Symptom Attenuation by a Satellite RNA in Vivo Is Dependent on Reduced Levels of Virus Coat Protein

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Many plant RNA viruses provide replication and encapsidation functions for one or more satellite RNAs (sat-RNAs) that can modulate the symptoms of the associated helper virus. Sat-RNA C, a virulent sat-RNA associated with turnip crinkle virus (TCV), normally intensifies symptoms but can attenuate symptoms if the TCV coat protein (CP) is replaced with that of cardamine chlorotic fleck carmovirus [Kong et al. (1995) Plant Cell 7, 1625–1634] or if TCV contains an alteration in the CP initiation codon (TCV-CPM) [Kong et al. (1997b) Plant Cell 9, 2051–2063]. To further elucidate the mechanism of symptom attenuation by sat-RNA C, the composition of the CP produced by TCV-CPm (CP\textsubscript{CPm}) was determined. Our results reveal that CP\textsubscript{CPm} likely has two additional amino acids at its N-terminus compared with wild-type TCV CP. TCV-CPm produces reduced levels of CP, and this reduction, not the two additional residues at the CP N-terminus, is responsible for symptom attenuation by sat-RNA C.© 1999 Academic Press

Key Words: turnip crinkle virus; satellite RNAs; symptom modulation; plant RNA viruses.

INTRODUCTION

Many plant RNA viruses are associated with one or more nonessential RNAs, such as defective interfering RNAs and satellite RNAs (sat-RNAs). These subviral RNAs depend on the helper virus for replication, encapsidation, and movement through the plant. The sat-RNAs range in size from 194 to 1500 nucleotides (nt) and usually have sequence unrelated to the viral genome. As molecular parasites of their helper viruses, sat-RNAs frequently modulate viral symptom expression. The smaller sat-RNAs (194–700 nt), including those of cucumber mosaic virus (CMV) and turnip crinkle virus (TCV), do not encode any functional open reading frames (ORFs) (Roossinck et al., 1992). However, despite the absence of gene products, these sat-RNAs can have dramatic effects on the symptoms induced by their helper viruses (for reviews, see Kaper and Collmer, 1988; Simon, 1988; Collmer and Howell, 1992). Many viral sat-RNAs attenuate disease (e.g., satellites of CMV and tobacco ringspot virus), a property of interest for viral disease control (Gerlach et al., 1987; Harrison et al., 1987; Tien and Wu, 1991). However, some sat-RNAs exacerbate symptoms of their helper virus. One such sat-RNA is sat-RNA C associated with TCV (Li and Simon, 1990).

Some viruses are associated with several sat-RNAs that differentially modulate symptoms (Murant and Kumar, 1990; Blok et al., 1994; Celix et al., 1997). Identical sat-RNAs can also have different effects on symptoms when associated with different helper virus strains (Kaper et al., 1990; Sleat and Palukaitis, 1990b; Roossinck et al., 1992; Sleat et al., 1994; Kong et al., 1997a; Militao et al., 1998). Furthermore, the same sat-RNA/helper virus combination can have different effects in different hosts. For example, symptoms of CMV are intensified by some CMV sat-RNAs, resulting in either chlorosis in tomato and tobacco (Palukaitis, 1988) or necrosis in tomato (Takanami, 1981; Sleat et al., 1994). Therefore, sat-RNA-mediated symptom modulation is determined by a tripartite interaction among the satellite, helper virus, and host plant. Many sat-RNA sequences that mediate symptom modulation have been defined (Simon et al., 1988; Palukaitis, 1988; Baulcombe et al., 1988; Kurath and Palukaitis, 1989; Masuta and Takanami, 1989; Jaegle et al., 1990; Sleat and Palukaitis, 1990a, 1990b; Naidu et al., 1992; Sleat et al., 1994; Zhang et al., 1994; Oncino et al., 1995; Kong et al., 1997a; Rodriguez-Alvarado and Roossinck, 1997; Taliansky and Robinson, 1987). In contrast, the role of the helper virus and host plant is poorly understood.

