Symptom Production on Tobacco and Tomato Is Determined by Two Distinct Domains of the Satellite RNA of Cucumber Mosaic Virus (Strain Y)

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SUMMARY

Complementary DNAs of two different satellite RNA isolates from cucumber mosaic virus (CMV), Y from Japan and Ra from France, have been cloned in a transcription vector containing the Pr promoter. When inoculated on plants with CMV RNA (strain KIN), the transcripts of the cloned Y satellite cDNA elicit a bright yellow mosaic on tobacco and a lethal necrosis on tomato. Addition of the transcripts of the Ra satellite cDNA to an inoculum of CMV RNA resulted in symptom attenuation on both tobacco and tomato, in agreement with the characterized symptoms of the natural satellite. Recombinant molecules involving these two satellites have been constructed in order to determine which parts of the Y satellite RNA are involved in symptom induction. The determinant for symptom production on tobacco lies in the region between nucleotides 1 and 219. The domain for necrotic symptoms on tomato resides on the 3' half of the molecule beyond nucleotide 219.

INTRODUCTION

Cucumber mosaic virus (CMV) satellite is a small RNA molecule that replicates only in association with a helper virus and which modifies the disease symptoms (Francki, 1985). More than 20 satellite RNA variants have been isolated, characterized and sequenced (collated in Kaper et al., 1988; Hidaka et al., 1988; Jacquemond & Lauquin, 1988). All of them share at least 80% sequence homology. The type of symptom modification is an intrinsic feature of a particular satellite RNA (Mossop & Francki, 1979; Jacquemond & Lot, 1981; Waterworth et al., 1979; Gonsalves et al., 1982; Takanami, 1981). Some satellite RNA species reduce the effects of the virus, whereas others cause more severe symptoms. The type of symptom modification also depends on the host plant and the strain of helper virus. For instance, the satellite I17N, previously cloned in our laboratory (Baulcombe et al., 1986), reduces the symptoms of CMV on tobacco but induces necrotic death of tomato (Jacquemond & Lot, 1981). Palukaitis (1988) has shown that the helper virus influences the way in which symptoms are modified by the satellite RNA. Taken together, the observations on virus dependence and host specificity suggest that symptom production results from an interaction between the satellite RNA, the helper virus and the host plant.

In this paper, we describe an analysis of the satellite RNA from CMV strain Y (Hidaka et al., 1984). Co-inoculation of CMV and the Y satellite induces a distinctive yellow mosaic symptom on tobacco plants and a lethal necrosis on tomato seedlings (Takanami, 1981). In order to define the region of the Y satellite RNA involved in the induction of symptoms we have created recombinant forms of the satellite RNA. These recombinant molecules comprise parts of the Y satellite RNA with parts of a second satellite RNA from CMV strain R. The R satellite RNA attenuates symptoms on both tobacco and tomato (Jacquemond, 1982). The types of symptom induced by the recombinant satellite RNA demonstrate the existence of separate symptom-inducing domains in the satellite RNA molecule.
METHODS

Viral strains and satellite RNA. CMV, strain KIN was obtained from Dr B. D. Harrison (Scottish Crop Research Institute, Invergowrie, U.K.) as a sample of total nucleic acids from infected tobacco leaves. Y satellite RNA received as purified satellite RNA was a gift of Dr Y. Takanami of the Japanese Salt and Tobacco Company. CMV strain R was provided by Dr M. Jacquemond (INRA, Montfavet, France) as a dried leaf sample and was propagated on tobacco by Dr M. A. Mayo (Scottish Crop Research Institute) who supplied us with purified viral RNA. The accession received was designated Ra, in order to distinguish it from the R satellite RNA described by Jacquemond & Lauquin (1988) (see Results).

The inocula of CMV strain KIN used as the helper virus were prepared as a total nucleic acid fraction using the extraction method described below. This relatively impure fraction produced highly infectious inocula which could be stored stably under ethanol. The risk of laboratory contamination by extraneous satellite RNA was less than if purified viral RNA was prepared and used as the inoculum.

