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Determination of Sequence and Structural Requirements for Pathogenicity of a Cucumber Mosaic Virus Satellite RNA (Y-satRNA)

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We describe the use of biologically active cDNA clones to investigate genetic determinants of a satellite RNA that modulates symptoms normally induced by its helper virus, cucumber mosaic virus (CMV). For this purpose, we have investigated a CMV satellite RNA (Y-satRNA) that induces bright yellow symptoms on tobacco and necrosis on tomato. To determine the pathogenicity-modulating domain of Y-satRNA, several insertion and deletion mutants were created by using various restriction sites in the cDNA of Y-satRNA, and RNA transcripts derived from the clones were mixed with CMV and used to inoculate plants. Although the satellite RNA was able to tolerate small insertions (as much as 4 bases at present), small deletions were deleterious, indicating that the sequence requirements for viability of the satellite RNA are relatively inflexible. Biological activity assays of chimeric satellite RNAs between Y-satRNA and a non-necrogenic satellite RNA, T73-satRNA, suggested that only two (or at least one of two) specific bases (positions 318 and 325) in the 3′ region direct the necrogenic property of Y-satRNA. Sequences involved in production of yellow symptoms were investigated by constructing chimeras between Y-sat cDNA and cDNA of a satellite RNA designated S19-satRNA. S19-satRNA has considerable homology to Y-satRNA but does not elicit yellow symptoms on tobacco. Chimeric clones were constructed by using a BstXI site that cuts within a stable secondary structure in the region between positions 100 and 200 (region Y). The results of infectivity tests with RNA transcripts suggest that formation of a secondary structure in region Y may be involved in induction of yellow symptoms as well as viability of Y-satRNA.

INTRODUCTION

Satellite RNAs depend on helper viruses for replication and encapsidation, but often share no significant sequence homology with the respective helper viruses (Murant and Mayo, 1982). In most cases, satellite RNAs suppress symptoms induced by their helper viruses, although some intensify the symptoms. Cucumber mosaic virus (CMV) supports satellite RNAs consisting of single-stranded, linear RNA molecules of 330 to 390 nucleotides (Hidaka et al., 1984; Garcia-Arenal, Zaitlin, and Palukaitis, 1987; Hidaka et al., 1988; Kaper, Tousignant, and Steen, 1988). The satellite RNAs often attenuate the symptoms induced by CMV in certain host plants, but in some cases quite dramatic increases in symptom severity may occur. For example, some CMV satellite RNAs are known to cause necrosis on tomato plants infected with CMV (Kaper and Waterworth, 1977; Waterworth, Kaper, and Tousignant, 1979), and a satellite RNA (Y-satRNA) maintained in our laboratory induces a bright yellow mosaic on one host (tobacco) as well as necrosis on another (tomato) (Takanami, 1981; Masuta, Kuwata, and Takanami, 1988a).

To understand how satellite RNAs alter the symptoms caused by CMV, one strategy for identifying the regions responsible for the particular symptom responses has been to compare nucleotide sequences of naturally occurring satellite RNAs with different pathogenicity (Garcia-Arenal et al., 1987; Kurath and Palukaitis, 1987; Kaper et al., 1988). An alternative is to construct mutant satellite RNAs for analyses of sequence requirements for biological activity or possible involvement of potential protein products in pathogenicity (Collmer and Kaper, 1988). In this report, we have used both strategies to investigate the relationship between the nucleotide sequence and pathogenicity of Y-satRNA. The construction of chimeric satellite RNAs permitted us to determine specific bases (domains) governing yellow symptom response on tobacco and necrosis on tomato, and to propose a model whereby the dramatic yellow induction could be mediated.

RESULTS

Biological Activity of Y-satRNA Mutants

To determine the sequence domains and structural features governing the viability of Y-satRNA, site-specific

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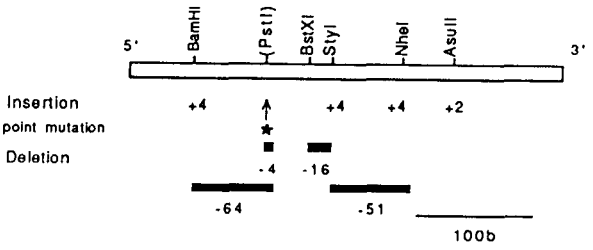

Figure 1. Structures of Y-satRNA Mutants.