We are studying symptom modulation of TCV by sat-RNA C in the host plant Arabidopsis thaliana. TCV is a member of the Carmovirus genus and is the only genus member that is associated with confirmed sat-RNAs. TCV has a single plus-sense RNA genome of ~4 kb (Carrington et al., 1989; Oh et al., 1995) and two subgenomic (sg) RNAs of 1.72 and 1.45 kb (Carrington et al., 1987; Wang and Simon, 1997) (Fig. 1A). The viral genomic RNA is the mRNA for p28 and its readthrough product p88, which are required for viral rep-
TCV systemically infects all ecotypes of *Arabidopsis* tested except for ecotype Di-0 (Simon et al., 1992). TCV can replicate in protoplasts of Di-0 but cannot move systemically in whole plants (Simon et al., 1992). When the CP ORF of TCV was replaced with that of the related carmovirus cardamine chlorotic flex, the resulting chimeric virus (TCV-CP<sub>CCFV</sub>) systemically infected Di-0 but cannot move in preparations of TCV-infected protoplasts (Kong et al., 1997a, 1997b). Virions, however, are nearly undetectable in preparations of TCV-CPm-infected protoplasts (Kong et al., 1997b). The inability to isolate comparable levels of virions based on the amount of CP present in cells could be due to CP levels being below the threshold required for virion formation. Alternatively, the TCV-CPm CP (CP<sub>CPm</sub>) may be incapable of efficient virion formation due to translation initiating near, but not at, the natural initiation codon, resulting in a mutant N-terminus. Furthermore, the ability of sat-RNA C to attenuate TCV-CPm symptoms could be due to the presence of reduced levels of CP, the absence of virions, and/or putative alterations to the N-terminus of CP<sub>CPm</sub>.

To distinguish between these possibilities and to further our understanding of symptom modulation by sat-RNA C, the possible initiation codon of CP<sub>CPm</sub> was characterized. Our results suggest that translation of CP<sub>CPm</sub> likely initiates at an in-frame non-AUG codon (CUG) upstream from the normal initiation codon resulting in two additional amino acids at the CP<sub>CPm</sub> N-terminus. In addition, reduced levels of CP, not alterations to the N-terminus, are the principal determinant of symptom attenuation by sat-RNA C. Possible models are proposed to explain how CP levels may be involved in symptom attenuation by sat-RNA C in the TCV/Arabidopsis system.

**RESULTS**

**TCV-CPm likely initiates translation of its CP from a noncanonical initiation codon resulting in two additional amino acids at its N-terminus**

Because CP<sub>wt</sub> and CP<sub>CPm</sub> migrate to similar positions on denaturing polyacrylamide gels, translation of CP<sub>CPm</sub>
was thought to initiate at or near the wt initiation codon. Because the nearest in-frame AUGs in the TCV CP mRNA are either 42 amino acids upstream followed by two stop codons or 40 amino acids downstream from the wt initiation codon and because all nearby out-of-frame AUG triplets are followed by stop codons, CPCPm is likely synthesized beginning with a noncanonical initiation codon such as the ACG at the wt initiation position or an in-frame CUG codon located six nucleotides upstream (Fig. 2A). Noncanonical translation initiation has been confirmed or suggested for several cellular mRNAs (Beams et al., 1991; Saris et al., 1991) and viral RNAs from plant (Schmitz et al., 1996) and animal (Mehdi et al., 1990; Reynolds et al., 1995) hosts (for reviews, see Gallie, 1993; Rohde et al., 1994). Translation was previously reported to initiate from ACG and CUG with 15% and 30% efficiency, respectively, compared with AUG in plant cells (Gordan et al., 1992).

