

# Satellite RNA–Mediated Resistance to Turnip Crinkle Virus in *Arabidopsis* Involves a Reduction in Virus Movement

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**Satellite RNAs (sat-RNAs) are parasites of viruses that can mediate resistance to the helper virus. We previously showed that a sat-RNA (sat-RNA C) of turnip crinkle virus (TCV), which normally intensifies symptoms of TCV, is able to attenuate symptoms when TCV contains the coat protein (CP) of cardamine chlorotic fleck virus (TCV-CP<sub>CCFV</sub>). We have now determined that sat-RNA C also attenuates symptoms of TCV containing an alteration in the initiating AUG of the CP open reading frame (TCV-CP<sub>m</sub>). TCV-CP<sub>m</sub>, which is able to move systemically in both the TCV-susceptible ecotype Columbia (Col-0) and the TCV-resistant ecotype Dijon (Di-0), produced a reduced level of CP and no detectable virions in infected plants. Sat-RNA C reduced the accumulation of TCV-CP<sub>m</sub> by <25% in protoplasts while reducing the level of TCV-CP<sub>m</sub> by 90 to 100% in uninoculated leaves of Col-0 and Di-0. Our results suggest that in the presence of a reduced level of a possibly altered CP, sat-RNA C reduces virus long-distance movement in a manner that is independent of the salicylic acid–dependent defense pathway.**

## INTRODUCTION

Plants exhibit different types of resistance to plant viruses, including induced resistance and satellite RNA (sat-RNA)–mediated resistance. In many cases of induced resistance, a host resistance gene product (R) is thought to interact directly or indirectly with a specific viral gene product (Avr), leading to localized cell death at the initial sites of infection (the hypersensitive response [HR]) and the accumulation of salicylic acid and pathogenesis-related (PR) proteins (White and Antoniwi, 1991; Dawson and Hilf, 1992; Whitham et al., 1994). Induced resistance, also known as *R-Avr* gene–dependent resistance (Hammond-Kosack and Jones, 1996), results in virus limitation to the inoculated leaf, which may be a consequence of the HR (Dawson and Hilf, 1992; Takahashi et al., 1994). However, activation of plant defenses is not always associated with a discernible HR (Köhm et al., 1993; Callaway et al., 1996), suggesting that factors other than localized cell death may be involved in virus restriction, such as salicylic acid–mediated interference with viral replication (Chivasa et al., 1997).

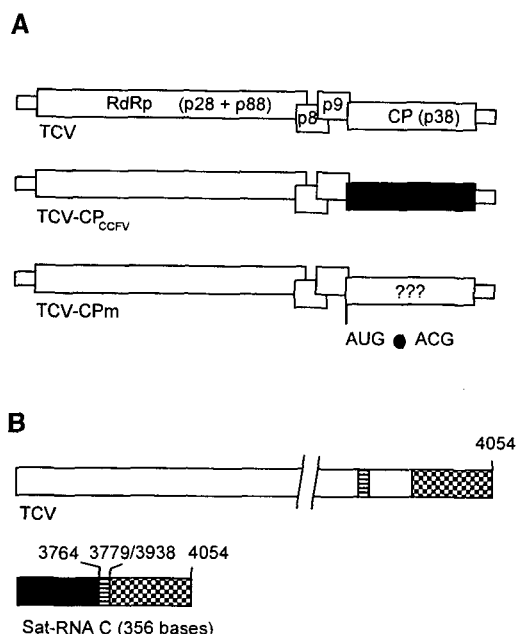
Sat-RNAs are small parasitic RNAs associated with some plant viruses and frequently are able to modify the symptoms induced by their helper virus (Roossinck et al., 1992). Small sat-RNAs (194 to 400 bases) do not encode proteins (Roossinck et al., 1992) and thus are completely dependent

on virus association for replication and spread in plants. Symptom attenuation (resistance) mediated by sat-RNAs is widely attributed to an inhibition of helper virus replication due to competition for limited replication factors between the helper virus genome and the sat-RNA (Collmer and Howell, 1992; Wu and Kaper, 1995). However, sat-RNA–mediated resistance is not always accompanied by reduced virus accumulation in plants (Harrison et al., 1987), indicating the possible involvement of more than one mechanism.

Turnip crinkle virus (TCV) is a single-stranded, positive-sense RNA virus that has a genome of 4054 bases (Carrington et al., 1989; Oh et al., 1995). As diagrammed in Figure 1A, TCV (use of “TCV” or viruses derived from TCV will refer to the viral genomic RNA) encodes five proteins: p28 and its readthrough product p88 are required for virus replication (Hacker et al., 1992; White et al., 1995); p8 and p9 are required for virus movement; p38 is the viral coat protein (CP), which appears to be required for both cell-to-cell and long-distance movement (Hacker et al., 1992; Laakso and Heaton, 1993).

TCV is pathogenic on all ecotypes of *Arabidopsis* tested, with the exception of ecotype Dijon (Di-0) (Li and Simon, 1990; Simon et al., 1992). Typical TCV symptoms on susceptible ecotypes include stunted, twisted stems and regions of chlorosis on rosette leaves. The resistant ecotype Di-0 contains a single dominant resistance gene that maps to chromosome 5 (Dempsey et al., 1997), and TCV-inoculated Di-0 plants accumulate increased levels of salicylic acid and PR proteins when compared with control plants (Uknes et al., 1993; Dempsey et al., 1997). TCV-CP<sub>CCFV</sub>, a TCV derivative

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**Figure 1.** Viral and Subviral RNAs Used in This Study.

