Replication of cucumber mosaic virus satellite RNA from negative-sense transcripts produced either \textit{in vitro} or in transgenic plants

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Both positive [(+)] and negative [(-)] sense versions of two satellite RNA (satRNA) genes from cucumber mosaic virus (CMV), the necrogenic I17N and the non-necrogenic R, have been introduced into the genome of tobacco plants. On infection with satRNA-free CMV, satRNA was amplified in plants expressing each of the four genes. All four genes confer protection against CMV infection. However, co-inoculation of plants with viral RNA and CMV satRNA transcripts synthesized \textit{in vitro} showed that [(-)] sense transcripts were less active than the corresponding [(+)] sense transcripts. This is the first report that [(-)] sense CMV satRNA transcripts can serve as a template for satRNA replication.

Introduction

Cucumber mosaic virus (CMV) is a positive [(+)] sense RNA virus infecting more than 800 monocotyledonous and dicotyledonous plant species (Douine et al., 1979), where it can be the cause of significant crop losses. CMV particles contain three genomic RNAs and a subgenomic RNA encoding the viral coat protein, which is derived from genomic RNA 3. Certain naturally occurring CMV strains include a fifth component, which has been shown to be a true satellite RNA (satRNA) of 330 to 386 nucleotides (nt) (Diaz-Ruiz & Kaper, 1977; Lot et al., 1977; Gould et al., 1978). In the presence of CMV as a helper virus, the satRNAs are replicated to high levels, reaching 95% of the encapsidated RNA molecules in infections of certain plant species; amounts of CMV genomic RNAs, in particular RNAs 1 and 2, are correspondingly decreased (Kaper & Tousignant, 1977; Mossop & Francki, 1979; Takanami, 1981; Jacquemond & Leroux, 1982). CMV satRNAs have attracted particular attention because of their ability to modulate symptoms of CMV infection. Most CMV satRNAs attenuate symptom intensity on nearly all plant hosts (Waterworth et al., 1979). However, in exceptional cases, specific satRNAs may intensify symptom expression. In tobacco, certain satRNAs provoke a bright yellow mosaic (Takanami, 1981). The host that permits greatest discrimination of satRNA strains based on symptomology is the tomato, which can develop lethal necrosis (Kaper & Waterworth, 1977), white leaf (Gonsalves et al., 1982), chlorosis (Palukaitis, 1988) or attenuated symptoms (Jacquemond & Lot, 1981; Kaper et al., 1981).

Collmer & Kaper (1986) have shown that CMV satRNA can be replicated in plants from transcripts synthesized \textit{in vitro} when co-inoculated with satRNA-free CMV. They showed that satRNA transcripts with a native 5' end could tolerate five non-satRNA nt at the 3' end, though with clear loss of activity when compared to native satRNA. In contrast, Kurath & Palukaitis (1987) found that a 57 nt extension of non-satellite sequences at the 5' end could be tolerated, but that two nt at the 3' end caused significant loss of activity, and that transcripts with eight 3' nt were no longer replicated. Masuta et al. (1988) showed that in transcripts with native 3' ends, the length of the 5' extension was important; up to 24 nt were tolerated, but biological activity (here defined as the ability to be replicated) decreased by 10³-fold compared to native satRNA.

Considering the extent of the observed reduction in biological activity of satRNA by additional 5' or 3' nt, it might not have been predicted that transgenic plants expressing genes with CMV satRNA flanked by heterologous sequences would synthesize satRNA on CMV infection. That they do was first shown by Baulcombe et al. (1986) using oligomeric CMV satRNA genes, and more recently by Jacquemond et al. (1988), Tousch et al. (1990) and several other groups (Masuta et al., 1989; McGarvey et al., 1990; Saito et al., 1992) using genes with strict monomers of CMV satRNA flanked by extensive 5' and 3' sequences.

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An important question that remains to be clarified is how the first steps in replication of CMV satRNA from such large precursor transcripts generate an initial population of unit-length satRNA molecules. We have hypothesized that this could occur by internal initiation by the replicase at the junction between satellite and flanking sequences, first on the (+) sense transcript, then on the negative (−) strand (Jacquemond et al., 1988). Another possibility is that unit-length molecules are simply generated by non-specific nuclease degradation of the initial transcript. In a cell-free in vitro CMV replicase assay, (+) sense satRNA transcripts with extensive flanking sequences had good template activity, showing that internal initiation on a (+) strand precursor RNA is possible (Hayes et al., 1992). It has also been predicted that (−) sense precursor molecules should have biological activity, though this was not observed in experiments using the in vitro replicase assay (Hayes et al., 1992), or in experiments where plants were co-inoculated with (−) sense satRNA transcripts synthesized in vitro and satRNA-free CMV (Collmer & Kaper, 1986; Kurath & Palukaitis, 1987).

