Reciprocal phenotype alterations between two satellite RNAs of cucumber mosaic virus

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Cucumber mosaic virus Y satellite RNA (Y-satRNA) induces distinctive yellow mosaic symptoms on tobacco, whereas S19 satellite RNA (S19-satRNA) causes an attenuated green mosaic on tobacco, although they show considerable sequence identity. Biological assays of infectious chimeric satellite RNA molecules synthesized from cDNA clones of Y-satRNA and S19-satRNA using common restriction sites showed that the determinant for the induction of yellow mosaic symptoms lies in the BstXI-NheI fragment, in which 14 nucleotide differences are found between the two satellite RNAs. To define more precisely the yellow mosaic determinant(s) in this fragment, several site-directed mutants of Y-satRNA were created. The replacement of AUU, at nucleotides 191 to 193 in Y-satRNA, with GC, which mimics the S19-satRNA sequence at the corresponding site, abolished the ability of Y-satRNA to elicit a yellow mosaic. Conversely, a mutant RNA molecule derived from S19-satRNA in which GC at nucleotides 192 and 193 was changed to AUU induced the yellow mosaic symptoms. Thus, the phenotypes of two satellite RNAs on tobacco can be altered reciprocally by changing the sequences in this limited region.

Introduction

Satellite RNAs are small RNA molecules which are completely dependent on coinfection with helper viruses for their replication and encapsidation (Francki, 1985). Satellite RNAs of cucumber mosaic virus (CMV) can influence the symptoms induced by their helper CMV infection in certain host plants; the type of symptom modification is an inherent property of a particular satellite RNA. Some satellite RNAs attenuate the symptoms induced by CMV (Waterworth et al., 1979; Mossop & Francki, 1979), whereas others elicit new symptoms that are quite different from those caused by the helper virus (Kaper & Waterworth, 1977; Waterworth et al., 1979; Jacquemond & Lot, 1981; Takanami, 1981; Gonsalves et al., 1982). Symptom modification also depends on the host plant (Waterworth et al., 1979; Jacquemond & Leroux, 1982; Masuta et al., 1988) and helper virus strain (Palukaitis, 1989; Sleat & Palukaitis, 1990a; Jaegle et al., 1990).

We have been studying the molecular basis of the symptom modification induced by Y satellite RNA (Y-satRNA), which causes bright yellow mosaic symptoms on tobacco and systemic necrosis on tomato (Takanami, 1981), although Kaper et al. (1986) have reported that it does not induce necrosis on tomato. Earlier studies indicated that a domain controlling necrosis on tomato is located within the 3' half of the satellite RNA (Kurath & Palukaitis, 1989; Masuta & Takanami, 1989; Devic et al., 1989); the necrogenic consensus sequence has been confirmed by site-directed mutagenesis (Masuta & Takanami, 1989; Devic et al., 1990; Sleat & Palukaitis, 1990b). It has been shown that the 5' half (Devic et al., 1989) or a central part (Masuta & Takanami, 1989; Jaegle et al., 1990) of Y-satRNA directs yellow mosaic induction on tobacco.

In this paper, we describe further analysis of the domain controlling the yellow mosaic phenotype by making chimeric and mutated satellite RNAs from Y-satRNA and S19 satellite RNA (S19-satRNA), the latter of which does not induce the yellow mosaic phenotype despite considerable sequence identity with Y-satRNA (Masuta & Takanami, 1989; Masuta et al., 1990).

Methods

Virus strain, satellite RNAs and cDNA clones. CMV-O (Hidaka & Tomaru, 1960) was used as a helper virus throughout this study. The origin of the satellite RNAs has been described previously (Takanami, 1981; Masuta & Takanami, 1989), as has a cDNA clone, pUT18GG-S, from which infectious Y-satRNA could be transcribed (Kuwata et al., 1988). A cDNA clone, pS19-sat, from which infectious S19-satRNA could be obtained was produced by the method described previously (Kuwata et al., 1988).