The restriction enzymes used for the construction of several Y-satRNA mutants are shown. A unique PstI site (*) was introduced by site-directed mutagenesis. Plus numbers with the arrows and minus numbers with the filled boxes indicate inserted and deleted bases, respectively.

mutations were introduced into cDNA clones containing inserts corresponding to the Y-satRNA sequence, as illustrated in Figure 1. The results of the biological activity assays of RNA transcripts derived from these mutated clones are summarized in Table 1. All three of the four base insertion mutants retained their biological activity and produced yellow mosaic symptoms. A 2-base insertion mutant at the AsuII site, which is in the 3′ region of the molecule, was not infectious, but a point mutation to introduce a PstI site at position 112 (G to C) did not affect the biological activity of Y-satRNA. In contrast to the viable insertion mutants, all the deletion mutants lost infectivity. For those mutants that replicated, progeny satellite RNAs were extracted from infected plants 2 weeks after inoculation, and cDNA clones were constructed by using the same synthetic primers used for synthesis of a double-stranded cDNA of Y-satRNA. Sequence analyses of three clones representing each mutant suggested that the progeny satellite RNAs maintained the introduced mutations without any sequence modifications. This evidence suggests that most of the progeny molecules were faithfully replicated during the infection cycle and that the observed phenotypes result from RNA used as a source of inoculum, rather than from revertant molecules generated during the infection process.

Biological Activity of Chimeric Satellite RNAs

Y-satRNA induces bright yellow symptoms on tobacco and necrosis on tomato. To investigate the relative effects of different sequence domains on the Y-satRNA pathogenicity, several chimeric satellites were constructed with cDNA clones derived from Y-satRNA and T73-satRNA or S19-satRNA, as shown in Figure 2. Y-satRNA, T73-satRNA, and S19-satRNA have 369, 334, and 370 nucleotides, respectively (Masuta, Kuwata, and Takanami, 1988b, and Figure 3). The sequence of Y-satRNA determined by Hidaka et al. (1984) was revised with three possible differences (residues 161, 167, and 173) and a nucleotide insertion at residue 234 (Masuta et al., 1988b). Thus, the positions for the residues given in this paper represent our revised sequence of Y-satRNA. Neither T73-satRNA nor S19-satRNA causes yellow symptoms on tobacco or necrosis on tomato, but both satellites attenuate CMV symptom severity on tobacco and tomato. The results of biological activity of RNA transcripts from the chimeric satellite clones are summarized in Table 2. The recombinant Y_B73_AY containing the 3′ region (about 100 bases from the 3′ end) from Y-satRNA induced lethal necrosis on tomato, whereas Y_B73 containing the 3′ region from T73-satRNA did not cause necrosis on tomato. The progeny satellite RNAs from these chimeric RNAs also retained the original chimeric sequences based on the nucleotide sequence of cDNA clones derived from progeny RNAs isolated 2 weeks after inoculation. From the limited sequence differences in the 3′ region (only 4 bases), we can specify the bases responsible for the necrogenic property of the satellite RNAs including Y-satRNA. Although the necrogenic Y_B73_AY contains C, A, G, and C residues at positions 270, 311, 318, and 325, respectively, the non-necrogenic Y_B73 contains A, G, A, and U at those positions. However, substitutions at positions 270 and 311 probably are incidental because nucleotides at these two positions vary between necrogenic and non-necrogenic satellite RNAs (Kaper et al., 1988). Although the context of nucleotides could be important, in this experiment we have focused on substitutions at residues 318 and 325 to correlate necrosis on tomato with Y-sat mutants. To dem-

Table 1. Biological Activity of Y-satRNA Mutants

Type of Mutation	Location of Mutation	Number of Affected Nucleotides	Biological Activity[a]
Point mutation	112	–	10/10
Insertions			
BamHI	50	4	12/12
StyI	169	4	10/10
NheI	223	4	10/10
AsuII	262	2	0/16
Deletions			
BamHI-(PstI)	50–113	64	0/12
(PstI)	110–113	4	0/12
BstXI-StyI	150–165	16	0/12
StyI-NheI	169–219	31	0/12

Each test plant was inoculated with 1 μg/mL transcript and 10 μg/mL CMV in 50 μL of 50 mM sodium phosphate buffer, pH 7.0.
[a] Number of plants showing yellow symptoms/number of inoculated tobacco plants. Satellite RNAs were readily detected by gel electrophoresis and dot-blot hybridization of all plants showing yellow symptoms 3 weeks after inoculation, but could not be detected in plants showing the typical green mosaic induced by cucumber mosaic virus alone.

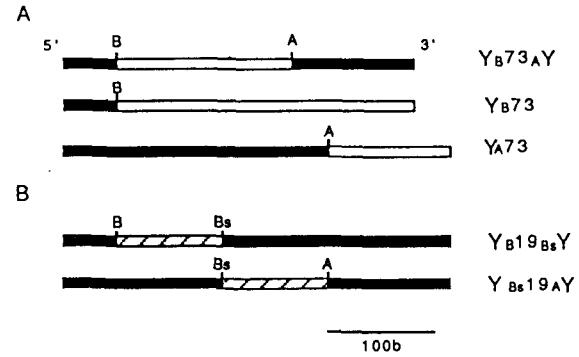

Figure 2. Schematic Diagram of Chimeric Satellite RNAs.