To determine whether the ACG or CUG might serve as initiation codons for CPm, mutations were introduced into the analogous positions of these nucleotides in TCV-CPm cDNA. As shown in Fig. 2A, the ACG codon

![Image](https://example.com/image.png)

**FIG. 2.** Characterization of the initiation codon of TCV-CPm CP. (A) Schematic representation of the CP ORFs in TCV, TCV-CPm (CPm), and the mutants (CPm-L, CPm-T, and CPm-O). The putative initiation codons are boxed, and the mutated nucleotides are indicated by lowercase letters. Two possible CP amino acid sequences are shown for TCV-CPm. The dashed lines in the CPm mutants indicate unaltered sequence. (B) RNA gel blots of total RNA and protein gel blots of total protein and virions accumulating in protoplasts 40 h after inoculation with transcripts of TCV, TCV-CPm (CPm), and CPm mutants as shown in (A). The A. thaliana protoplasts (5 × 10⁶) were inoculated with 20 μg of wt TCV or mutant transcripts. Total RNA was subjected to RNA gel blot analyses using a probe specific for TCV genomic RNA (gRNA) and sgRNAs (1.72 and 1.45 kb) (see Table 1) or rRNAs (Simon et al., 1992). CP and virions (v) were visualized by chemiluminescence using anti-TCV CP antibody. Each lane represents total protein or virions extracted from 2.5 × 10⁵ or 8.3 × 10⁵ protoplasts, respectively. Species corresponding to TCV genomic RNA and the 1.45- and 1.72-kb sgRNAs are indicated. (C) Symptoms of Col-0 or Di-0 plants inoculated with TCV, TCV-CPm (CPm), or CPm-L. Seedlings at the six- to eight-leaf stage were inoculated with the wt and mutant transcripts, with (+C) or without sat-RNA C as shown below the plants. Representative plants were photographed at 17 days p.i.. Mock plants were treated with inoculation buffer alone.
was altered to AGG generating CPm-L and the CUG codon was changed to UUG generating CPm-T. CPm-O was generated by combining both new mutations into a single construct. Neither of the resultant triplets (AGG or UUG) initiates translation in either mammalian or plant cells (Gallie, 1993; Rohde et al., 1994). Transcripts synthesized in vitro representing CPm-L, CPm-T, and CPm-O were inoculated onto A. thaliana protoplasts, and total RNA, protein, and virions were extracted 40 h p.i. for examination of viral RNA accumulation, CP synthesis, and virion formation.

RNA gel blot analysis revealed that CPm-L and CPm-T replicated to near wt levels, whereas TCV-CPm replicated to a lower level (Fig. 2B) as previously described (Kong et al., 1997b; note, however, that the TCV-CPm sample is underloaded according to the corresponding rRNA levels in Fig. 2B). It is not known why CPm-L and CPm-T had higher levels of genomic RNA replication compared with the parental mutant TCV-CPm. Surprisingly, no viral RNA was detected in CPm-O-infected protoplasts. This was unexpected because the mutations introduced into CPm-O are outside the viral p28 and p88 ORFs and are not within promoters known to be required for replication (Hacker et al., 1992; Wang and Simon, 1997). In addition, previous studies have shown that TCV genomic RNA accumulates in the absence of the majority of the CP ORF, including this region (Kong et al., 1995). However, the possibility cannot be ruled out that there is an additional mutation in some other critical location within the genome of CPm-O.

Western blot analysis using antibodies raised against CPW revealed that protoplasts infected with TCV-CPm accumulated 23% of wt levels of CP and no detectable virions as previously reported (Fig. 2B; Kong et al., 1997b). Protoplasts infected with CPm-L, which maintains the CUG codon but not the ACG codon, accumulated 35% of wt levels of CP. In addition, like TCV-CPm, no virions were detected in CPm-L-infected protoplasts (Fig. 2B). In contrast, no CP was detected in protoplasts infected with CPm-T, which maintains the ACG codon but not the CUG codon (Fig. 2B). As expected, no CP was synthesized in CPm-O-infected protoplasts due to the lack of viral RNA accumulation (Fig. 2B). These results suggest that the CUG is likely the initiation codon for translation of CPm-C.