Cloning and nucleotide sequence determination. Complementary DNA molecules of Y and Ra satellite RNA species were synthesized from purified satellite RNA and from viral RNA preparations, respectively. The RNA was polyadenylated at the 3' end using Escherichia coli poly(A) polymerase (Bethesda Research Laboratories) (Sippel, 1973). Synthesis of cDNA was primed with oligo(dT) and carried out according to the protocol of a cDNA synthesis kit (Amersham). The cDNA was tailed with dCTP in 100 mM-potassium cacodylate, 25 mM-Tris base, 10 mM-CoCl2, 0.2 mM-DTT, 0.1 mM-dCTP and 10 mMCl of [α-32P]dCTP for 15 min at 37°C with 25 units of terminal deoxynucleotidyl transferase (Bethesda Research Laboratories). Annealing with an equimolar ratio of dG-tailed pUC9 vector was carried out in 100 mM-NaCl, 10 mM-Tris-HCl pH 7.8, 0.1 mM-EDTA at 65°C for 10 min and cooled to 4°C overnight. Clones of satellite cDNA were identified by hybridization with a cDNA probe of I17 N satellite (Baulcombe et al., 1986) and those with the longest cDNA insertions were sequenced directly from the plasmid DNA (Murphy & Kavanaugh, 1988). The 5'-terminal sequence of the Ra satellite RNA was determined by chemical degradation of primer-extended cDNA (Hamilton & Baulcombe, 1989).

Oligonucleotide-directed mutagenesis. Satellite cDNA clones were subcloned in M13 RF DNA and the mutagenesis was carried out with the Muta-Gen M13 in vitro mutagenesis kit (Bio-Rad).

In vitro transcription. For each transcription assay 2.5 μg of SmaI-cut plasmid DNA was used. The reaction mixture contained 0.2 mM-Tris-HCl pH 8.0, 5 mM-MgCl2, 150 mM-NaCl, 40 units of RNAsin (Promega Biotec) and 8 units of E. coli RNA polymerase (BioLab) in 40 μl. In test experiments, the cap analogue meGpppG was added at 0.5 mM. After 1 h of incubation at 37°C, the reactions were frozen or used directly for inoculation. Aliquots of 2 μl were analysed on Northern blots to confirm that transcription had occurred.

Infectivity assay. Four-week-old tobacco plants (Nicotiana tabacum var. Samsun NN) and 2-week-old tomato (Lycopersicon esculentum var. Ailsa Craig) were dusted with carborundum and inoculated with total nucleic acids (20 μg) extracted from leaves infected with CMV strain KIN (as described below). The inoculum was in 200 μl of 20 mM-Na2HPO4 and was supplemented with satellite transcripts as described in the text. The plants were propagated in a heated glasshouse. In winter, sodium lamp illumination was provided for 16 h/day. Leaf samples were collected after 15 days for tobacco, or just prior to the death of the plant for tomato and homogenized in 50 mM-Tris-HCl pH 9.0, 100 mM-NaCl, 10 mM-EDTA, 2% SDS, 0.1 mg/ml Proteinase K (5 ml/g). After two extractions with phenol-chloroform, nucleic acids were precipitated with ethanol and recovery was assayed by absorbance at 260 nm. Aliquots of each sample (4 μg) were fractionated by electrophoresis on a formaldehyde-agarose gel and the gel was blotted onto the nitrocellulose membrane. The membrane was hybridized with a cDNA insert from satellite RNA of CMV strain I17 N or a cDNA clone of CMV RNA 3 (Baulcombe et al., 1986). The cDNA inserts were annealed with random hexanucleotides and radiolabelled as described by Feinberg & Vogelstein (1984).