(A) Chimeric satellite RNAs between Y-satRNA and T73-satRNA. Y-sat and T73-sat sequences are represented as filled and open boxes, respectively.

(B) Chimeric satellite RNAs between Y-satRNA and S19-satRNA. The S19-sat sequence is hatched.

The biological activity of the chimeric satellite RNAs is given in Table 2. The cleavage sites used for the construction of chimeric satRNAs are shown on the plasmid structure (see Figure 1). B, BamHI; A, AsuII; Bs, BstXI.

onstrate that the two nucleotides actually condition the necrogenic response, site-specific modifications of Y-satRNA were made to create two mutants, Y-318A and Y-325U, which contain transitions from a G to an A at position 318 and from a C to a U at position 325, respectively. As shown in Table 3 and Figure 4, Y-325U did not cause any necrogenic response when coinoculated with CMV and even attenuated the symptoms induced by CMV, but Y-318A lost its biological activity.

Y-satRNA is unusual because it induces bright yellow symptoms on tobacco. Comparative studies of Y-satRNA and other satellite RNAs revealed that Y-satRNA contains a unique sequence domain between residues 100 and 200, which we designate region Y. This region of Y-satRNA, extending from nucleotide 101 to 220, is compared with similar regions of T73-satRNA, S19-satRNA, and OY2-satRNA, as shown in Figure 5. Relatively short satellite RNAs ranging in size from 330 to 340 nucleotides do not contain a sequence domain corresponding to region Y. However, both the 370-nucleotide S19-satRNA and the 386-nucleotide OY2-satRNA (Hidaka et al., 1988) contain a conserved sequence that can be folded into a secondary structure, as shown in Figures 5 and 6, similar to that proposed by Hidaka et al. (1988) from enzymatic accessibility of Y-satRNA in vitro. The proposed model of Y-satRNA was obtained by computer analysis incorporating major cleavage sites by nucleases into the program. When we deleted 16 internal bases (from position 150 to position 165) in Y-satRNA to disrupt the folded structure, the deletion mutant lost its biological activity (Figure 1 and Table 1). To correlate the structure with biological activity (especially yellow symptom induction) of Y-satRNA, we constructed two chimeric satellite RNAs, $Y_B19_{Bs}Y$ and $Y_{Bs}19_AY$. A convenient BstXI site in region Y permitted us to exchange only the half side of the stem-loop structure between Y-satRNA and S19-satRNA (Figure 6). The $Y_B19_{Bs}Y$ chimera elicited yellow symptoms in all inoculated plants, whereas inoculation with $Y_{Bs}19_AY$ produced attenuated green mosaic symptoms (Table 2), similar to those induced by many other satellite RNAs, except for one plant that showed a trace of yellow. At this stage, we do not know why only faint yellow symptoms appeared in this plant, but several possibilities exist, including variations in the physiology of the plant or some genetic aberration associated with this particular plant. To determine whether the same secondary structure directly influences biological activity, some site-specific changes were created in region Y of Y-satRNA. As shown in Table 3, a G insertion at position 158 and a point mutation at position 161 did not alter biological activity, and the resulting infected plants developed bright yellow symptoms. Again, we found no sequence changes in the progeny after replication of the chimeric satellite RNAs.

DISCUSSION

Y-satRNA induces dramatic bright yellow symptoms on tobacco and necrosis on tomato. To determine the nucleo-

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            10         20         30         40         50         60
Y    GUUUUGUUUGAUGGAGAAUUGCGUAGAGGGGUUAUAUCUGCGUGAGGAUCCAUCACUCGG
S19  ------------------------------------------------------------
T73  ------------------------------------A-------------------

            70         80         90        100        110
Y    CGGUGUGGGAUACCUCCCUGCUAAGGCGGGUUGAG*AG*UGUAUCUCGGACUGGAGGCGGG
S19  ----------------------C-----------*U-AC-------------C---A--
T73  ---------U-------------C-----------UC-AC-C-C----------G-A-C-*

           130        140        150        160        170
Y    AUGUCUGCGGGUGUUCCGUCUGCUGCCCACGAUGGU*GGGAGUCACCCAAGGGGUGACUU
S19  *-U--------C-CG-U-C-G---------------G-----C---UAU-U-U-*-CUA-
T73  C--G---*U-C-A-C-AU---C---**********************----**********

           190        200        210        220        230
Y    UUUCAGCUCUGCAUUUCUCAUUUGAGCCCCCGCUCAGUUUGCUAGCAAAACCCGGCACAU
S19  -------------GC-----------------------------------G----------
T73  **-----A--A-*GCG-------------------------------------------

           250        260        270        280        290
Y    GGUUCGCCGUUACUAUGGA*UUUCGAAAGAAACACUCUGUUAGGUGGUAUCGUGGAUGAC
S19  -------------C-----U-------------------------------------------
T73  -------------C-----*C-----------A-----------------------------

           310        320        330        340        350
Y    GCACGCAGGGAGAAGCUAAGGCUUAUGCUAUGCUGAUCUCCGUGAAUGUCUAUACAUUCC
S19  -------------G------A------U-----------------------C-------
T73  -------------G------A------U---------------------------------

Y    UCUACAGGACCC
S19  ------------
T73  ------------
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Figure 3. Nucleotide Sequences of Y-satRNA, S19-satRNA, and T73-satRNA.

