis in tomato (Table 1).

Construction of recombinant satellite RNA genomes. To begin to characterize and localize the satellite RNA sequence domains responsible for the different symptoms induced in tomato, we constructed recombinant satellite RNA genomes *in vitro* from the infectious pDsat4, pBsat5, and pWLsat47 clones. The common *Nhe*I site at nucleotide 185 of each parental clone was used to construct six recombinant satellite sequences comprised of 5′ and 3′ regions from different satellite RNAs. This strategy and the final structure of each recombinant are presented in Table 1. The nomenclature of the recombinants designates, in order, the source of the 5′ region, the source of the 3′ region, and the site of recombination. Thus, pBWsat185 is a plasmid with a recombinant satellite RNA sequence in which the 5′ region of the pBsat5 clone and the 3′ region of the pWLsat47 clone have been recombined at nucleotide 185 of the 335-nt satellite RNA sequence.

Replication of recombinant satellite RNAs in tobacco. T7 RNA transcripts of each recombinant were initially inoculated onto tobacco to assess their viability. All six recombinants replicated to similar levels with both L_2-CMV and WL-CMV, as indicated by dot blot hybridization analyses done 2 wk after inoculation (results not shown). One recombinant, pWDsat185, replicated in a relatively small proportion of the plants (2/15 and 2/16 in separate experiments), whereas the other five recombinants replicated in almost all of the plants inoculated. Virus was purified from the tobacco plants for characterization of the satellite RNA progeny and inoculation of tomatoes. None of the recombinants induced any visible change of the mild mosaic symptoms caused by the CMV helper strains in tobacco, and the proportions of the recombinant satellite RNAs in the virion preparations were similar to those of the parental satellite RNAs.

Biological activity of recombinant satellite RNAs in tomato. Tomatoes were inoculated with purified virus containing each of the six recombinant satellite RNAs with either the L_2-CMV or the WL-CMV helper virus strains. Dot blot analysis at 2 wk after inoculation indicated that almost all of the plants were replicating satellite RNA, including those with the pWDsat185 recombinant, which had replicated in only a small number of tobacco plants; however, the latter recombinant was not passaged sequentially through tobacco to reassess its specific infectivity. Symptoms were recorded for up to 4 wk after inoculation and are summarized in Table 1. In all cases, the symptoms observed in each plant correlated with satellite RNA replication, as determined by dot blot hybridizations of extracts of individual plants (results not shown). Once again, the proportions of the satellite RNAs in the virion preparations were similar for all of the recombinants.

With L_2-CMV, two of the recombinants induced chlorosis and two induced necrosis. The pBWsat185 and pBDsat185 (Fig. 1A) recombinants caused veinal chlorosis typical of the parental pBsat5 clone. For pBDsat185, this chlorosis appeared concurrently with severe stunting at approximately 8–10 days and was very bright until necrosis began at about 20 days after inoculation. The plants in which the pWDsat185 recombinant replicated were severely stunted and developed necrosis also at about 20 days after inoculation (Fig. 1B). The dramatic difference in symptoms induced by the pWDsat185 and the reciprocal pDWsat185 recombinant satellite RNAs is shown in Figure 1B.

The amelioration phenotype was difficult to assess conclusively with L_2-CMV because the fern-leaf symptoms induced by this virus strain alone are relatively mild. The more severe fern-leaf induced by WL-CMV facilitated definite determinations of amelioration, and results are again summarized in Table 1. The four recombinant satellite RNAs that did not induce necrosis all induced visible amelioration of the viral symptoms. In addition, the pBDsat185 recombinant also ameliorated the fern-leaf

symptoms of the helper virus, but the plants were still very stunted, and necrosis began at about 20 days after inoculation as with L_2-CMV. As expected, none of the recombinants induced chlorosis with WL-CMV. Virus was purified from tomato plants for analyses of the progeny satellite RNAs.