**(A)** Viral genomic RNAs. Thick rectangles represent ORFs and thin rectangles represent untranslated regions. TCV sequences are in white and CCFV sequences are in black. TCV-CP<sub>CCFV</sub> has the CP ORF from CCFV. TCV-CPm has a point mutation in the initiating codon (AUG to ACG) of the TCV CP ORF; the question marks indicate the undetermined sequence at the N terminus of the TCV-CPm CP. RdRp, RNA-dependent RNA polymerase.

**(B)** Sequence composition of sat-RNA C. Similar sequences between sat-RNA C and TCV are shaded alike. The broken rectangle in TCV denotes that the representation of TCV is not drawn to scale. The blackened portion of sat-RNA C is very similar to a second TCV sat-RNA, sat-RNA D. Numbers denote positions of the TCV-related sequence.

that contains the CP of the related virus cardamine chlorotic fleck (Figure 1A; Kong et al., 1995), is able to systemically infect Di-0, indicating that the TCV CP or the CP open reading frame (ORF) is the viral component involved in the resistance response (Oh et al., 1995).

TCV is associated with a family of subviral RNAs that includes sat-RNA C (356 bases), a chimeric RNA composed of a second TCV sat-RNA (sat-RNA D; 194 bases) at the 5' end, and two regions of TCV at the 3' end (Figure 1B; Simon and Howell, 1986). By containing a substantial portion of genomic RNA sequence, sat-RNA C is atypical of most sat-RNAs, which share little sequence similarity with their helper virus (Roossnick et al., 1992). Sat-RNA C intensifies the symptoms of TCV on all hosts for which TCV induces visible symptoms (Li and Simon, 1990). For example, when TCV is inoculated with sat-RNA C onto ecotype Columbia (Col-0) plants, young leaves fail to expand, and plants develop a

spreading necrosis that results in plant death within 21 days postinoculation (DPI). Surprisingly, sat-RNA C eliminated the symptoms of Col-0 and Di-0 plants inoculated with TCV-CP<sub>CCFV</sub>, and no viral genomic RNA was detected in uninoculated leaves (Kong et al., 1995). These results indicate that the CP or the CP ORF, in addition to being the viral factor involved in induced resistance, is also involved in sat-RNA-mediated resistance.

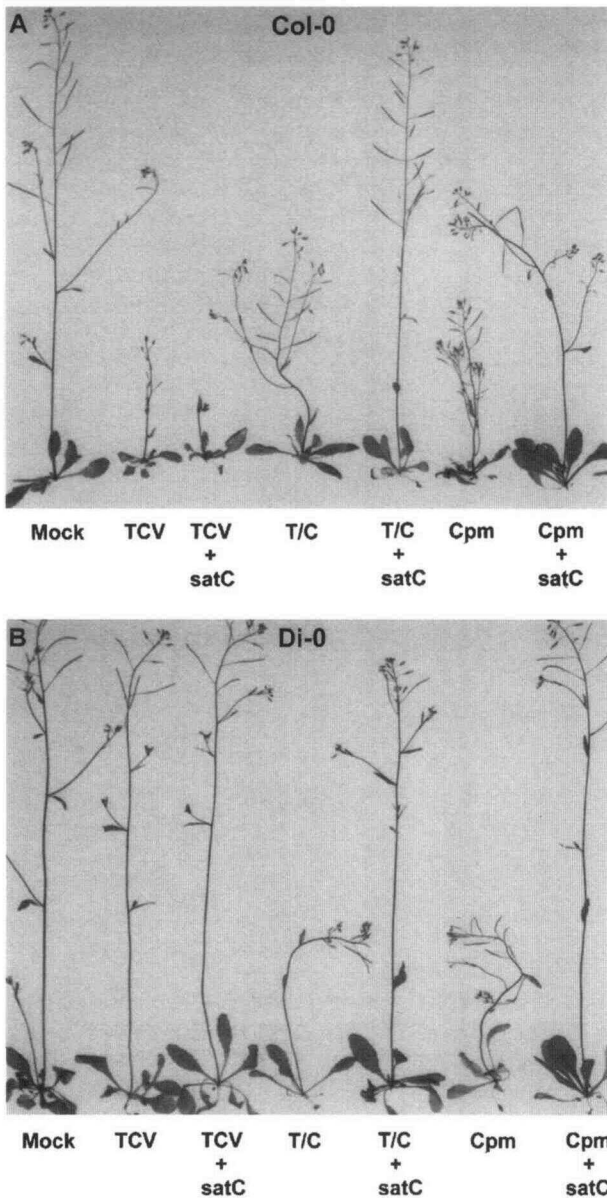
In this study, we determined that TCV containing an alteration in the initiating AUG of the CP ORF (TCV-CPm) produced a reduced level of CP in infected plants. The mutant CP did not assemble into virions that could be isolated by using standard procedures, although TCV-CPm was able to move systemically in the TCV-susceptible ecotype Col-0. TCV-CPm was able to overcome the resistance of Di-0 to TCV, and symptoms on both Col-0 and Di-0 were attenuated by sat-RNA C. Our results suggest that sat-RNA C-mediated symptom attenuation of TCV-CPm involves a reduction in virus long-distance movement, whereas resistance of Di-0 to TCV involves a restriction in cell-to-cell movement. In addition, unlike the resistance of Di-0 to TCV, sat-RNA C-mediated resistance does not involve the salicylic acid-dependent defense pathway.