Asterisks indicate a deletion at a given position with respect to other satellite RNAs.

Table 2. Biological Activity of Chimeric Satellite RNAs

Chimeric sat-RNAs	Tobacco		Tomato	
	Infectivity	Symptoms	Infectivity	Symptoms
Y_B73_AY	10/10[a]	Mild mosaic	10/10	Necrotic
Y_B73	10/10	Mild mosaic	10/10	Mild mosaic
Y_A73	5/5	Yellow	5/5	Mild mosaic
$Y_B19_{Bs}Y$	12/12	Yellow	NT[b]	-
$Y_{Bs}19_AY$	12/12	Mild mosaic	NT	-

Inoculation conditions are found in Table 1.
[a] Number of plants containing satellite RNA/number of inoculated plants.
[b] NT, not tested.

tide sequence or specific bases responsible for the pathogenicity of Y-satRNA, we first investigated the stringency of the sequence and the structural requirements necessary for biological activity of Y-satRNA. The results shown in Table 1 suggest that even small deletions can inactivate Y-satRNA, although several insertion mutants containing as many as 4 bases were still infectious. Although we do not know the reason(s) for loss of infectivity of a mutant with 2 additional bases at residue 262 (AsuII site), one possibility could be destruction of the structural features required for replicase recognition or systemic movement in plants. These findings contrast with the observations for the satellite RNA (RNA C) of turnip crinkle virus which, despite its small size, was able to tolerate insertions up to 60 bases and deletions as large as 22 bases, although mutations in the 3′ domain of the satellite RNA C also destroyed biological activity (Simon et al., 1988). Thus, CMV satellite RNAs, at least Y-satRNA, seem to be sensitive to minor modifications induced by in vitro mutagenesis. Observations similar to ours have been reported for viroids whose sequence requirements for viability are very stringently conserved (Meshi et al., 1985; Schnölzer et al., 1985; Visvader and Symons, 1986), although CMV satellite RNAs are somewhat more tolerant to mutation than viroids.

Because it is difficult to use site-specific mutants for analysis of the pathogenicity domains, we exchanged structural domains between two satellite RNAs. To investigate necrosis induction by Y-satRNA, we constructed two chimeric satellite RNAs (Y_B73_AY and Y_B73) between Y-satRNA and non-necrogenic T73-satRNA. The results shown in Table 2 suggest that the nucleotide sequence governing necrosis on tomato is located between the AsuII site and the 3′ end of the Y-satRNA molecule. There are only four nucleotide differences in the region between the two chimeric satellite RNAs, and two of these substitutions (positions 270 and 311) vary between other necrogenic and non-necrogenic strains (Kaper et al., 1988). When site-specific modifications were introduced to yield Y-318A, the resulting RNA transcript had no biological activity. The other mutant, Y-325U, retained biological activity and lost its ability to induce necrosis. In a previous study, Collmer and Kaper (1988) eliminated the possible involvement of a protein encoded by an open reading frame (ORF 1, beginning at an AUG at position 11 to 13) in the necrosis induction on tomato by D-CARNA5. Palukaitis (1988) suggested that the nucleotide sequence domain specifying necrosis on tomato lay outside a sequence domain (residues 70 to 160) that specifies the induction of chlorosis in tomato. In the recent publications by Kurath and Palukaitis (1989) and Jacquemond and Lauquin (1988), the nucleotide sequence(s) for necrosis induction was located within the 3′ region (in the 3′ 150 nucleotides in the former paper). Our observations extend these results because we were able to destroy the necrosis-inducing property of Y-satRNA by making a point mutation. Therefore, we conclude that the base at position 325 is essential for the necrogenic response, but we are unable to explain why the nucleotide change at position 318 destroyed the biological activity of Y-satRNA.