To determine whether CPm-L mimics TCV-CPm in infectivity and symptom modulation by sat-RNA C, A. thaliana ecotypes Col-0 and Di-0 were inoculated with TCV, TCV-CPm, and CPm-L, with or without sat-RNA C. Symptoms were assessed visually at various times up to 17 days p.i. As shown in Fig. 2C, CPm-L, like TCV-CPm, was infectious on both Col-0 and Di-0, and unlike TCV, which only infects Col-0. In addition, symptoms of CPm-L were delayed by 1–2 days compared with wt TCV, similar to the symptom delay from inoculation with TCV-CPm. Coinfection with sat-RNA C resulted in 70% of the plants inoculated with CPm-L remaining either symptomless (Col-0) or exhibiting attenuated symptoms (Di-0) at 17 days p.i. (Fig. 2C; plants that did not have attenuated symptoms exhibited systemic symptoms similar to those of plants inoculated with only the genomic RNA). No symptoms developed in either CPm-T or CPm-O-infected plants, with or without sat-RNA C (data not shown). Because TCV requires its CP for systemic movement in plants (Hacker et al., 1992), the inability of CPm-T to infect plants was most likely due to the lack of CP synthesized during the infection.

Taken together, these results suggest that the CUG six nucleotides upstream of the wt initiation codon is the most likely initiation codon used in translating CPm-C. Because methionine appears to be the initiating amino acid in all noncanonical translation initiations investigated so far (Gupta and Patwardhan, 1988; Curran and Kolakofsky, 1988; Hann et al., 1988; Peabody, 1989), there probably are two additional amino acids (glutamic acid and threonine) at the N-terminus of CPm-C compared with CPW (Fig. 3A). These additional amino acids could account for the slightly slower migration of CPm-C compared with CPW, which is visible on most polyacrylamide gels (see Fig. 3B, for example).

### TCV producing wt levels of CP with two additional N-terminal amino acids abolishes symptom attenuation by sat-RNA C

To determine whether lack of detectable virions and symptom attenuation by sat-RNA C when associated with TCV-CPm is due to the two putative amino acids at the N-terminus of the CPs and/or the reduced levels of CP, the effect of wt levels of mutant CP on symptom modulation and virion formation were investigated. To obtain wt levels of this mutant CP, nucleotides specifying the two extra amino acids likely found in CPm-C were inserted after the initiator AUG of the CP ORF in wt TCV generating TCV-CPm3 (Fig. 3A).

Transcripts synthesized in vitro were inoculated onto A. thaliana protoplasts, and total RNA extracted at 40 h p.i. was subjected to RNA gel blot analysis. As shown in Fig. 3B, TCV-CPm3 replicated to near wt levels (note that the TCV-CPm3 sample was underloaded according to the level of rRNA). Western blot analyses of total protein and virion preparations at 40 h p.i. revealed that TCV-CPm3 produced levels of CP comparable to wt TCV and virions were synthesized (Fig. 3B). The CP of TCV-CPm3 comigrated with CPW at a position slightly slower than CPW (Fig. 3B), supporting the hypothesis that CPm-C contains similar additional amino acids at its N-terminus. Because CPm-C was capable of forming virions, the additional residues at the N-terminus do not by themselves abrogate virion formation.

TCV-CPm3 caused more severe stunting on ecotype Col-0 than wt TCV or TCV-CPm and was able to overcome the resistance of Di-0 to wt TCV (Fig. 3C). Symp-
toms developed concomitantly with those of wt TCV (1–2 days earlier than those of TCV-CPm), probably from either increased CP levels or the presence of virions. Although TCV-CPm3 mimicked TCV-CPm in overcoming the resistance of Di-0 to TCV, coinoculation with sat-RNA C neither attenuated nor intensified the symptoms of TCV-CPm3 (Fig. 3C). These results indicate that the N-terminus of the CP is important in the resistance of Di-0 to TCV and suggest that the N-terminal mutations by themselves do not abrogate virion formation or cause symptom attenuation of TCV-CPm by sat-RNA C.