## RESULTS

### TCV with a Mutation in the Initiation Codon of the CP ORF Can Systemically Infect Di-0 Plants

The TCV CP, in addition to its role in encapsidation and movement, inhibits the replication of sat-RNA C while enhancing the accumulation of sat-RNA D (Kong et al., 1995, 1997). To study the effect of CP on replication, the initiation codon of the TCV CP ORF was changed from AUG to ACG, producing TCV-CPm (Figure 1A). TCV-CPm produced systemic symptoms on the TCV-susceptible ecotype Col-0 and the TCV-resistant ecotype Di-0, as shown in Figures 2A and 2B. This result was unexpected in light of studies indicating a need for the CP in both short- and long-distance movement (Hacker et al., 1992; Laakso and Heaton, 1993). To examine whether the AUG-to-ACG mutation had reverted in progeny RNAs accumulating in TCV-CPm-infected plants, total RNA was isolated from infected leaves and subjected to reverse transcription-polymerase chain reaction. Of the 47 clones sequenced from Di-0 and Col-0 plants, all maintained the AUG-to-ACG alteration, and there were no mutations in the flanking 300 bases (data not shown). In addition, transcripts from a second independent clone of full-length TCV-CPm cDNA produced similar symptoms on Col-0 and Di-0 plants, suggesting that any second-site mutations in the TCV-CPm cDNA putatively produced during the cloning process were not responsible for the infectivity of TCV-CPm on Di-0 (data not shown).

TCV-CPm symptoms on Col-0 and Di-0 plants were similar to TCV symptoms, except that plants were frequently



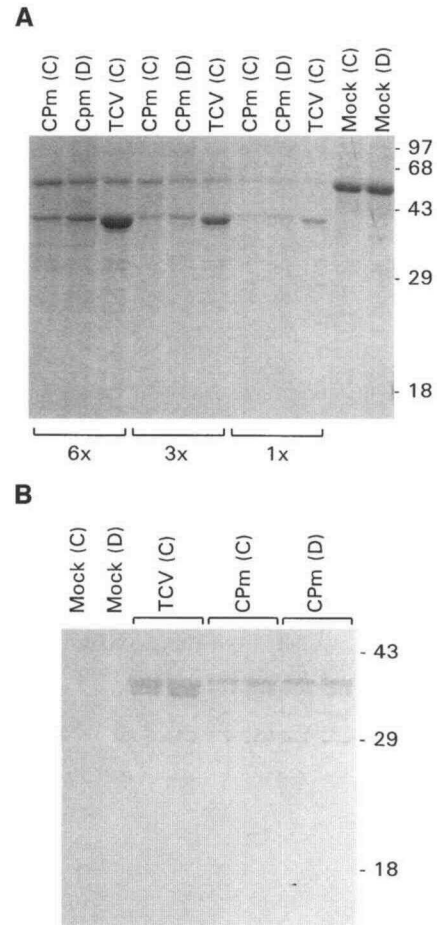
**Figure 2.** Symptoms of Plants Inoculated with TCV or TCV-Derived Viruses, with or without Sat-RNA C.

**(A)** Col-0 plants.

**(B)** Di-0 plants.

Seedlings at the six- to eight-leaf stage were inoculated with the helper virus/sat-RNA C combinations listed below the plants. Mock plants were treated with inoculation buffer alone. Representative plants were photographed at 17 DPI. T/C, TCV-CP<sub>CCFV</sub>; Cpm, TCV-CPm; satC, sat-RNA C.

bushy with tightly curled cauline leaves. Symptoms appeared on TCV-CPm-infected Col-0 and Di-0 plants 1 to 2 days later than on TCV-infected Col-0 plants, suggesting that TCV-CPm moved more slowly than did TCV. Because TCV and TCV-CPm differ by only a single nucleotide, the TCV CP, as opposed to the RNA encoding the CP, is the most likely elicitor of resistance to wild-type TCV in Di-0 plants.

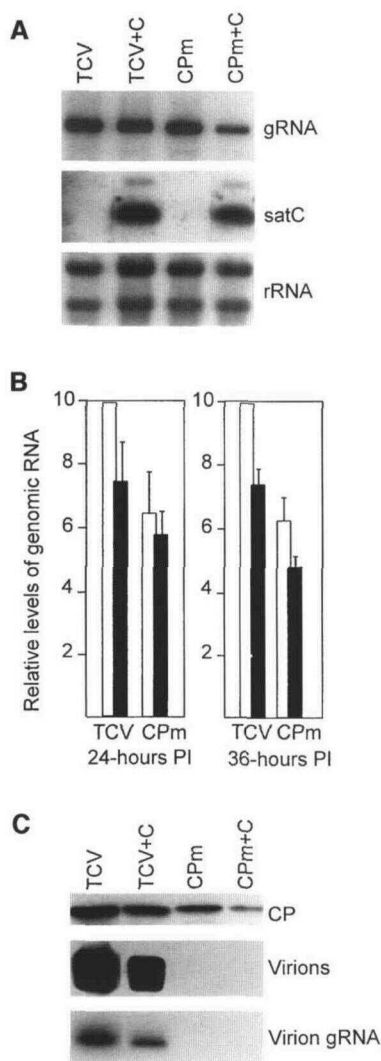


**Figure 3.** Accumulation of TCV and TCV-CPm CPs in Plants.

Total protein was isolated from individual Col-0 (C) or Di-0 (D) plants 21 DPI with transcripts of TCV or TCV-CPm (CPm) and subjected to electrophoresis. The migration positions and molecular mass of marker proteins (in kilodaltons) are shown at right. Letters within parentheses denote proteins that were isolated from Col-0 or Di-0 plants.