It is of great interest to determine the molecular determinants of the yellow symptom induction by Y-satRNA

Table 3. Site-Specific Modifications in the Bases Responsible for Pathogenicity of Y-satRNA

Transcripts	Mutations (Nucleotide Position)	Biological Activity[a]		Symptoms	
		Tobacco	Tomato	Yellowing on Tobacco	Necrosis on Tomato
Y-318A	G→A (318)	0/5	0/5	-	-
Y-325U	C→U (325)	7/7	7/7	7/7	0/7
Y-158G	G insertion (158)	8/8	NT[b]	8/8	NT
Y-161C	U→C (161)	8/8	NT	8/8	NT
Y-satRNA (control)		5/5	5/5	5/5	5/5

Inoculation conditions are found in Table 1.
[a] Assayed 1 month after inoculation. Number of plants containing the satellite/number of inoculated plants.
[b] NT, not tested.

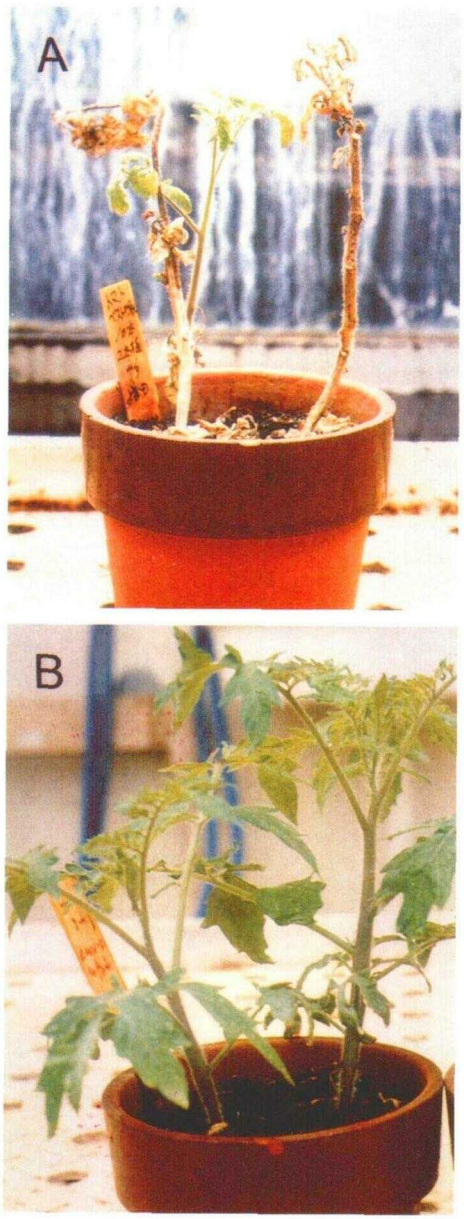


Figure 4. Symptoms on Tomato by Y-satRNA and Y-325U Transcripts in the Presence of CMV-O.

(A) Necrosis on the stem and leaves induced by Y-satRNA.
(B) Mild mosaic symptoms on tomato plants infected with Y-325U, which contains a base substitution from a C to a U at position 325.

because, as far as we are aware, Y-satRNA is the only satellite RNA that elicits such dramatic brilliant yellow symptoms. First we investigated the correlation between potential protein products encoded by Y-satRNA and the yellow symptoms. Y-satRNA contains two open reading frames that are potentially expressed. The first open reading frame (ORF 1) begins at the 5′-proximal AUG (positions 11 to 13) and ends with a UGA (positions 92 to 94). The second open reading frame (ORF 2) begins at positions 151 to 153 and ends with a UGA (331 to 333). These ORFs were interrupted by the insertions of 4 bases at the BamHI (position 50) for ORF 1 and at the StyI (position 169) for ORF 2. As shown in Table 1, both insertion mutants were able to replicate, and induced yellow symptoms on tobacco, indicating that maintenance of the reading frames is not required for symptom expression by Y-satRNA or replication of the satellite RNA. Thus, the induction of yellow symptoms as well as necrotic symptoms (Collmer and Kaper, 1988) can occur independently of the expression of the ORF.