Sequencing of the progeny satellite RNA molecules. Five μg of a crude extract of nucleic acids from tobacco and tomato plants was used as a template for cDNA synthesis. Synthesis of the first strand was primed with an 18-mer oligonucleotide complementary to the 3' extremity of the Y and Ra satellite RNAs. The reaction mixture of 20 μl (50 mM-Tris-HCl pH 8.3 at 42°C, 40 mM-KCl, 8 mM-MgCl2, 1 mM-dATP, -dGTP and -TTP, 0.01 mM-dCTP, 10 μCi [α-32P]dCTP, 4 pmol of primer) was incubated at 42°C for 90 min with 10 units of avian myeloblastosis virus reverse transcriptase (Bio-Rad). The cDNA was amplified by the polymerase chain reaction (Scharf et al., 1986), which was performed on 5% of the first strand cDNA synthesis reaction mixture in 50 μl of 0.5 mM-dATP, -dGTP, -TTP and -dCTP, 67 mM-Tris-HCl pH 8.8 at 25°C, 17 mM-(NH4)2SO4, 10 mM-MgCl2, 10 mM-2-mercaptoethanol, 0.2 mg/ml gelatine, 50 pmol of 3' end primer and 50 pmol of 3' primer (18-mer). Two units of Taq DNA polymerase (Perkin Elmer Cetus) were added and the reaction was subjected to 25 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 2 min. To prevent evaporation, the mixture was overlaid with 50 μl of paraffin oil. The polymerase chain reaction products were analysed on a 2% agarose gel and if they migrated as a single component, 10 μl of the reaction was used directly for sequencing the double-stranded fragment using the deoxyoxynucleotide method. Otherwise, DNA fragments were cloned in the dG-tailed pUC9 vector as described above and then sequenced (Murphy & Kavanaugh, 1988). At least three independent clones were sequenced from each cDNA sample.
Symptom determination by CMV satellite RNA

Fig. 1. Nucleotide sequence comparison of Y and Ra satellite RNA. The arrows and numbers indicate the position of the four differences between the Y satellite RNA sequence and the original published sequence (Hidaka et al., 1984). The box delineates the recognition sequence of the restriction enzyme Nhel in the cDNA used for the construction of the recombinant molecules.

RESULTS

Nucleotide sequence of Y and Ra satellite RNA

Fig. 1 shows the aligned nucleotide sequence of the Y and Ra satellite RNAs derived in part from the cDNA clones. The cDNA clones were actually short of the full length by 11 (Ra satellite) or six (Y satellite) nucleotides at the 5' end. The missing Y satellite RNA sequence was known from the published sequence (Hidaka et al., 1984) and the 5' end of the R satellite RNA was sequenced directly from the RNA as described by Hamilton & Baulcombe (1989).

The version of the Y satellite sequence shown in Fig. 1 differs in four positions from the published sequence (Hidaka et al., 1984): a nucleotide insertion at position 234 and nucleotide
changes at positions 161 (C→T), 167 (C→A) and 173 (C→T). These three latter differences were also described by Kaper et al. (1986).

The sequence of the R satellite RNA has been described previously by Jacquemond & Lauquin (1988). However, the sequence they described differs at 36 positions from the sequence of our cDNA clones. For this reason and to avoid confusion, we refer to our variant as the Ra satellite RNA.

As shown in Fig. 1, the major difference between the Y and Ra satellite RNA sequences is a block insertion of 35 nucleotides in the Y sequence at position 133. Surrounding this insertion, the border regions (95 to 132 and 166 to 184) share restricted homology with the Ra satellite RNA, particularly on the 3' side. Most of the other CMV satellite RNA isolates shared at least 80% homology with the Ra sequence shown in Fig. 1. Only the Y and 0Y2 satellite RNAs (Hidaka et al., 1988) are significantly longer than 335 nucleotides.
Symptom determination by CMV satellite RNA