**(A)** Coomassie Brilliant Blue R 250-stained gel. One, three, or six volumes of the extracts were loaded per lane, as indicated below. The amount of mock extract loaded corresponds to the amount in the lanes containing six volumes of extract.

**(B)** Protein gel blot analysis. Equal volumes of total protein extracts were subjected to gel blot analysis using antibodies raised against the TCV CP. Duplicate samples were analyzed.



**Figure 4.** Effect of Sat-RNA C on the Accumulation of TCV and TCV-CPm RNA, CP, and Virions in Protoplasts.

Col-0 protoplasts were inoculated with transcripts of TCV or TCV-CPm (CPm) in the presence or absence of sat-RNA C (C), and samples were collected at 24 or 36 hr PI.

**(A)** RNA gel blot analysis. Two micrograms of total RNA isolated from protoplasts at 36 hr PI was subjected to RNA gel blot analysis and sequentially hybridized with probes for TCV genomic RNA (gRNA), sat-RNA C (satC), and rRNA. Sat-RNA C is present in monomeric (major species) and dimeric forms.

**(B)** Levels of genomic RNA and sat-RNA C accumulating in protoplasts at 24 or 36 hr PI. Data from five (left) or four (right) independent experiments were averaged, and standard errors are indicated. White bars and black bars indicate viral genomic RNA levels in the absence or presence of sat-RNA C, respectively.

**(C)** Protein gel blots of total proteins (top) or virions (center) accumulating in protoplasts 36 hr PI with transcripts of TCV or TCV-CPm, with or without sat-RNA C. CP and virions were visualized by chemiluminescence using the anti-TCV CP antibody. Each lane represents total proteins or virions extracted from  $2.5 \times 10^5$  or  $8.3 \times 10^5$  proto-

### TCV-CPm Produces Reduced Levels of CP and No Detectable Virions

To analyze the nature of the CP synthesized in TCV-CPm plants, total proteins were extracted from TCV-CPm-infected Col-0 and Di-0 plants and compared with total proteins extracted from TCV-infected Col-0 plants. As shown in Figure 3A, protein from TCV-CPm-infected Col-0 and Di-0 plants included an abundant species of  $\sim 38$  kD that migrated to a position similar to that of the wild-type TCV CP. This protein, present at  $\sim 20\%$  of the wild-type level, cross-reacted with antibodies raised against the TCV CP (Figure 3B). To determine whether virions could be isolated from TCV-CPm-infected cells, Arabidopsis protoplasts were prepared from Col-0 callus cultures and inoculated with transcripts of TCV or TCV-CPm with or without sat-RNA C. As shown in Figures 4A and 4B, TCV-CPm RNA accumulation was 63% of that of the wild-type TCV at 36 hr postinoculation (PI), whereas the amount of TCV-CPm CP accumulating was  $\sim 30\%$  of that of the wild type (Figure 4C, top), which is similar to the percentage found in plants. Virions containing encapsidated RNA were readily isolated from TCV-infected protoplasts, whereas virions were not detected in samples isolated from TCV-CPm-infected protoplasts (Figure 4C, center and bottom). The inability to isolate a comparable level of TCV-CPm virions based on the level of CP in cells suggests that the CP produced by TCV-CPm is not wild type in nature but rather contains an altered N terminus. Because the nearest in-frame AUG in the TCV CP ORF is located 40 amino acids downstream and any upstream AUG triplets in all three frames are followed by stop codons, CP synthesis probably initiates in the vicinity of the ACG that replaced the wild-type initiation codon or at another non-AUG codon (see Discussion).

### Sat-RNA C Attenuates Symptoms Produced by TCV-CPm

Sat-RNA C intensifies the symptoms of TCV and attenuates the symptoms of TCV-CP<sub>CCFV</sub>, suggesting that the CP or CP ORF is involved in sat-RNA C-mediated symptom modulation (Kong et al., 1995; Oh et al., 1995). To determine the effect of sat-RNA C on the symptoms of TCV-CPm, Col-0 and Di-0 plants were inoculated with transcripts of sat-RNA C and TCV, TCV-CP<sub>CCFV</sub>, or TCV-CPm as the helper virus. Inclusion of sat-RNA C in the inoculum substantially lessened symptoms produced by TCV-CPm on  $\sim 70\%$  of Col-0 and Di-0 plants (Figure 2; plants that did have attenuated symptoms exhibited symptoms similar to plants inoculated with only TCV-CPm). As expected, all Col-0 or Di-0 plants inocu-

plasts, respectively. At bottom, a duplicate of the gel shown at center was treated with 6% formaldehyde and subjected to RNA gel blot analysis to visualize the genomic RNA encapsidated by the virions.

lated with TCV-CP<sub>CCFV</sub> were nearly or completely symptomless when sat-RNA C was included in the inoculum (Figure 2; Kong et al., 1995). Sat-RNA C attenuation of TCV-CPm and TCV-CP<sub>CCFV</sub> symptoms was in marked contrast to the normal intensification of TCV symptoms by sat-RNA C on all inoculated Col-0 plants. As described above, Di-0 plants were resistant to TCV in the presence or absence of sat-RNA C. Because TCV, TCV-CPm, and TCV-CP<sub>CCFV</sub> differ only in their CPs, the CP must be an important factor in determining whether sat-RNA C intensifies or attenuates the symptoms of the helper virus.