Because the 369-nucleotide Y-satRNA has a unique domain (region Y) between positions 100 and 200 that does not exist in the smaller 330- to 340-nucleotide satellite RNAs and because the nucleotide sequence in the corresponding region of 386-nucleotide OY2-satRNA is totally different from region Y of Y-satRNA, we speculated that region Y contained the nucleotide sequence responsible for yellow symptom induction. To focus on the function of region Y, we constructed chimeric mutants with cDNA clones derived from Y-satRNA and the 370-nucleotide S19-satRNA. Although $Y_B19_{Bs}Y$ could induce the yellow phenotype, $Y_{Bs}19_AY$ could not, suggesting that any or all of the 16-nucleotide differences between Y-satRNA and S19-satRNA in region 150 to 260 could be responsible for the yellow phenotype. S19-satRNA does not cause yellow symptoms, but otherwise has a nucleotide sequence similar to Y-satRNA (Figure 3). Between Y-satRNA and S19-satRNA, there are several changes clustered in the vicinity of nucleotide 170 (Figure 3). When we created a mutant containing 4-base insertions at position 169 (StyI site), which totally changes the sequence around StyI site, the mutant induced the yellowing phenotype, as shown in Table 1. Therefore, it seems that the specific sequence around position 170 is not required for the yellow phenotype, but that secondary or tertiary structures may be involved. The corresponding regions of both Y-satRNA and S19-satRNA contain similar nucleotide sequences, but differ by several bases on both sides of the conserved sequence underlined in Figure 5. Within this region, we have used computer analysis to detect a possible secondary structure similar to that proposed by Hidaka et al. (1988) that lies in the genomes of S19-satRNA, $Y_B19_{Bs}Y$, and $Y_{Bs}19_AY$. Although we do not yet have any direct evidence that this region is actually folded in vivo into the structure illustrated in Figure 6, the nucleotides within this structure narrow down the region involved in induction of the yellow phenotype. Because only four nucleotide differences exist in the region between the AsuII sites and the 3′ ends of the genomes of Y-satRNA and S19-satRNA, and because the regions between the BamHI sites and the

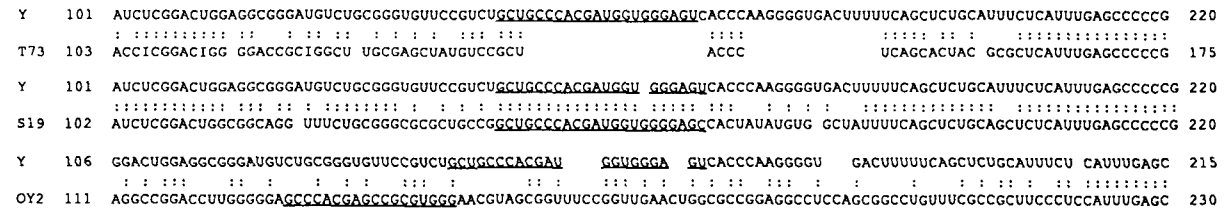


Figure 5. Comparison of the Regions Corresponding to the Y-satRNA Sequence between Residues 101 and 220 (Region Y).

The conserved sequences capable of forming a stem-loop structure (see Figure 6) are underlined. The sequence of OY2-satRNA is from Hidaka et al. (1988).

5′ ends of the two RNAs are identical (Figure 3), we postulate that these differences alter the conformation of the secondary structure and that resultant differential interaction of the structure with host components could control the phenotype elicited by these two strains. As shown in Figure 6, the structure in Y-satRNA is composed of a loop of 4 nucleotides at the tip of the hairpin structure and a stem of 8 bp with a bulge of a G residue (Figure 6B, part II). Furthermore, the hairpin structure is stabilized at the bottom with another 5 bp (Figure 6B, part I). If we draw a hairpin loop (Figure 6B, part II) in region Y using a computer software called DNASIS (Hitachi, Japan) for prediction of RNA secondary structure, based on the calculation method proposed by Zuker and Stiegler (1981), the I stems (see Figure 6) orienting the hairpin structure to a fixed position are conserved in Y-satRNA and $Y_B19_{Bs}Y$ but not in S19-satRNA and $Y_{Bs}19_AY$. The part I stems of S19-satRNA and $Y_{Bs}19_AY$ are energetically unstable because of distortion by a G-C pair at the bottom of the hairpin loop, and, thus, the hairpin structure should be more flexible. When we introduced site-specific mutations within the hairpin loop (Y-158G, Y-161C in Table 3), the secondary structures are found to be more stable by the computer analysis. This is consistent with the fact that neither mutation destroyed the yellow phenotype induced by Y-satRNA.

To investigate more directly the extent to which stability of the putative secondary structure actually correlates with yellow symptom induction by Y-satRNA, we are now constructing site-specific derivatives that contain a hairpin structure mimicking those of S19-satRNA and $Y_{Bs}19_AY$. However, so far we have been unable to isolate mutants that do not elicit yellow symptoms. Because the concentration of these progeny satellite RNAs in infected plants was similar to that of authentic Y-satRNA, as estimated by comparing the intensity of the ethidium bromide fluorescence of the samples after gel electrophoresis, we believe that, except for the lethal modification at position 318, the site-specific changes have not altered the ability of Y-satRNA to replicate.

METHODS

Source of CMV Satellite RNAs

The origin of Y-satRNA has been described before (Takanami, 1981). In addition to Y-satRNA, two additional satellite RNAs, designated S19-satRNA and T73-satRNA, were isolated from field-infected spinach and tobacco plants, respectively. These satellite RNAs were propagated in tobacco plants (*Nicotiana tabacum* cv Xanthi nc) or in tomato plants (*Lycopersicon esculentum* cv Best of All).