Construction of chimeric and mutated forms of satellite RNA. Chimeric cDNAs containing the Y-satRNA and S19-satRNA sequences were

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constructed from pUT118GG-S and pS19-sat using common cleavage sites for BstXI and NheI (Fig. 1). Mutations were introduced by oligonucleotide-directed mutagenesis, as described by Kunkel (1985), using the following oligonucleotides: dACGATGGTGAGGCTAC-TAAGGGTGACTTTT for MY1, dACGATGGGTAGGATG-GGGAAGGGGTAGACTTTT for MY2, dGGTGAGGCTCAC-CCTAGTGTGGCTTTACCGCTGCAT for MY3, dGGTGAGGCTCATATACTGTGGCATTTCAGCTCTG for MY4, dTTTTACAGCTCTGCTCATTTTGATCC for MY5, dTTTTACAGCTGCTCATTTTGATCC for MY6 and dTTTTACAGCTCTGATTTCATTTGACCCC for MS1 (mutations are shown in italics).

Assessment of biological activity of clones. EcoRI-linearized cDNA clones were transcribed using T7 RNA polymerase (Pharmacia) as described (Kuwata et al., 1988). A transcription mixture containing approximately 20 µg/ml transcript was inoculated together with 20 µg/ml of CMV-O onto 1.5-month-old tobacco (Nicotiana tabacum cv. Xanthi-nc) seedlings. For each transcript, six tobacco plants were inoculated in at least two independent experiments. Symptoms were noted 2 to 3 weeks after inoculation, and the accumulation of single- and double-stranded satellite RNAs was confirmed by agarose gel electrophoresis (Masuta et al., 1990).

Cloning and sequencing of progeny satellite RNA molecules. Total nucleic acid was extracted from two leaves from different tobacco plants and separated on a 2% agarose gel. The band containing progeny satellite RNA was excised from the gel and RNA was recovered by centrifugation using Ultrafree C3 HV (Millipore). Progeny satellite RNA was annealed with an oligonucleotide, dCCCCAAGCACTGGGTCCTGTAGAGGAATGT, which is complementary to 19 nucleotides (italics) at the 3' end of the satellite RNA and contains a 3' non-viral extension of 10 nucleotides to create a HindIII site (bold) for cloning, and first-strand DNA was synthesized using Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories). After the template RNA had been digested with RNase A, second-strand DNA was synthesized using the Klenow fragment of DNA polymerase I (Takara) using the oligonucleotide dCCCCGATTCTGTGGCTAGGAGAATGT, which is complementary to the 5' 18 nucleotides of the full-length first-strand DNA (italics) and contains an EcoRI site (bold), as a primer. The resulting double-stranded DNA was digested with EcoRI and HindIII, and then cloned between the EcoRI and HindIII sites of plBluescript II SK(+) (Stratagene). At least three independent clones from each progeny satellite RNA were sequenced using an automated sequencing system, Genesis 2000 (DuPont).

Results and Discussion

Biological activity of chimeric satellite RNAs in tobacco

The sequences of the two independent full-length cDNA clones of S19-satRNA were identical and differed at six positions from the sequence of S19-satRNA published previously (Masuta & Takanami, 1989); there were a nucleotide deletion at position 19, insertion of a C residue at position 120, and nucleotide changes at positions 24 (U to C), 25 (A to G), 32 (U to A) and 58 (C to A). As shown in Fig. 1, the major difference between Y-satRNA and S19-satRNA was in the central part of Y-satRNA (positions 100 to 200), designated region Y (Masuta & Takanami, 1989).