Reduced levels of CP<sub>wt</sub> cause symptom attenuation by sat-RNA C

Because the presence of additional amino acids at the N-terminus of the CP do not lead to symptom attenuation by sat-RNA C, the reduced amount of CP synthesized from a noncanonical initiation codon may be the cause of symptom attenuation. If so, then reducing the synthesis of CP in wt TCV infections should affect sat-RNA C symptom modulation.

To reduce the synthesis of CP<sub>wt</sub>, a GC base pair in the 1.45-kb sgRNA promoter (Wang et al., 1999) was replaced with a weaker GU base pair generating TCV-SGm (Fig. 4A). The mutation did not alter the p9 ORF where the promoter is located. Five percent of wt levels of the 1.45-kb sgRNA was synthesized according to RNA gel blots of total RNA extracted from TCV-SGm-infected protoplasts 40 h after inoculation with transcripts of TCV, CPm, and CPm3 (Fig. 4B). Western blot analysis of total protein extracted from TCV-SGm-infected protoplasts at 40 h p.i. revealed CP<sub>wt</sub> levels similar to those produced by TCV-CPm (Fig. 4B). This result suggests that translation initiation from the wt initiator AUG is more efficient than the nearby noncanonical initiator CUG. Virions were also markedly reduced in TCV-SGm-infected protoplasts (Fig. 4B), suggesting that the amount of CP accumulating in cells is near a threshold level for virion assembly.

To determine whether TCV-SGm mimics TCV-CPm in
infectivity and symptom modulation by sat-RNA C. *Arabidopsis thaliana* ecotypes Col-0 and Di-0 were inoculated with transcripts of TCV, TCV-CPm, and TCV-SGm, with or without sat-RNA C. Like TCV-CPm, TCV-SGm infected both Col-0 and Di-0 plants, suggesting that virus with reduced levels of CP overcomes the resistance of Di-0 (Fig. 4C). Symptoms of TCV-SGm on both ecotypes developed concomitantly with TCV-CPm and were slightly more severe than symptoms of TCV-CPm; Di-0 plants infected with TCV-SGm were more stunted and Col-0 plants exhibited increased chlorosis than when infected with TCV-CPm. Although sat-RNA C attenuated the symptoms of 80% of plants inoculated with TCV-SGm at 17 days p.i. in both ecotypes (plants that did not have attenuated symptoms exhibited TCV-SGm-like symptoms; data not shown), symptomless plants never recovered. This result was in contrast with TCV-CPm/sat-RNA C-infected Col-0, in which 70% of plants were symptomless at 17 days p.i.. This result suggests that although reduced CP levels strongly influence sat-RNA-mediated symptom attenua-

**FIG. 4.** Effect of reduced levels of wt CP on symptom modulation by sat-RNA C. (A) Schematic representation of the mutation (C → U) introduced into the 1.45-kb sgRNA promoter to produce TCV-SGm (SGm). The core promoter sequence defined previously (Wang et al., 1999) is boxed. The transcription initiation site for the CP mRNA is indicated by an arrow. (B) Comparison of the accumulation of viral RNAs, CP, and virions in protoplasts infected with transcripts of TCV, TCV-CPm (CPm), or TCV-SGm (SGm). Abbreviations are described in legend to Fig. 2. Infection of protoplasts and protein gel blots of CP or virions was performed as described in legend to Fig. 2. (C) Symptoms of Col-0 or Di-0 plants inoculated with transcripts of TCV-SGm (SGm), with (+C) or without sat-RNA C. Inoculation and photography of the plants were performed as described in legend to Fig. 2.
tion, the effect is more pronounced if the CP contains additional amino acids at its N-terminus.