### Sat-RNA C-Mediated Symptom Attenuation Involves a Reduction in Virus Long-Distance Movement

To determine whether sat-RNA C attenuation of TCV-CPm symptoms in Col-0 and Di-0 plants was due to an effect of the sat-RNA on virus replication, the effect of sat-RNA C on accumulation of virus genomic RNA in protoplasts was determined. At 24 hr PI, the presence of sat-RNA C reduced the level of TCV-CPm by an average of 10% compared with an average reduction in TCV levels of 25% (Figure 4B, left). At 36 hr PI, sat-RNA C reduced the level of TCV-CPm by 23% compared with a reduction of TCV levels of 25% (Figures 4A and 4B, right). These results suggest that sat-RNA C-mediated attenuation of TCV-CPm symptoms was not due to a substantial reduction in the replication/stability of TCV-CPm.

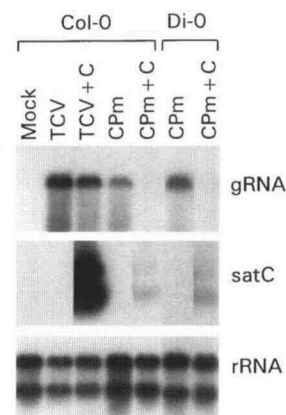
To determine whether sat-RNA C-mediated attenuation of TCV-CPm symptoms could be correlated with a decrease in virus accumulation in plants, Col-0 and Di-0 seedlings were inoculated on the oldest leaf pair with transcripts of TCV or TCV-CPm, with or without sat-RNA C. As shown in Figure 5, no TCV-CPm was detected in extracts of whole Col-0 or Di-0 plants at 16 DPI in the presence of sat-RNA C. However, a trace amount of sat-RNA C was found in Col-0 and Di-0 plants, suggesting that a low level of TCV-CPm was present in at least some of the pooled plants. Attenuation of TCV-CPm symptoms mediated by sat-RNA C is therefore correlated with a substantial reduction in the level of TCV-CPm accumulating in whole plants. Because the presence of sat-RNA C reduced TCV-CPm levels in protoplasts by <25%, these results together suggest that sat-RNA C attenuates symptoms by affecting TCV-CPm movement.

Virus movement involves two distinguishable phases: (1) cell-to-cell movement through plasmodesmata, which involves viral-encoded movement proteins and may require the CP; and (2) long-distance (vascular) movement through the phloem sieve tube network, which may require a second virus movement protein (Cronin et al., 1995; Scholthof et al., 1995), the CP, and host factors independent from cell-to-cell movement (Carrington et al., 1996; Gilbertson and Lucas, 1996; Séron and Haenni, 1996). To determine whether sat-RNA C affects cell-to-cell and/or long-distance movement, the effect of the sat-RNA on virus accumulation in inoculated and uninoculated leaves at early times after inoculation

was examined. Transcripts of TCV, TCV-CPm, or TCV-CP<sub>CCFV</sub> in the presence or absence of sat-RNA C were used to inoculate the oldest leaf pair of Col-0 and Di-0 seedlings at the six- to eight-leaf stage. Inoculated and uninoculated leaves from 15 to 30 plants were separated into pools at 2 to 10 DPI, and total RNA was extracted and subjected to RNA gel blot analysis using probes specific for genomic RNA, sat-RNA C, and rRNA.