<table>
<thead>
<tr>
<th>Satellite</th>
<th>Phenotype</th>
<th>Cloning sites</th>
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<tbody>
<tr>
<td>Y-satRNA</td>
<td>BstXI</td>
<td>NheI</td>
</tr>
<tr>
<td>S19-satRNA</td>
<td>BstXI</td>
<td>NheI</td>
</tr>
<tr>
<td>Y_B 19_N</td>
<td>BstXI</td>
<td>NheI</td>
</tr>
<tr>
<td>Y_B 19_N</td>
<td>BstXI</td>
<td>NheI</td>
</tr>
</tbody>
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Fig. 1. Comparison of the nucleotide sequences of Y-satRNA and S19-satRNA. Identical nucleotides are indicated by dashes. Gaps in the aligned sequences are indicated by asterisks. The positions of the restriction sites in cDNA clones used for the construction of chimeric satellite RNAs are underlined.

Fig. 2. Phenotypes induced by the transcripts of the chimeric satellite cDNAs. The hatched and black boxes indicate Y-satRNA and S19-satRNA sequences, respectively.

To localize the active domain(s) in Y-satRNA that induces the brilliant yellow mosaic symptoms, we constructed chimeric forms of satellite RNA molecules from infectious pUT118GG-S and pS19-sat clones in vitro by exchanging the BstXI–NheI fragments (Fig. 2). The transcribed chimeric satellite RNAs with the BstXI–NheI fragment derived from either Y-satRNA or S19-satRNA were designated 19_B Y_N19 or 19_B Y_N19. All tobacco plants inoculated with 19_B Y_N19 in the presence of CMV-O showed yellow mosaic symptoms indistinguishable from those of tobacco plants inoculated with Y-satRNA. By contrast, all the tobacco plants inoculated with 19_B Y_N19 exhibited attenuated green mosaic symptoms (Fig. 2 and 3). Gel electrophoretic analysis of the progeny satellite RNAs indicated that the chimeric satellite RNAs accumulated to the same level as the parental satellite RNAs (data not shown). Sequencing three cDNA clones of the progeny satellite RNA of each chimera confirmed that the parental chimeric satellite RNAs had been exactly reproduced.
Table 4. Mutations introduced in Y-satRNA and S19-satRNA. The region between nucleotides 150 and 196 of Y-satRNA and the corresponding regions of S19-satRNA and the mutant satellite RNAs are aligned. Nucleotides identical to those of Y-satRNA are indicated by dashes. Base substitutions are indicated by the corresponding capital letters. Gaps in the aligned sequences are indicated by asterisks. The phenotype of each mutant is shown to the right.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>MS1</td>
<td>Yellow</td>
</tr>
<tr>
<td>MY6</td>
<td>Green</td>
</tr>
<tr>
<td>MY3</td>
<td>Yellow</td>
</tr>
<tr>
<td>MY4</td>
<td>Yellow</td>
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<tr>
<td>MY2</td>
<td>Yellow</td>
</tr>
<tr>
<td>MY1</td>
<td>Yellow</td>
</tr>
<tr>
<td>Y 150</td>
<td>Yellow</td>
</tr>
<tr>
<td>S19 151</td>
<td>Green</td>
</tr>
</tbody>
</table>

**Fig. 3.** Symptoms on tobacco plants elicited by various in vitro transcripts from satellite cDNA clones. The plants were inoculated with buffer as a control (f), with CMV-O alone (c), or with CMV-O plus in vitro transcripts of either S19-satRNA clone (a), 19B,Y,s19 clone (b), Y-satRNA clone (d), or YB,19s,Y clone (e).

**Biological activity of mutated forms of Y-satRNA and S19-satRNA in tobacco**

Various mutations were introduced in the BstX1–NheI fragment of Y-satRNA and S19-satRNA, in which 14 nucleotide differences were present, to define the yellow mosaic determinant (Fig. 4). Mutants MY1 to MY6 were derived from Y-satRNA, MS1 was a derivative of S19-satRNA.