**DISCUSSION**

Previous studies (Kong et al., 1995) demonstrated that sat-RNA C was able to attenuate the symptoms of TCV when the CP initiation codon was changed from AUG to ACG (TCV-CPm; Kong et al., 1997b). Because lower levels of possibly mutant CP were produced, it was not clear if the attenuation of symptoms by sat-RNA C was due to the reduced levels of CP or the mutant nature of the CP. To address the nature of CP_{CPm}, mutations were introduced into two possible noncanonical in-frame initiation codons in TCV-CPm: the ACG that replaced the wt AUG initiation codon and an upstream in-frame CUG. Converting the ACG to a codon not used for translation initiation (construct CPm-L) did not affect the synthesis of CP, whereas mutating the CUG triplet (construct CPm-T) eliminated CP synthesis (Fig. 2B). This suggests that CP_{CPm} most likely initiates from the upstream CUG and contains additional glutamic acid and threonine residues at its N-terminus (Fig. 3A). The presence of these two additional residues correlates with the slightly slower mobility of CP_{CPm} compared with CP_{WT} (Figs. 2B, 3B, and 4B). TCV-CPm3, a mutant virus with glutamic acid and threonine codons inserted after the wt CP initiation codon, synthesized wt levels of a CP that likely has the same two residues at its N-terminus as CP_{CPm} and comigrates with CP_{CPm} on denaturing polyacrylamide gels (Figs. 3A and 3B). Although TCV-CPm3 mimicked TCV-CPm in overcoming the resistance of Di-0 to TCV, symptoms were no longer attenuated by sat-RNA C (Fig. 4C). This suggests that the two additional residues are not sufficient to induce symptom attenuation by sat-RNA C.

An alternative explanation, which cannot be ruled out, is that we have not yet determined the correct initiation codon for CP_{CPm} and that alteration of the CUG destabilizes the CP. However, this possibility is unlikely because no known noncanonical initiation codons are found any farther upstream before encountering an in-frame termination codon.

To determine whether the reduced levels of CP found in protoplasts infected with TCV-CPm and CPm-L are responsible for symptom attenuation by sat-RNA C, wt TCV genomic RNA was altered to produce less CP_{WT} mRNA (the 1.45-kb sgRNA). TCV-SGm synthesized reduced levels of CP_{WT} in protoplasts, and symptoms were partially attenuated by sat-RNA C in both ecotypes of A. thaliana (Fig. 4C). This was in slight contrast with TCV-CPm, whose symptoms could be completely attenuated by sat-RNA C in Col-0 and partially attenuated in Di-0 (Figs. 2C, 3C, and 4C). These results suggest that the mutant nature of CP_{CPm} contributes to the ability of sat-RNA C to completely attenuate viral symptoms.

The TCV CP is dispensable for replication in pro-
sat-RNA region involved in symptom modulation. The disappearance or amelioration of TCV symptoms in the presence of sat-RNA C may therefore seem to echo the cosuppression phenomenon found in transgenic plants. Cosuppression occurs when the introduction of a transgene encoding part of or the entire coding sequence of a particular host gene can suppress expression of the transgene and the endogenous host gene (reviewed by Montgomery and Fire, 1998). However, the mechanism underlying symptom attenuation by sat-RNA C is unlikely to be due to cosuppression for the following reasons: first, sat-RNA C-mediated resistance of A. thaliana to TCV-CPm only moderately affects the level of genomic RNA in inoculated leaves (Kong et al., 1997b), which is different from the substantial amount of RNA degradation during cosuppression (Lee et al., 1997); second, another normally virulent subviral RNA, defective interfering (DI) RNA G, has greater sequence similarity (94%) with the TCV genomic RNA sequence in the 3′-terminal region (Kong et al., 1997a). However, although DI RNA G attenuates the symptoms of TCV-CPm, it does not attenuate the symptoms of TCV-CPm.