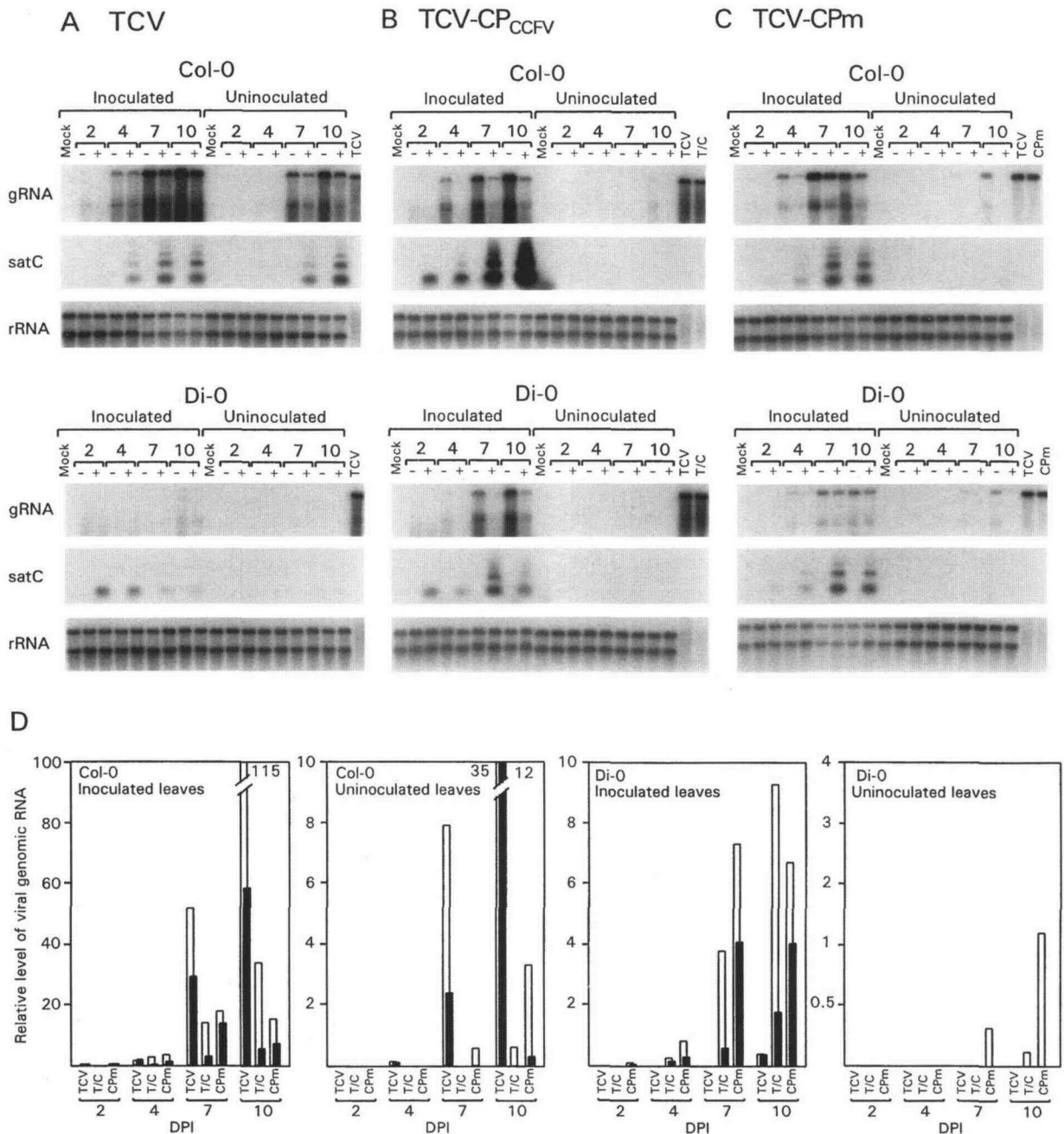
The levels of TCV and sat-RNA C in plants at different days after inoculation are presented in Figure 6A. TCV RNA was detected in inoculated Col-0 leaves at 2 DPI (visible after longer exposure of the autoradiogram) and in uninoculated leaves by 4 DPI. Sat-RNA C reduced the levels of TCV RNA by ~50% in inoculated leaves and 70% in uninoculated leaves at 10 DPI (Figures 6A and 6D), even though symptoms were exacerbated by the sat-RNA C (Figure 2). Only low levels of full-length TCV were detected in inoculated leaves of Di-0 plants by 10 DPI. There was no detectable TCV in uninoculated leaves of Di-0 plants by 10 DPI (Figure 6A), which is consistent with previous reports (Kong et al., 1995; Oh et al., 1995).

TCV-CP<sub>CCFV</sub>-infected Col-0 plants displayed less severe symptoms than did those associated with TCV. These symptoms consisted mainly of mild stunting and bolt curling (Figure 2; Kong et al., 1995). Full-length TCV-CP<sub>CCFV</sub> RNA was detected at 4 DPI in inoculated leaves and at 10 DPI in uninoculated leaves (Figure 6B). Sat-RNA C attenuated TCV-CP<sub>CCFV</sub> symptoms and reduced virus genomic RNA accumulation by 80% in inoculated leaves and 100% in uninoculated leaves. Similar results were found for TCV-CP<sub>CCFV</sub>-infected Di-0 plants, except that virus genomic RNA levels



**Figure 5.** Effect of Sat-RNA C on the Accumulation of TCV and TCV-CPm RNA in Whole Plants.

Two micrograms of total RNA isolated from three pooled Col-0 or Di-0 plants inoculated 16 days previously with TCV or TCV-CPm (CPm) transcripts with or without sat-RNA C (C) was subjected to RNA gel blot analysis. Blots were sequentially hybridized with probes for TCV genomic RNA (gRNA), sat-RNA C (satC), and rRNA.



**Figure 6.** RNA Gel Blot Analysis of Viral RNA and Sat-RNA C Accumulation at Early Times after Inoculation of Col-0 and Di-0 Plants.

(A) Col-0 and Di-0 plants inoculated with TCV.

(B) Col-0 and Di-0 plants inoculated with TCV-CP<sub>CCFV</sub>.

(C) Col-0 and Di-0 plants inoculated with TCV-CPm.

(D) Data normalized from (A) to (C).