We have investigated these questions using three approaches. In a previous report, (+) and (−) sense satRNA transcripts synthesized in vitro either with or without flanking sequences were tested in an in vitro CMV replicase assay (Hayes et al., 1992). In the experiments described here, both (+) and (−) sense CMV satRNA genes corresponding either to the necrogenic satRNA, I17N, or the non-necrogenic satRNA, R (Jacquemond & Lauquin, 1988), have been introduced into the genome of tobacco plants. In addition, the biological activity of (+) and (−) transcripts has been explored in experiments in which satRNA precursor transcripts synthesized in vitro were co-inoculated onto tomato plants with satRNA-free CMV.

Methods

Gene cloning and associated molecular biology techniques. Molecular biology techniques were as described by Sambrook et al. (1989). All enzymes were used according to the supplier’s instructions.

Construction of satRNA genes for expression in plants. The BamHI inserts in pAT153 bearing cDNAs corresponding to either R or I17N were inserted into the BamHI site of pMarcel70, to yield the four genes shown in Fig. 1. The vector pMarcel70 is a variant of the previously described pMarcel vectors (Robaglia et al., 1987) which uses a cauliflower mosaic virus 35S promoter with a double enhancer region (Kay et al., 1987).

Cloning satRNA cDNAs in a vector allowing transcription in vitro. The BamHI insert bearing the R satellite cDNA used above was cloned in the corresponding site of the pBluescript KS+ plasmid (Stratagene) to yield pBL. R. A cDNA fragment corresponding exactly to the R satRNA was amplified by PCR from pAT153R using the oligonucleotides 5' GGGTCCTGTGGAGGAATGAT 3' (complementary to the 5' end) and 5' GTTTTGTGATGGAGAATT 3' (complementing to the 3' end). The incubation conditions used were 30 cycles of 1 min at 95 °C, 2 min at 45 °C and 1 min at 72 °C. The amplified fragment was purified from a 5% polyacrylamide gel in TBE buffer (Sambrook et al., 1989), and cloned in pBluescript KS+, which had been linearized with EcoRV, to yield pBL. M+ and pBL. M− (Fig. 2).

In vitro transcription. Plasmids were purified by CsCl gradient centrifugation (Sambrook et al., 1989), linearized with an appropriate restriction enzyme and incubated with either T7 or T3 RNA polymerase to yield the transcripts shown in Fig. 2. In vitro transcription with T7 RNA polymerase was carried out for 1 h at 37 °C in a 50 μl reaction volume containing 10 units of RNA polymerase, 1 μg of DNA, 0.4 mM of each rNTP, 40 mM-Tris–HCl pH 8.0, 8 mM-MgCl2, 2 mM-spermidine, 25 mM-NaCl and 10 mM-DTT. Transcription with T3 RNA polymerase was carried out under similar conditions, but at pH 7.4 with 6 mM-MgCl2 and 4 mM-spermidine, in the absence of NaCl. The concentration of RNA transcripts was estimated on 12% agarose gels using purified satRNA as standards.

Transformation of tobacco. The pMarcel70 recombinants were conjugated into Agrobacterium rhizogenes A4 in triparental matings. The resulting four Agrobacterium strains were used to induce transformed roots on leaf explants of tobacco (Nicotiana tabacum cv. Xanthi nc, line XHFD8) from which transformed plants were regenerated as described previously (Teper & Casse-Delbart, 1987).

Analysis of replication of virus and satRNA after infection of tobacco plants. Virus was purified according to Lot et al. (1972) 8 to 10 days after inoculation. RNA was extracted from the virions by the phenol-SDS method and analysed on non-denaturing 24% polyacrylamide gels (Jacquemond & Lot, 1981). The amount of CMV coat protein in crude leaf extracts 10 days after inoculation was quantified by double-antibody sandwich ELISA (Clark & Adams, 1977) as described previously (Jacquemond et al., 1988).