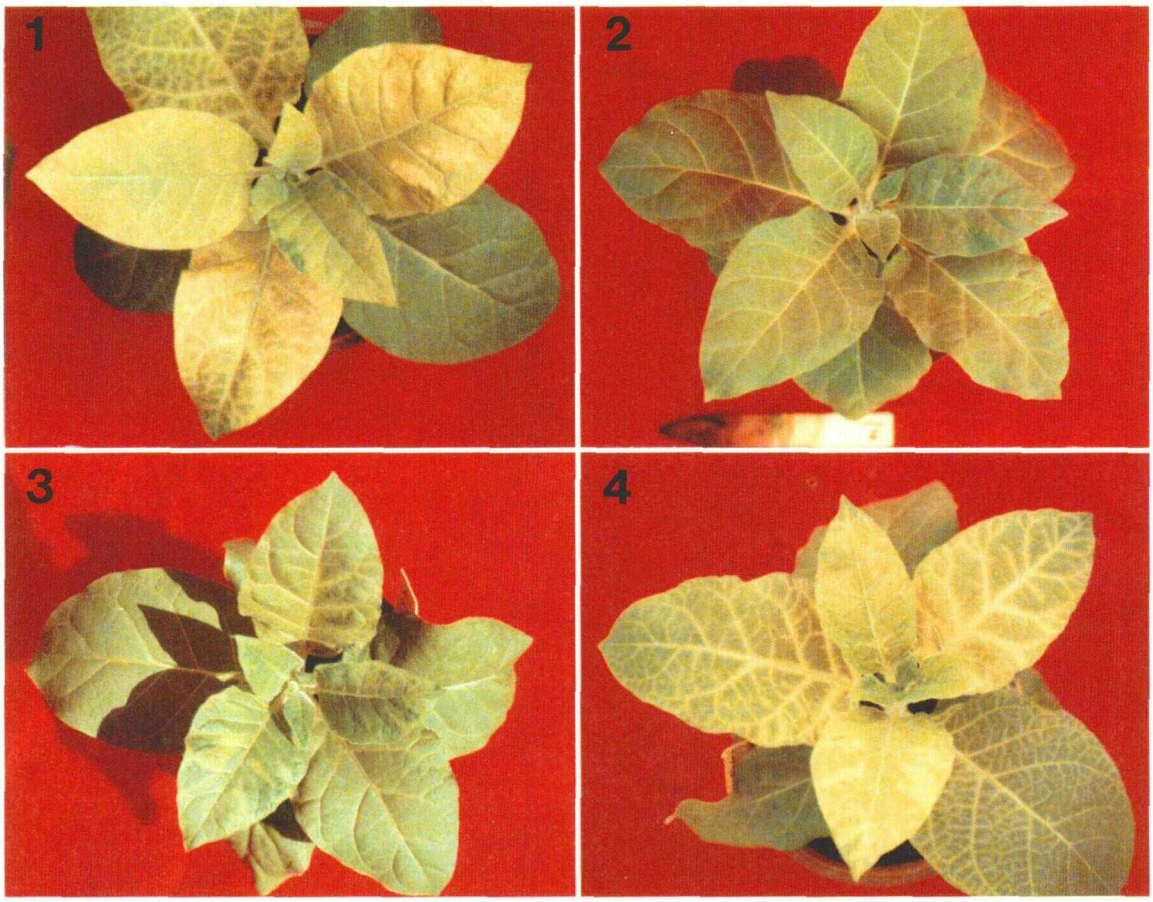
Construction of Site-Specific Mutants

The original transcription plasmid containing the full-length cDNA of Y-satRNA used to construct mutants and chimeric satellite RNAs was pUT119GG-S, which was a modified plasmid of pUT118GG-S (Kuwata, Masuta, and Takanami, 1988). Run-off transcripts from SmaI-cut pUT119GG-S with T7 RNA polymerase were designed to contain two additional Gs at the 5′ end and exactly match the sequence at the 3′ end. Insertion mutants (at the BamHI, StyI, NheI, and AsuII sites) were constructed by filling in the 5′ overhangs with the Klenow fragment (Maniatis, Fritsch, and Sambrook, 1982) after cleavage by the restriction enzymes. Deletions were created by digesting pUT119GG-S with two unique restriction enzymes, and the overhanging nucleotides were removed with the Klenow fragment or T4 DNA polymerase. By religating the filled-in or degraded restriction sites with T4 DNA

Figure 6. Differences in the Secondary Structure in Region Y and Pathogenicity on Tobacco of Y-satRNA, S19-satRNA, and Chimeric RNAs.

(A) Symptoms on tobacco plants 10 days after inoculation with CMV-O plus Y-satRNA (1), S19-satRNA (2), $Y_{Bs}19_AY$ (3), and $Y_B19_{Bs}Y$ (4).
(B) Secondary structure model in region Y of Y-satRNA (1), and S19-satRNA (2), $Y_{Bs}19_AY$ (3), and $Y_{Bs}19_{Bs}Y$ (4). The bases derived from Y-satRNA and S19-satRNA are designated by capital and lower case letters, respectively.