Assay of in vitro transcripts

The inserts of the Ra and Y satellite cDNA clones were transferred from the pUC9 cloning vector to a pUC19 vector containing the Pr promoter of the plasmid pPM1 (Ahlquist & Janda, 1984) as described in Fig. 2. The final constructions could be linearized with Smal and transcribed in vitro into an RNA molecule which was identical to the natural satellite RNA at both termini, resulting in highly infectious transcripts. In a dilution experiment, it was shown that transcripts of 70 pg template DNA are enough to induce symptom formation (data not shown). The addition of the m'GpppG cap analogue to the in vitro transcription reaction had no effect on the biological activity of the satellite RNA transcripts (data not shown), as described previously by Collmer & Kaper (1986). Plants were inoculated routinely with uncapped transcripts of 2.5 µg of template DNA. As a control, 2.5 µg of plasmid containing the satellite cDNA clone had been inoculated on plants but no symptoms were observed and no progeny satellite RNA was detected in plant extracts. Infectious cDNA molecules were described by Van Emmelo et al. (1987) for the satellite RNA of tobacco necrosis virus and by Jacquemond & Lauquin (1988) for the R satellite RNA of CMV. The presence of homopolymeric dG/dC tails are essential for its infectivity. In our constructions and in the one of Collmer & Kaper (1986) these tails are absent and the cDNA is not infectious.

To assess the ability of the in vitro transcripts to produce symptoms, a series of tobacco plants were inoculated with CMV RNA or a combination of CMV RNA and the in vitro transcripts of satellite cDNA (Fig. 3). All plants inoculated with CMV showed viral symptoms on the inoculated and first systemically infected leaves at approx. 5 days post-infection (p.i.). Later, at 10 days p.i., on plants inoculated with CMV RNA plus in vitro transcripts of satellite cDNA, the effects of satellite were evident on systemically infected leaves. These effects were identical to those caused by natural satellite RNA. Thus, tobacco plants inoculated with transcripts of the Y satellite cDNA showed yellow mosaic symptoms (Fig. 3 a). Infection with the transcripts of the Ra satellite cDNA caused the attenuation of viral symptoms (Fig. 3 a). On tomato plants the development of symptoms took longer than on tobacco but showed the same sequence of events: CMV-associated symptoms first, followed by the superimposition of the symptoms caused by the satellite RNA. The Ra satellite transcripts reduced the severity of the fern leaf symptoms of the virus (Jacquemond, 1982). The Y satellite transcripts inoculated with CMV RNA caused a lethal necrotic disease of the tomato plants, as described by Takanami (1981) (Fig. 3 b).

The presence of satellite RNA in infected plants was detected by Northern blot analysis (Fig. 4) using probes specific for satellite RNA or CMV RNA. Controls with mock-inoculated or CMV-inoculated plants confirmed that the experiments were not contaminated by exogenous CMV or satellite RNA (Fig. 4 a, lanes 1 and 2). In the experimental samples, satellite RNA was detected only in plants inoculated with in vitro transcripts and CMV RNA (Fig. 4 a, lanes 3 and 4). These Northern blots resolved the difference in size of the Y and Ra satellite RNA species. It was therefore possible to confirm that the transcripts of the two cDNA clones replicated faithfully in plants to produce progeny molecules of the expected size.

The analysis of CMV RNA in satellite RNA-infected plants showed an influence of satellite RNA on the accumulation of CMV RNA (Fig. 3 b). In all samples containing satellite RNA there was a reduction in the amount of viral RNA in systemically infected leaves. The symptoms were observed reproducibly. Of a total of 18 tobacco plants in six independent inoculations with CMV RNA and Y satellite RNA transcripts, 17 plants showed yellow chlorosis and contained satellite RNA, one was symptomless and did not contain satellite RNA and CMV RNA. All 15 plants inoculated with CMV RNA and Ra satellite transcripts showed attenuation of viral symptoms and contained progeny satellite RNA. On 18 tomato plants inoculated with CMV RNA and Y satellite RNA, 16 died from necrosis and two were symptomless. These last plants did not contain satellite RNA or CMV RNA. All 14 tomato plants inoculated with CMV RNA and Ra satellite RNA transcripts showed symptom attenuation and contained progeny satellite RNA.