Characterization of the recombinant progeny satellite RNAs. Preliminary examination of the satellite RNA progeny from each experiment was done by denaturing and semidenaturing gel electrophoresis of RNA extracted from purified virus (results not shown). For more detailed characterization, RNA protection assays were used to determine whether the satellite RNAs replicating in the experiments described had recombinant sequences characteristic of the transcript RNA used as inocula. In these assays, probes that were ^{32}P-labeled minus-sense transcripts of the satellite cDNA clones were annealed either with plus-sense transcript RNA or with progeny RNA extracted from purified virions. The RNA duplexes were then digested with RNases under conditions in which sequence mismatches will be recognized and cleaved (Winter *et al.* 1985). The sizes of the protected ^{32}P-labeled RNA species thus provides information about the sequence relationship between the RNA tested and the clone used to make the probe. Although the RNase treatment in these assays does not cleave all mismatches to completion, this method has been used to detect single-base mismatches (Winter *et al.* 1985), and it will certainly detect any major sequence rearrangements or contamination with intact parental satellite RNAs (unpublished results).

For each of the six recombinant satellite RNAs, probes complementary to the two parental RNAs and the recombinant RNA were used in assays with the recombinant transcript RNA and with viral RNAs from tobacco and tomato in which the recombinant had presumably replicated. The results of two of these assays for the reciprocal pBWsat185 and pWBsat185 recombinants are shown in Figure 2, and assays of the other four recombinants showed essentially the same results. In all cases, the patterns of the probe fragments protected by the transcript RNA were the same as the patterns protected by satellite RNA from both tobacco and tomato. This indicates that the satellite RNAs replicating in the experiments described had the expected recombinant genomes. There was no evidence for the presence of any full-length parental satellite RNAs, which would have protected a complete 335-nt length of the parental clone probes used in the assays. However, the presence of a band of 220 nt in lane B of Figure 2B indicates that not all mismatches between the B5-sat probe and the WB-sat RNA were cleaved. Thus, we cannot conclude that minor sequence changes did not occur during passage of the recombinants in these hosts.

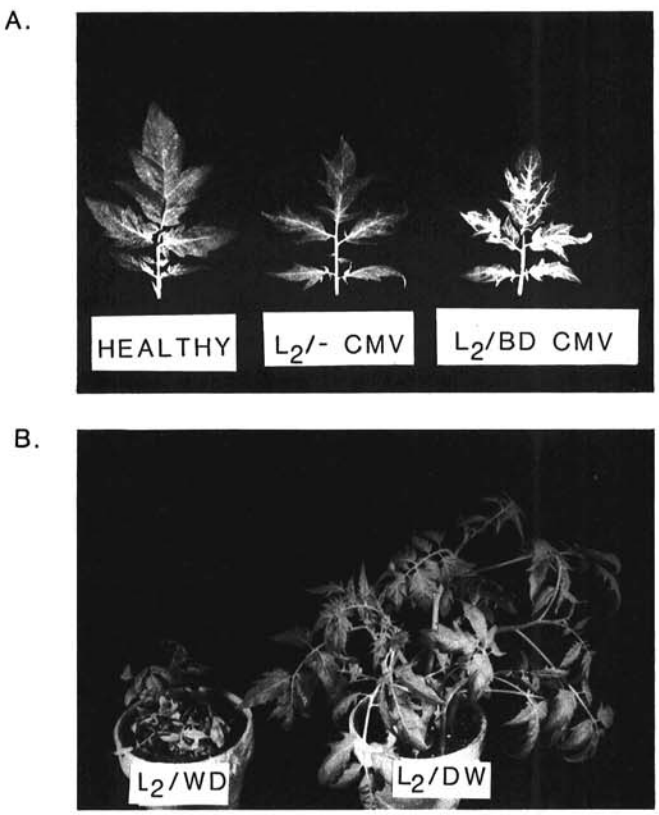

Fig. 1. Symptoms induced in tomato by replication of recombinant satellite RNAs. **A**, Bright yellow chlorosis induced by the pBDsat185 recombinant (right). Also shown are fern-leaf symptoms induced by L_2-CMV alone (center), and a mock-inoculated tomato leaf (left). **B**, Stunting and early necrosis induced by replication of the pWDsat185 recombinant (left), in comparison with the lack of symptom modification by the reciprocal pDWsat185 recombinant (right) in tomato.