Inoculations were with (+) or without (–) sat-RNA C. Mock plants were treated with inoculation buffer alone. Total RNA was isolated from inoculated and uninoculated leaves at 2, 4, 7, and 10 DPI, as indicated. One hundred and fifty nanograms of TCV, TCV-CP<sub>CCFV</sub> (T/C), or TCV-CPm (CPm) transcripts was loaded in the right-hand lane(s), as indicated. Blots were hybridized sequentially with probes specific for genomic RNA (gRNA), sat-RNA C (satC), or rRNA. The slowest migrating species that hybridizes with the gRNA probe is the full-length viral genomic RNA. Sat-RNA C is present in both monomeric and multimeric species. Levels of sat-RNA C were high when TCV-CP<sub>CCFV</sub> was the helper virus, which has



were four- to eightfold lower than in Col-0 plants (Figures 6B and 6D). Because sat-RNA C caused a 70% decrease in the accumulation of TCV-CP<sub>CCFV</sub> RNA in protoplasts (Kong et al., 1995), the substantial decrease in TCV-CP<sub>CCFV</sub> levels in inoculated leaves mediated by sat-RNA C can be attributed mainly to a restriction in virus replication or stability.

TCV-CPm was detected in inoculated leaves of Col-0 plants by 2 DPI (after longer exposure of the autoradiograms) and in uninoculated leaves by 7 DPI, consistent with the timing of symptom appearance on younger leaves. Sat-RNA C reduced TCV-CPm levels by 24% at 7 DPI and 55% at 10 DPI in inoculated leaves. In uninoculated leaves of Col-0, sat-RNA C reduced the levels of TCV-CPm RNA by at least 90% (Figures 6C and 6D). The presence of trace levels of TCV-CPm in pooled uninoculated leaves of Col-0 10 DPI with TCV-CPm and sat-RNA C is consistent with the observation that only ~70% of the plants were symptomless at 16 DPI.

The overall levels of TCV-CPm in Di-0 plants were two- to threefold lower than in Col-0 plants (Figures 6C and 6D), although symptoms were very similar (Figure 2). Sat-RNA C reduced the level of TCV-CPm RNA in inoculated Di-0 leaves by ~40% and in uninoculated leaves by 100%. The reduced levels of TCV-CPm in inoculated leaves of Col-0 and Di-0 plants due to the presence of sat-RNA C cannot by itself account for the loss of systemic virus movement, because at 7 DPI, TCV-CPm levels in the presence of sat-RNA C were similar to or greater than TCV-CP<sub>CCFV</sub> levels in the absence of sat-RNA C (Figure 6D). This result suggests that a reduction in long-distance movement is also involved in sat-RNA C-mediated resistance to TCV-CPm.

#### Resistance Mediated by Sat-RNA C Does Not Involve the Salicylic Acid-Dependent Defense Pathway

*R-Avr* gene-dependent resistance is frequently correlated with an increase in salicylic acid accumulation in inoculated leaves shortly after pathogen attack and later in uninoculated tissues (Hammond-Kosack and Jones, 1996; Ryals et al., 1996). The increase in salicylic acid levels results in the expression of PR proteins (Bol et al., 1990) and other defense-related products (Crute et al., 1994). The systemic accumulation of salicylic acid and plant defense proteins is believed to confer resistance to further attacks by a broad spectrum of pathogens, a phenomenon known as systemic acquired resistance (SAR; Ryals et al., 1996).

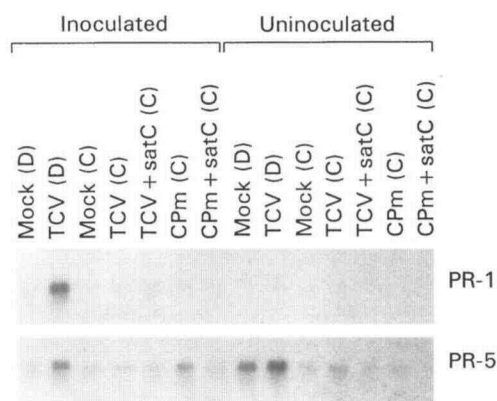
Di-0 plants inoculated with TCV accumulated increased levels of salicylic acid and PR proteins in inoculated leaves and exhibited SAR to subsequent infection by selected viral and bacterial pathogens (Uknes et al., 1993). We examined whether sat-RNA C-mediated resistance involves the same salicylic acid-dependent defense pathway by assaying for (1) SAR against further virus inoculation, (2) PR protein gene expression, and (3) effects of mutants in the salicylic acid-dependent pathway on sat-RNA-mediated resistance.

To examine whether sat-RNA C-mediated resistance confers systemic resistance, Col-0 and Di-0 seedlings at the five- to six-leaf stage were inoculated on the first leaf pair with transcripts of sat-RNA C and either TCV-CPm or TCV-CP<sub>CCFV</sub>. At 4 DPI, the second leaf pair was superinoculated with transcripts of the virus that was not used for the primary inoculation, without sat-RNA C. As a control, plants mock treated on the first leaf pair were inoculated on the second leaf pair with transcripts of one of the two viruses. Symptoms were monitored daily for 16 days after the second inoculation. The results of duplicate experiments indicated that Col-0 and Di-0 plants preinoculated with sat-RNA C and either TCV-CPm or TCV-CP<sub>CCFV</sub> exhibited identical symptoms when compared with control plants pretreated with inoculation buffer (data not shown). Therefore, sat-RNA C-mediated resistance to TCV-CP<sub>CCFV</sub> or TCV-CPm was manifested locally and did not result in SAR against subsequent virus infection.