Construction and assay of recombinant satellite RNA

To localize the active domains of the Y satellite RNA, recombinant RNA molecules were prepared which involved the construction of hybrid cDNA molecules as described in Fig. 5.
Fig. 3. Symptoms on tobacco (a) and tomato (b) plants elicited by the different *in vitro* transcripts from satellite cDNA clones. The plants were inoculated with buffer as a control, or with CMV RNA alone or with CMV RNA plus *in vitro* transcripts of either the Y satellite clone (Y), the Ra satellite clone (Ra), with the Y + Ra recombinant (Y + Ra) or the Ra + Y recombinant (Ra + Y).
Symptom determination by CMV satellite RNA

Fig. 4. Northern blot analysis of satellite progeny RNA and viral RNA production in infected tobacco and tomato plants. The nitrocellulose filters were hybridized with 32P-labelled probes specific for either satellite RNA (a) or CMV RNA (b). The plants were inoculated with buffer (mock) (lane 1), CMV RNA (lane 2) and CMV RNA plus in vitro transcripts of Y satellite clone (lane 3) or Ra satellite (lane 4) or Y + Ra recombinant (lane 5) or Ra + Y recombinant (lane 6). The electrophoretic migration was from top to bottom on the figure. Monomer satellite RNA was detected most strongly by the satellite probe. In some samples, a dimeric satellite form was detected. The CMV RNA probe strongly detected RNA 3 and RNA 4. RNA 1 and RNA 2 comigrated and hybridized weakly with this probe.

These hybrid cDNAs were obtained by ligating the 5' half of the Y satellite with the 3' half of the Ra satellite (construction Y + Ra) or vice versa (construction Ra + Y). In each construction the 5' Nhel fragment of the satellite preserved the Pr promoter.

In vitro transcripts of the recombinant clones were assayed on seven tobacco and seven tomato plants by co-inoculation with CMV RNA. On all plants, the Y + Ra construction produced the same symptoms as the Y satellite RNA on tobacco but on tomato an attenuation of viral symptoms was observed, as seen with the Ra satellite RNA infection (Fig. 3). Transcripts of the Ra + Y construction had reciprocal effects: the viral symptoms were attenuated on tobacco as would be observed for the Ra satellite RNA, and on tomato the necrotic disease of the Y satellite RNA was produced. The presence of progeny satellite RNA in the infected plants was verified by Northern blot analysis (Fig. 4a). This test suggested that the recombinant satellite RNAs were replicated faithfully because the progeny satellite RNA migrated slightly more slowly
DISCUSSION

There has been some controversy about the type of symptoms produced by the Y satellite RNA that was first described by Hidaka et al. (1984) as producing a necrotic disease on tomato. Kaper et al. (1986) found two components in an inoculum containing Y satellite RNA and suggested that a 335, rather than the 368 nucleotide component caused the necrotic disease on tomato. The isolate we have cloned is similar to the 368 nucleotide satellite RNA but it produces, without doubt, a lethal necrosis on tomato, as shown by the symptoms induced by the in vitro transcripts of cDNA clones. A possible explanation for the discrepancy between our data and those of Kaper et al. (1986) may be a single nucleotide at position 234 which is absent in the accession of Y satellite RNA used by Kaper et al. (1986) (Fig. 1). Alternatively, the different helper CMV used by the latter group may not support the formation of necrotic symptoms in conjunction with Y satellite RNA. Palukaitis (1988) and Masuta et al. (1988) have shown that the nature and the extent of symptoms depend on both the satellite RNA and the strain of CMV helper virus.