A



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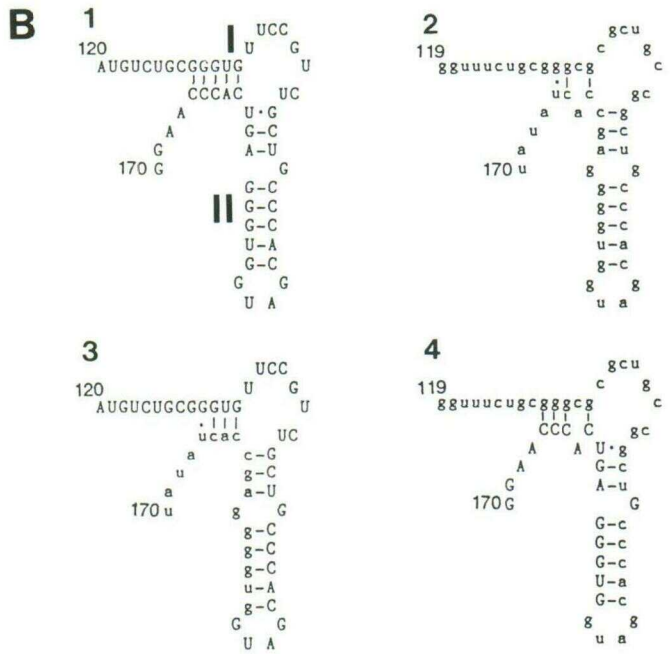

ligase, insertion or deletion mutants were constructed (Maniatis et al., 1982). A point mutation at position 112 and an insertion of a G residue at position 158 were introduced by oligonucleotide-directed in vitro mutagenesis essentially as described by Kunkel (1985). The single-stranded template DNA was generated from pUT119GG-S, which contains a single-stranded phage origin of replication. pUT119GG-S can produce single-stranded DNA having uracil residues on a *dut*$^-$ *ung*$^-$ host (*Escherichia coli* BW 313) upon superinfection with a helper phage. After phosphorylation, the mutant oligonucleotides were annealed to single-stranded DNA template in 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 25 mM NaCl, 0.5 mM dithiothreitol at 65°C for 15 min, followed by the incubation at 37°C for 15 min. Synthesis and ligation of the mutant strand were then conducted in 50 mM Tris-HCl, pH 8.0, 60 mM ammonium acetate, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM NAD, 0.5 mM each of deoxynucleotide triphosphates at 25°C for 2 hr. The resultant plasmids were used to transform *E. coli* BMH 71-87 *mut*S (ung$^+$ host), which inactivate the template strand but permit survival of the mutant strand.

Construction of Chimeric Satellite RNAs

The fragments generated by cleavage of full-length cDNA clones of T73-satRNA and S19-satRNA with two unique restriction enzymes were purified by gel electrophoresis. The region between the corresponding restriction sites in pUT119GG-S was replaced with fragments from Y-sat, T73-sat, or S19-sat cDNAs to create Y_B73_AY (BamHI-AsuII), Y_B73 (BamHI-SmaI), Y_A73 (AsuII-SmaI), $Y_B19_{Bs}Y$ (BamHI-BstXI), and $Y_{Bs}19_AY$ (BstXI-AsuII) (Figure 2). Subsequently, run-off transcripts from SmaI-cut plasmids with chimeric satellite RNAs were synthesized from a T7 promoter originally ligated upstream of Y-satRNA cDNA.

Cloning and Sequencing of Progeny Satellites

Total RNA was extracted from infected leaves essentially according to Bourque, Hagiladi, and Naylor (1973), and the nucleic acids were separated on a 4% agarose gel. The band containing satellite RNA was excised from the gel, and the RNA was eluted by using an analytical electroeluter (IBI). Full-length cDNA of the satellite RNA was then synthesized by priming with synthetic oligonucleotides used for synthesis of the parental satellite RNAs. After cloning the cDNAs into the SmaI site of pUC119, the recombinant plasmids were sequenced as described by Chen and Seeburg (1985) for double-stranded DNA, and the inserts were sequenced by the dideoxy chain termination method originally developed by Sanger, Nicklen, and Coulson (1977).

Biological Activity Tests

Biological activity of the in vitro transcripts was assayed according to Masuta et al. (1988b). One-and-a-half-month-old tobacco plants or young seedlings of tomato were inoculated with CMV-O (Hidaka and Tomaru, 1960) and transcripts from SmaI-cut plasmids, and scored 3 weeks later for yellow symptoms on tobacco or necrosis on tomato.

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