DISCUSSION

The strategy of constructing recombinant genomes *in vitro* has been used to investigate many viral and subviral functional domains, including the pathogenicity-modulating domain of citrus exocortis viroid (Visvader and Symons 1986). This strategy requires the availability of infectious clones of closely related variants with different biological properties. Therefore, to investigate the symptom-inducing domains of CMV satellite RNAs, we have used cDNA clones of D-sat, B-sat, and WL_1-sat RNAs. RNA transcripts of these cloned satellite sequences are infectious and induce necrosis, chlorosis, and amelioration, respectively, in tomato.

Six recombinant sequences were constructed from these three parental satellite cDNA clones. Each recombinant genome was viable in tobacco and tomato, and the symptoms they induced in tomato (Table 1) permitted the delimitation of two pathogenic domains. The two recombinants that induced necrosis in tomato (pWDsat185 and pBDsat185) were those that derived their 3′ region from the D-sat, whereas the two that had the 5′ region of the D-sat (pDWsat185 and pDBsat185) showed no symptom exacerbation. Thus, we can conclude that the sequence feature(s) responsible for necrosis induction in D-sat RNA is located within the 3′ region of the molecule. The physical limits of the domain involved may extend beyond the recombination site to include some common sequences 5′ of nt 185, but the necrosis-determining sequence(s) must lie in the 3′ 150 nt. From recent studies by Baulcombe *et al.* (1988) and Jacquemond and Lauquin (1988), the necrosis-inducing domain appears to involve sequences between nucleotides 266 and 311; WLsat47 differs from pDsat4 in only three positions in this region.

The two recombinants that induced chlorosis (pBWsat185 and pBDsat185) derived their 5′ region from the B-sat clone, whereas the two that had the 3′ region of the B-sat clone

(pWBsat185 and pDBsat185) showed no chlorosis. Therefore, the sequence feature(s) responsible for chlorosis is in the 5′ 185 nt of the B-sat RNA. This is consistent with the previous suggestion, from sequence comparisons of several satellite RNAs, that the chlorosis-inducing domain is between residues 85 and 195 (Palukaitis 1988). pWLsat47 differs from pBsat5 in six positions between residues 85 and 185, and pDsat4 differs from pBsat5 in 12 positions in this region.