Sequence analysis of satRNA progeny. Single-stranded cDNA of satRNA progeny was synthesized using a 9-mer oligonucleotide complementary to the 3' ends of both R and I17N satRNAs (5' GGGTCCTGT 3'). The 5'-labelled primer was hybridized with 5 μg of viral RNA purified from infected tobacco plants. cDNA was synthesized using avian myeloblastosis virus reverse transcriptase under conditions suggested by the manufacturer. RNA was denatured
with 30% DMSO and the cDNA corresponding to full-length copies was purified by electrophoresis through an 8 % polyacrylamide gel. The cDNA was sequenced according to Maxam & Gilbert (1980).

**Inoculation of tomato plants with CMV and in vitro transcripts and detection of satRNA.** SatRNA transcripts (1 μg/ml) were assayed by co-inoculation of 14-day-old tomato (*Lycopersicon esculentum* cv. Monalbo) seedlings with 10 μg/ml RNA of satRNA-free CMV I17F. Two weeks later, plants were superinfected with 50 μg/ml RNA of CMV I17N bearing a necrogenic satRNA. Plants that had replicated satRNA from the transcript were protected against necrosis when superinfected with CMV I17N. Biological activity is expressed as the percentage of plants protected. Values reported are the sums of five independent experiments, with a total of 60 to 90 individuals in each treatment. Control plants, inoculated with viral RNA alone, all developed severe fernleaf symptoms; all superinfected controls died of necrosis.

### Results

**Creation of transgenic plants expressing CMV satRNA genes**

*BamHI* restriction fragments bearing cDNAs corresponding to the CMV satRNAs I17N or R (Jacquemond & Lauquin, 1988) were cloned, in either orientation, in the unique *BamHI* site of pMarcel70. This resulted in four satRNA genes, in which (+) or (−) sense monomer satRNA was flanked by extensive heterologous sequences (Fig. 1). The I17N + gene is essentially identical to the one previously introduced into tobacco (Jacquemond et al., 1988), and has also been introduced into the tomato genome (Tousch et al., 1990). Genetic analysis of progeny obtained by self-crossing the initial transformants was consistent with there being a single locus of insertion of the satRNA gene (data not shown). Detailed analysis of copy number was not carried out, since this is not correlated with expression level. The presence of satRNA precursor transcripts of the expected size (0·95 kb) was verified by Northern blot analysis of total RNA extracted from transformed plants (data not shown). A representative transformed line for each gene was selected for further study.

**Response of satRNA transformants to infection with CMV**

Initial transformants were self-crossed, and transformed individuals among the resulting progeny were used in inoculation experiments, in which non-transformed plants were used as controls. Tobacco plants at the five-leaf stage were inoculated with satRNA-free CMV I17F. As expected from previous experiments (Jacquemond et al., 1988), plants expressing the I17N + gene were essentially free of symptoms of CMV infection. Equivalent symptom attenuation was observed in plants expressing the other three genes, R +, I17N − or R − (data not shown).

The sequence of encapsidated progeny satRNA produced in plants expressing either a (+) or a (−) I17N gene was as expected (Jacquemond & Lauquin, 1988). The sequence of progeny RNA obtained from plants expressing R satRNA genes was different from the published sequence at positions 224 and 225. Inspection

<table>
<thead>
<tr>
<th>Recombinant plasmid</th>
<th>Cleavage site/ promoter</th>
<th>RNA polarity</th>
<th>Features of RNA transcripts</th>
<th>Plants protected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBL.R</td>
<td><em>HindIII</em>/<em>T7</em> +</td>
<td></td>
<td>49 nt</td>
<td>SAT (+) 10A 30G 222 nt</td>
</tr>
<tr>
<td>pBL.R</td>
<td><em>XbaI</em>/<em>T3</em> −</td>
<td>273 nt</td>
<td>30C 10T</td>
<td>SAT (−) 16G 15 nt</td>
</tr>
<tr>
<td>pBL.M+</td>
<td><em>XbaI</em>/<em>T3</em> +</td>
<td>56 nt</td>
<td>GAT</td>
<td>SAT (+) ATC 35 nt</td>
</tr>
<tr>
<td>pBL.M−</td>
<td><em>XbaI</em>/<em>T3</em> −</td>
<td>56 nt</td>
<td>GAT</td>
<td>SAT (−) ATC 35 nt</td>
</tr>
</tbody>
</table>