MY1 and MY2 were designed to examine the possibility that the stability of the putative secondary structure in the Y-satRNA (Hidaka et al., 1988) might correlate with yellow mosaic symptom induction, as suggested by Masuta & Takanami (1989). MY1, which mimics the part I stem structure of the chimeric satellite YB,19s,Y (Masuta & Takanami, 1989), showed the yellow mosaic phenotype on tobacco (Fig. 5). Furthermore, MY2, into which a mutation was introduced to disrupt the putative part I stem structure, caused yellow mosaic symptoms on tobacco (Fig. 5). Therefore, the putative secondary structure does not seem to be involved in the induction of the yellow mosaic phenotype.

Masuta & Takanami (1989) have introduced two mutations into Y-satRNA, a G residue insertion at position 158 and a point mutation at position 161 (U to C), proving that they do not affect the ability to elicit yellow mosaic symptoms. Mutants MY3 and MY4 contained modifications of the downstream regions of the putative stem (positions 168 to 178 for MY3; 166 to 178 for MY4) and mimic the S19-satRNA sequence (Fig. 4). Tobacco plants inoculated with MY3 or MY4 developed yellow mosaic symptoms (Fig. 5). Of the 14 nucleotide differences in the BstXI–NheI fragments of the satellite RNAs, 11 were shown not to be related to the induction of the yellow mosaic phenotype. Thus, the three nucleotides at positions 191 to 193 of Y-satRNA are the possible candidates for the function of directing the induction of the yellow mosaic phenotype.

Based on the results described above, MY5 and MY6 were designed so that the nucleotides at positions 191 to 193 in the 3' region of the BstXI–NheI fragment of Y-satRNA mimic the sequences of S19-satRNA and T43-satRNA (Masuta et al., 1990), respectively. MY5 produced attenuated green mosaic symptoms on tobacco (Fig. 4 and 5), and MY6 also showed a green mosaic phenotype (Fig. 4). These mutants clearly demonstrated that the sequence from positions 191 to 193 in Y-satRNA is important for eliciting yellow mosaic symptoms on tobacco. This finding, therefore, extends our previous work (Masuta & Takanami, 1989) and the work of Jaegle et al. (1990).

To confirm the importance of the sequence from positions 191 to 193 in Y-satRNA, we tried to introduce the ability to elicit yellow mosaic symptoms into S19-
satRNA by replacing nucleotides GC at positions 192 and 193 with AUU, creating MS1 (Fig. 4). Tobacco plants inoculated with MS1 showed brilliant yellow mosaic symptoms which were indistinguishable from those induced by Y-satRNA (Fig. 5). It is surprising that S19-satRNA, which does not induce yellowing, could be converted to a yellow mosaic-inducing satellite RNA by changing a few nucleotides in this region. The results with mutants MY5, MY6 and MS1 lead to the conclusion that nucleotides 191 to 193 of Y-satRNA, and 192 and 193 of S19-satRNA, can reciprocally alter the phenotypes of the two natural satellite RNAs.

Although the actual boundary of the domain for induction of yellow mosaic has not been determined in these experiments because we have modified only a part of the sequences of Y-satRNA and S19-satRNA so that they mimic each other, the available data show that the sequence in the vicinity of nucleotides 178 to 193 in Y-satRNA is functionally involved in the induction of yellow mosaic symptoms (see Fig. 4). This is partly supported by the work of Jaegle et al. (1990), in which an alteration of nucleotides 185 and/or 186 has been shown to affect the yellow mosaic-inducing ability of Y-satRNA.

It has been reported that some mutations introduced into satellite RNA completely abolish biological function and, in some cases, secondary mutations arise in progeny satellite RNA (Collmer & Kaper, 1988; Masuta & Takanami, 1989; Devic et al., 1990; Jaegle et al., 1990). All the mutated satellite RNAs we have created here are infectious and the levels to which they accumulate in tobacco are almost the same as those of the authentic satellite RNAs. Moreover, we have not observed any secondary mutations in any of the progeny satellite RNAs. This may be due to our strategy in which mutations are introduced in two very similar satellite RNAs. Since they are of similar length and are about 90% identical, mutated forms of satellite RNA could retain essential structures or intramolecular interactions for replication.

**References**


Phenotype exchange between CMV satellite RNA


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