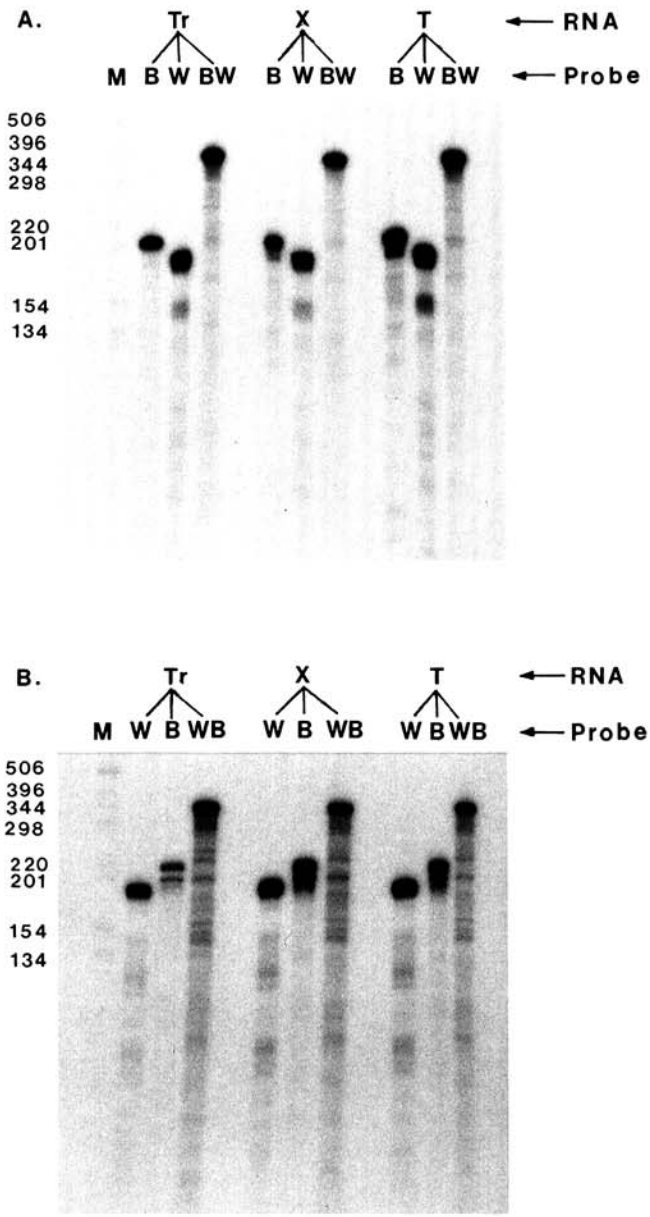

Fig. 2. Characterization of recombinant transcript RNA and progeny satellite RNAs by RNA protection assays. Full-length, ^{32}P-labeled, minus-sense transcripts of parental and recombinant clones were used as probes to anneal with recombinant transcripts and progeny RNA. These duplexes were digested with RNases under conditions in which most sequence mismatches will be recognized and cleaved (Winter *et al.* 1985). The cleavage products were separated by electrophoresis on denaturing 6% polyacrylamide gels and visualized by autoradiography. Panel **A** shows assays of pBWsat185-related RNAs, and panel **B** shows assays of pWBsat185-related RNAs. Lanes marked M contain ^{32}P-labeled DNA fragments with sizes (in nucleotides) indicated on the left. The probe and test RNA in each reaction are indicated above each lane; Tr is plus-sense transcript RNA of either pBWsat185 (**A**) or pWBsat185 (**B**), X is progeny RNA from the replication of each recombinant in tobacco, and T is the progeny RNA from the replication of each recombinant in tomato. Due to sequence conservation between the parental satellite RNAs surrounding position 185, the sum of the fragments produced is greater than the whole satellite RNA (335 nucleotides).

The interpretation of experiments designed to localize the domain responsible for the amelioration of the virus-induced fern-leaf distortion proved to be more complex. In the presence of WL-CMV, amelioration of the fern-leaf symptom was evident for five of the six recombinants. These results precluded the simple interpretations that allowed assignment of the chlorotic and necrotic domains to one of the two regions of the molecule exchanged to make the recombinants. Although amelioration was considered to be mainly a trait of the WL_1-sat RNA, the induction of amelioration by the pBDsat185 and pDBsat185 recombinants showed that this characteristic is not unique to the WL_1-sat RNA sequence. The amelioration associated with these recombinants may be contributed by the B-sat RNA sequences, because mild amelioration was noted for the parental B-sat clone. However, if this is the case, then both the 3′ and 5′ regions of the B-sat RNA sequence would have to be independently capable of inducing amelioration. It is also possible that there is an amelioration domain in the 5′ region of the D-sat RNA sequence that is not evident due to the presence and expression of the dominant necrosis-inducing domain. These hypotheses suggest that the sequence features responsible for amelioration could be spread over several areas of the genome, as was found for the attenuation phenotype of poliovirus type 1 (Omata *et al.* 1986). Alternatively, the domain could be split by the recombination site into two portions that are both functional. In any case, characterization of the amelioration domain is complex and will require further investigation.

The construction of genomes with regions of sequence that are not found together in natural satellite RNAs allows some observation of the interaction between domains on the same molecule. In the pBDsat185 and pWDsat185 recombinants, the domain for necrosis clearly was expressed, but there was a qualitative difference in the expression relative to the parental Dsat4 clone. The onset of necrosis was delayed in pBDsat185-infected plants (3–6 wk) relative to pDsat4-infected plants (2–4 wk). Necrosis in tomatoes infected with pWDsat185 was also delayed relative to the pDsat4-induced necrosis, with some plants taking up to 6 wk for complete necrosis. The delay could be due to the presence of an amelioration domain in the 5′ region of the recombinants, which influences the expression of the necrosis-inducing domain in the 3′ region.

The six recombinant satellite RNAs described here have shown that the domains responsible for inducing chlorosis and necrosis in CMV satellite RNAs are physically separate. This is in contrast to the single pathogenesis-modulating domain with different sequence variations found in potato spindle tuber viroid (Schnölzer *et al.* 1985). These CMV satellite RNA domains are apparently "expressed" by independent mechanisms that may modify each other but do not prevent the ultimate development of each symptom. Future studies with other recombinants and site-directed mutagenesis should allow us to define more precisely the sequence elements that comprise each domain and ultimately to discern the mechanisms by which these domains have their effects.

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