In this paper we have taken advantage of the distinctive symptom-inducing capability of Y and Ra satellite RNAs in order to locate symptom-forming domains. In previous studies, it has been impossible to correlate specific sequence changes, secondary structure characteristics or peptides potentially encoded by these satellite RNAs with their pathogenic response (Garcia-Arenal et al., 1987). The failure to find sequences responsible for specific symptoms may be due to the fact that symptom determinants might involve few nucleotides and that their localization could be hampered by the natural polymorphism in the satellite RNA population. The properties of the Y and Ra recombinant satellite RNAs provide direct evidence for the location of symptom-inducing domains in the CMV satellite RNA.

The nucleotides associated with the bright yellow mosaic symptoms on tobacco of the Y satellite RNA are located between positions 1 and 219. Within this region there is a stretch of 35 nucleotides which are not found in any other satellite RNA species and which may be involved in the induction of this symptom. Further types of recombinant satellite RNA will be created in order to test whether the symptom induction involves a satellite-encoded peptide or a more direct effect of the satellite RNA. Both are possible, although it should also be noted that the
only evidence for the presence of CMV satellite RNA-encoded proteins in vivo is provided by their synthesis in a cell-free translation system (Avila-Rincon et al., 1986; Hidaka et al., 1988). An RNA-mediated effect might involve the stem–loop structure determined by computer analysis (Zuker & Stiegler, 1981) which can be formed by the 35 nucleotides unique to the Y satellite RNA (Hidaka et al., 1988).

If a satellite RNA-encoded protein is involved in the induction of yellow mosaic symptoms, it would be encoded by one of three open reading frames (ORFs) (Fig. 6). The extreme 5' ORF, ORF I as described by Kaper et al. (1988) is present in a similar form in other satellite RNAs that do not induce yellow mosaic symptoms. On the other hand, ORF IIA' is found only in the Y satellite RNA. For ORF IIB, although it comprises a sequence of 22 amino acids similar to other satellite RNAs, most of the peptide contains features unique to the Y satellite RNA. These last two ORFs could be implicated in the formation of yellow mosaic.

Similarly, the necrosis-inducing function of the satellite RNA may involve protein-mediated or RNA-mediated interactions. By mutation of the AUG initiating codon, Collmer & Kaper (1988) eliminated the possibility that the 5' ORF I is involved in necrosis, and this finding is consistent with data reported here. Only ORF IIB which overlaps the NheI site (Fig. 6) could be implicated in the production of necrotic symptoms.

Although the mechanism of satellite RNA action remains undetermined, it is clear that the mere presence and accumulation of satellite RNA is not sufficient to bring about its effects. The effects caused by satellite RNA (symptom induction or viral attenuation) are seen only in systemically infected leaves and never in the inoculated leaves although the satellite RNA does accumulate there to a high level (Baulcombe et al., 1986; unpublished results). A possible explanation for this effect may be the requirement for a high ratio of satellite to viral RNA, which is brought about by an inhibitory effect of satellite RNA on viral RNA accumulation in systemically infected leaves. The inhibitory effect has been noted previously for a symptom-attenuating satellite RNA (Harrison et al., 1987) but is also observed when CMV infections with Y satellite RNA have spread systematically and elicit the yellow mosaic or the lethal necrotic diseases (Fig. 3b).

In this paper we have described separate symptom-inducing domains in the satellite RNA molecule. However we cannot conclude that these domains are the only part of the satellite RNA involved in symptom production. As the satellite RNA has a high degree of secondary structure (Gordon & Symons, 1983) it is possible that these domains interact with other parts of the molecule which may be common to the Y and Ra satellite RNA.

When the symptom-inducing domains have been located more precisely than described here it will be possible to probe for plant and viral components which interact to bring about the symptoms in the plant. The information may be useful also in the production of a disabled satellite RNA for use in the genetic engineering of resistance to CMV (Harrison et al., 1987). Kurath & Palukaitis (1989) have described recombinant molecules constructed from other strains of satellite RNA. Their results are in agreement with ours, showing that the necrosis of tomato is determined by the 3' segment of the molecule.

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