Different sat-RNAs may use different mechanisms to attenuate symptoms. In many hosts, disease attenuation by CMV sat-RNAs is accompanied by a reduction in virus accumulation (Kaper and Tousignant, 1977; Kaper and Collmer, 1988). In contrast, CMV sat-RNA symptom attenuation of tomato aspermy virus is not always accompanied by a noticeable decrease in the level of viral RNAs (Moriones et al., 1992). Although the attenuation of TCV-CPCCFV symptoms by sat-RNA C was associated with a substantial reduction in virus replication (Kong et al., 1997b), sat-RNA C attenuation of TCV-CPm symptoms did not involve a large reduction in virus accumulation but rather was associated with a reduction in virus movement (Kong et al., 1997b). Because virions are not detected in TCV-CPm-infected protoplasts, some other type of viral RNA-CP complex could be engaged in virus movement. Symptom attenuation by sat-RNA C of TCV variants producing reduced levels of CP could therefore result from sat-RNA C competing with the viral genomic RNA for limited amounts of CP, thus reducing or eliminating genomic RNA–CP complexes required for systemic movement of the virus.

A second possibility for how sat-RNA C may attenuate symptoms involves sequestration of factor or factors required for virus movement. Recently, studies have led to suggestions that host factors are involved in trafficking viruses from phloem parenchyma cells into phloem sieve elements and back out, steps required for long-distance virus movement (Schaad and Carrington, 1996; Gilbertson and Lucas, 1996). The 3′ end region of sat-RNA C that has recently been shown to be responsible for symptom attenuation (Wang and Simon, manuscript in preparation) may be targeted by either the viral CP or a putative host factor (X) that is involved in virus long-distance movement (Fig. 5). Regardless of the nature of the interaction between the CP and the 3′ region of sat-RNA C (i.e., direct or indirect), the presence of high levels of viral CP (as in TCV- or TCV-CPm3-infected plants) may exclude the binding of X to the 3′ end of sat-RNA C but not sat-RNA D (satD) and competes for binding with a putative host factor "X" (indicated by large black circles) that is involved in virus long-distance movement. (A) When high levels of CP are present, CP outcompetes "X" for binding to the 3′ end of satC, leaving "X" available for virus long-distance movement (i.e., systemic infection). (B) When low levels of CP are present, "X" outcompetes the CP for binding to the 3′ end of satC, and sequestration of "X" by satC restricts virus movement and results in attenuation of symptoms. (C) "X" outcompetes non-TCV CP (indicated by small hatched circles), as present in TCV-CPCCFV-infected plants, for binding to the 3′ end of satC, resulting in similar symptom modulation as shown in B.

The susceptibility of Di-0 to TCV-CPm and TCV-CPm3 suggests that the N-terminus of the CP is important for the resistance of Di-0 to TCV. In addition, the susceptibility of Di-0 to TCV-SGm suggests that reducing the levels of wt CP can also eliminate resistance. In addition to its role in resistance, the TCV CP also contributes to expression of symptoms. The symptoms of TCV-CPm3
were more severe than those of TCV in Col-0, even though similar levels of viral RNA and CP were synthesized in protoplasts (Fig. 3). This suggests that mutations at the N-terminus of the CP exacerbate symptoms. The multifunctional nature of viral CPs may be due to multiple determinants that can be hidden (or exposed) by changes in the structure or quantity of the protein. This property of CPs has been demonstrated for tobacco mosaic virus: a reduction of subunit interactions was hypothesized to expose a site on the CP that interacts with the N" gene product of tobacco, resulting in hypersensitive resistance (Culver et al., 1994). It is also possible that virosomes themselves elicit more severe symptoms, whereas the CP monomer does not elicit such a response. This could explain why TCV, TCV-CPm3 (Fig. 3), and TCV-SGm (Fig. 4) caused more severe symptoms than TCV-CPm and CPm-L (Fig. 2).