Fig. 2. Structural features and biological activity of satRNA transcripts synthesized *in vitro*. DNA fragments bearing cDNA corresponding to the non-necrogenic satRNA, R, either with or without homopolymeric 5' and 3' flanking sequences, were cloned into the *BamHI* and *EcoRV* sites, respectively, of p Bluescript KS + to yield three recombinant plasmids. Digestion with appropriate restriction enzymes and use of either *T7* or *T3* RNA polymerase gave the various transcripts shown. In transcripts synthesized with pBL R, the satRNA sequences were flanked with oligohomopolymeric sequences and also nucleotides (shown as X nt) derived from the initial cloning vector (pAT153) and from the polylinker of p Bluescript KS +. In those synthesized from pBL. M + or pBL. M −, the satRNA was flanked only with the polylinker sequences. In all cases, promoter sequences of p Bluescript KS + were absent. The ability of satRNA to be synthesized from the transcripts was assessed by co-inoculation of tomato plants with transcripts and satRNA-free CMV I17F. Plants that had replicated satRNA from the transcript were protected against necrosis when superinfected with CMV I17N. Biological activity is expressed as the percentage of plants protected. Values reported are the sums of five independent experiments, with a total of 60 to 90 individuals in each treatment. Control plants, inoculated with viral RNA alone, all developed severe fernleaf symptoms; all superinfected controls died of necrosis.
of the original sequence of the cDNA showed that this was due to a typographical error in the previous publication (Jacquemond & Lauquin, 1988). The correct sequence for R satRNA is with T and C at these positions, rather than A and T as previously published.

Quantification of virus and analysis of encapsidated RNA in the different transformed and control plants are presented in Table 1. A significant decrease in virus yield and coat protein concentration was observed in plants expressing any of the four genes. Such decreases are consistent with the marked attenuation of the symptoms developed by all the transformed plants. As has been shown previously (Jacquemond et al., 1988), plants expressing a (+) sense gene accumulated large amounts of satRNA during infection. However, the proportion of encapsidated satRNA was considerably lower in plants expressing a (−) sense gene. Correspondingly, the virus yield and coat protein concentration were not reduced as much in these plants.

**Table 1. Analysis of progeny virus and satRNA in transformed tobacco after inoculation with CMV I17F**

<table>
<thead>
<tr>
<th>Plant genotype</th>
<th>Capsid concentration (μg/g of leaf tissue)*</th>
<th>Virus yield (mg/kg of leaf tissue)</th>
<th>SatRNA † (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4277±578</td>
<td>614±68</td>
<td>ND ‡</td>
</tr>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>I17N+</td>
<td>613±791</td>
<td>127±10</td>
<td>32.2±1.3</td>
</tr>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 9)</td>
<td></td>
</tr>
<tr>
<td>I17N−</td>
<td>890±160</td>
<td>330±55</td>
<td>81±0.8</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4277±578</td>
<td>457±62</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>R+</td>
<td>893±101</td>
<td>147±20</td>
<td>414±1.8</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>R−</td>
<td>2398±373</td>
<td>219±41</td>
<td>84±1.4</td>
</tr>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 6)</td>
<td></td>
</tr>
</tbody>
</table>

* Estimated by ELISA using a purified preparation of CMV I17F virions, diluted in an crude extract of healthy tobacco plants, as a standard.
† Proportion of satRNA as percentage of the mass of encapsidated viral RNA, estimated by densitometry (260 nm) of purified viral RNA on a polyacrylamide gel.
‡ ND, Not detected, by Northern blot experiments.
§ n, Number of plants analysed.
¶ Probability of equality less than 5/10000 when values obtained with transformants and control plants were compared by the unpaired t-test.
* Probability of equality on comparison by the unpaired t-test is less than 5/1000.

Biological activity of satRNA transcripts synthesized in vitro when co-inoculated with satRNA-free CMV