MATERIALS AND METHODS

Virus strains and plasmid constructions

Plasmids containing full-length cDNAs of TCV (pT7TCVms; Oh et al., 1995), sat-RNA C (pT7satC(+) ; Song and Simon, 1994), and TCV-CPm (pT7TCV-CPm; Kong et al., 1997) downstream from a T7 RNA polymerase promoter have been described.

For construction of plasmids CPm-L, CPm-T, and CPm-O, oligonucleotide CPm-LTO, containing degenerate nucleotides in its sequence (all the oligonucleotides are listed in Table 1), was used with oligonucleotide OL3270C(+) in a polymerase chain reaction (PCR) using pT7TCV-CPm as template. A second PCR was performed with oligonucleotide OL2736C(−) as template. A second PCR was performed with oligonucleotide OL3270C(−) containing degenerate nucleotides in its sequence (all the oligonucleotides are listed in Table 1), was used with oligonucleotide OL3270C(+) in a polymerase chain reaction (PCR) using pT7TCV-CPm as template. A second PCR was performed with oligonucleotides 1.7 LB and OL2736C(+) . The PCR products were digested with BsmI and EcoRI, respectively, and the larger fragments were purified and combined. The mixture was then ligated to pT7TCVms that had been previously digested with BsmI and EcoRI. Plasmid TCV-CPm3 was generated in a similar fashion except that oligonucleotide CPm-LTO was replaced with CPm3C(−). Plasmid TCV-SGm was generated in a similar fashion except that the template and oligonucleotide CPm-LTO were replaced with pT7TCVms and OL2604C(−), respectively, and the second PCR was performed with oligonucleotides SGmC(+) and RV-1C(−).

Plant growth and inoculations

Plants (A. thaliana) ecotypes Col-0 and Di-0 were grown in growth chambers at 20° C as described by Li and Simon (1990). Plant seedlings at the six- to eight-leaf stage were mechanically inoculated on the oldest leaf pair as described previously (Kong et al., 1997b) with 0.1 mg/ml full-length transcripts synthesized in vitro from cloned cDNAs using T7 RNA polymerase (Carpenter et al., 1995). For experiments examining the effects of sat-RNA C on symptom modulation, 0.01 mg/ml of full-length transcripts synthesized from the cloned cDNA of sat-RNA C was included in the inoculum.

Preparation and inoculation of A. thaliana protoplasts

Protoplasts were prepared from Col-0 callus cultures as described (Kong et al., 1997b). Protoplasts (5 × 10⁶) were inoculated with 20 μg of genomic RNA transcripts synthesized in vitro as previously described (Kong et al., 1997b).

Protein gel blot analysis

Total proteins were extracted from protoplasts by vortexing the cells in an equal volume of extraction buffer [125 mM Tris-HCl, pH 6.8, 0.1% SDS, and 20% glycerol (v/v)] followed by centrifugation at 10,000 rpm for 5 min in a microcentrifuge to collect the supernatants. Total protein or isolated virosomes were separated on either 12% SDS-polyacrylamide gels or 1% agarose gels, respectively, containing 50 mM Tris base/38 mM glycine, pH 8.3, as previously described (Heaton, 1992). Total protein or virosomes were transferred to NitroPlus membrane (Micron Separations Inc., Westborough, MA) as previously de-
scribed (Kong et al., 1997b). Protein gel blot analysis was performed as described by Ausubel et al. (1987) with some modifications as described by Kong et al. (1997b).

**Virion isolation and analysis**

Viruses were isolated from infected protoplasts as previously described (Qu and Morris, 1997) with modifications described by Kong et al. (1997b). Virus particles were analyzed by electrophoresis through 1% agarose gels prepared in 50 mM Tris base/38 mM glycine, pH 8.3, as previously described (Laakso and Heaton, 1993) followed by protein gel blot analysis as described above.

**RNA gel blot analysis**

Four micrograms of total RNA isolated from protoplasts (Simon et al., 1992) were denatured by heating in 50–70% formamide and then subjected to electrophoresis through 1.5% agarose gels. RNA was followed by protein gel blot analysis as described above.


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