In order to study further differences between (+) and (−) sense transcripts, two fragments bearing a single copy of cDNA corresponding to R satRNA, with or without flanking homopolymeric sequences, were cloned in pBluescript KS+ to yield pBL.R, pBL.M+ and pBL.M− (Fig. 2). Insertion was verified by sequencing. After cleavage with an appropriate restriction enzyme, either T7 or T3 polymerase was used to synthesize a total of four transcripts differing both in the polarity of the satRNA sequence and in the presence or absence of homopolymeric sequences flanking the satRNA. The ability of satRNA to be replicated from these transcripts was tested in several assays, using 10 to 20 plants infected with a given transcript in each assay. The presence of non-necrogenic satRNA in plants inoculated with the different transcripts was shown by its ability to protect against necrosis on superinfection with a necrogenic satRNA. Mean percentages of plants protected against necrosis are shown in Fig. 2. All the control plants developed severe fernleaf symptoms, showing that no necrogenic satRNA was present in the inoculum. The absence of non-necrogenic satRNA in the initial, satRNA-free inoculum (CMV I17F) was shown by superinfection with necrogenic CMV I17N, which led to the necrosis of all control plants. Biological activity of the transcripts was at most 0.1% to 0.01% of that found with authentic satRNA. Increasing the concentration of RNA did not enhance this activity, but could lead to partial inhibition of infection by the viral RNAs (data not shown).
Transcripts obtained using T7 polymerase with HindIII-cleaved pBL.R were most similar to those used in the transgenic plants. Under the conditions described here, 40 to 50% of the plants inoculated with this transcript produced satRNA, and were protected against superinfection (Fig. 2). In contrast, the corresponding (−) sense transcript, produced with T3 polymerase and XbaI-cleaved pBL.R, had much lower biological activity, but this was still detectable. When the satRNA cDNA was isolated from the flanking homopolymeric tails and recloned in the EcoRV site, the (+) sense transcripts produced (pBL.M + cleaved with XbaI and using T3) had activity equivalent to the previous (+) transcript. Once again, the corresponding (−) transcript (pBL.M cleaved with XbaI and using T3) had much lower biological activity. Total nucleic acids purified from protected tomato plants that had been inoculated with each of the different transcripts contained a large amount of ss and ds satRNA (Fig. 3).

Discussion

The ability of satRNA to be replicated from similar (+) and (−) sense precursor transcripts was tested in RNA inoculation experiments and in transgenic plants. In all cases (+) sense transcripts with at least 50 to 60 nt of flanking sequence were biologically active. The results described here are the first showing that (−) sense satRNA transcripts can also serve as a template for satRNA replication, both in transgenic plants and in RNA inoculation experiments. Synthesis of the complementary strand from similar (−) sense satRNA templates was not detected in vitro (Hayes et al., 1992). This difference between the in vivo and in vitro observations could be due to numerous factors, for example the in vitro system could be inherently less sensitive. It is also possible that additional factors, such as a cellular RNA-dependent RNA polymerase, could also play an in vivo role. The presence or absence of homopolymeric flanking sequences had no detectable effect on satRNA replication from either polarity of precursor transcripts. In transcript inoculation experiments, the (−) sense transcripts were less active than (+) sense ones, in that replication of satRNA occurred in fewer of the plants inoculated (Fig. 2). But, when replication did occur, equivalent levels of ss and ds satRNA were synthesized (Fig. 3). This suggests that the limiting factor is synthesis of an initial population of unit-length satRNA from which replication then proceeds normally. Both (+) and (−) sense CMV satRNA genes conferred protection against CMV in transgenic plants, although (−) sense genes seem to be less efficiently expressed. Perhaps continuous gene expression at a modest level, such as that obtained in transgenic plants, gives more opportunity for (−) sense transcripts to be recognized before being degraded, delaying rather than preventing synthesis of an initial population of unit-length satRNA, which is then replicated normally.

Several hypotheses can be advanced to explain the lower activity of (−) sense transcripts. From transcript inoculation experiments, it could be proposed that they might be less stable than (+) sense transcripts. However, this is not likely to be the only factor since (−) transcripts, even ones corresponding exactly to the satRNA, have no detectable substrate activity in an in vitro replicate assay (Hayes et al., 1992). A second possibility is that the (−) sense transcripts tested were inherently less efficient templates for the CMV replicase. This is surprising in its own right, since it has been shown by replicate studies in vivo that when (+) sense satRNA is used as substrate, both strands of satRNA are synthesized. Further work with an in vitro CMV replicate assay (Hayes & Buck, 1990; Wu et al., 1991) should make it possible to study in more detail the template activity of (−) sense satRNA transcripts. This could also determine whether the template for synthesis of (+) sense satRNA requires an additional G residue at the 3′ end, as suggested by Collmer & Kaper (1985). In this regard it is significant that, in the transcript inoculation experiments reported here, a (−) sense transcript with 16 3′ G residues was not a better template than one with other 3′ flanking nucleotides. It is also possible that the optimum template for (+) strand synthesis is in fact ds satRNA, in which case synthesis of (−) and (+) sense RNAs would be linked processes.

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