Di-0 plants inoculated with TCV produce PR proteins in both inoculated and uninoculated leaves (Uknes et al., 1993), which is typical for virus-host combinations that induce SAR (Dempsey and Klessig, 1994; Ryals et al., 1994; Dong, 1995). To determine whether sat-RNA C-mediated resistance involves the expression of PR protein genes, Col-0 seedlings were inoculated with TCV or TCV-CPm in the presence or absence of sat-RNA C. Total RNA isolated from either inoculated or uninoculated leaves at 5 DPI was subjected to RNA gel blot analysis with probes specific for Arabidopsis PR-1 or PR-5 mRNAs (Uknes et al., 1992). As shown in Figure 7, none of the inoculations induced accumulation of either PR mRNA above basal levels, even though most plants were resistant to TCV-CPm because of the presence of sat-RNA C. In control TCV-inoculated Di-0 plants, expression of PR-5 was induced in both inoculated and uninoculated leaves, and expression of PR-1 was induced in inoculated leaves. The lack of increased PR-1 mRNA accumulation in systemically infected Di-0 leaves differs from a previous report (Uknes et al., 1993); this may be

Figure 6. (continued).

been attributed to the absence of the TCV CP (Kong et al., 1995). Densitometric scans of each blot (**D**) were normalized to the rRNA levels of each sample and the TCV transcript level on the same blot. The white and black bars represent full-length viral genomic RNA levels in the absence and presence of sat-RNA C, respectively.



**Figure 7.** Accumulation of PR-1 and PR-5 mRNAs.

Col-0 (C) or Di-0 (D) seedlings were inoculated as indicated or treated with inoculation buffer (Mock). Six micrograms of total RNA isolated from inoculated or uninoculated leaves was loaded per lane and hybridized with probes specific for PR-1 or PR-5 mRNA. CPm, TCV-CPm; satC, sat-RNA C.

due to differing environmental factors that result in the absence of local lesions on most inoculated leaves of Di-0 under our growth conditions (Simon et al., 1992), unlike the study by Uknes et al. (1993), in which local lesions were produced on all inoculated leaves. Nonetheless, our results indicate that although sat-RNA C mediates resistance to TCV-CPm in Col-0 plants, PR protein gene expression is not induced to detectable levels in inoculated or uninoculated leaves, thus supporting the lack of apparent SAR response.

To examine further whether the salicylic acid-dependent defense pathway is involved in sat-RNA C-mediated symptom attenuation, the following Col-0 lines altered in this pathway were tested: (1) transgenic Col-0 plants that express the bacterial *nahG* gene, which encodes the enzyme salicylic acid hydroxylase that degrades salicylic acid to catechol (*nahG* plants accumulate substantially less salicylic acid during an HR and are defective in SAR induction; Gaffney et al., 1993); (2) *npr1* plants that do not express PR proteins even when induced by biological or chemical elicitors (Cao et al., 1994); and (3) *cpr1* and (4) *cpr6* plants that constitutively express PR proteins (Bowling et al., 1994). Plants of these lines and parental control lines (see Methods) were inoculated with TCV, TCV-CP<sub>CCFV</sub>, or TCV-CPm in the presence or absence of sat-RNA C. All lines responded in an identical fashion to control Col-0 plants; that is, all helper viruses produced systemic symptoms, with sat-RNA C intensifying the symptoms of TCV and attenuating the symptoms of TCV-CP<sub>CCFV</sub> and TCV-CPm (data not shown). Taken together, these results strongly suggest that sat-RNA C-mediated symptom intensification or attenuation does not involve the salicylic acid-dependent defense pathway, PR proteins, or SAR.

## DISCUSSION

### The CP Determines TCV Infectivity on Di-0 Plants and Mediates Symptom Modulation by Sat-RNA C

Resistance to pathogens that involves an induction of the host defense pathway requires an interaction between host- and pathogen-encoded products (Ryals et al., 1994; Staskawicz et al., 1995). Restriction of TCV movement in Di-0 plants was obviated by TCV-CPm, which differs from wild-type TCV by a single alteration in the nucleotide sequence of the CP ORF, and TCV-CP<sub>CCFV</sub>, which has the CP ORF from CCFV, a related carmovirus. These results indicate that the TCV CP, and not the RNA encoding the CP, is the elicitor of the host defense response in Di-0 plants. The involvement of viral CPs as elicitors of resistance has also been found in other virus systems (Kavanagh et al., 1992; Köhm et al., 1993; Culver et al., 1994; Taraporewala and Culver, 1996). In addition, CPs have been found to be involved in symptom expression of a number of viruses (Petty and Jackson, 1990; Neeleman et al., 1991; Lindbeck et al., 1992; Shintaku et al., 1992; Takahashi et al., 1993; Banerjee et al., 1995; Rao and Grantham, 1995; Suzuki et al., 1995), including TCV (Heaton et al., 1991; Heaton and Laasko, 1995). This report shows that the CP is also involved in subviral RNA-mediated resistance.

The alteration(s) in the N-terminal region of the CP synthesized from TCV-CPm genomic RNA has not yet been defined. The protein cross-reacted with polyclonal antibodies raised against the TCV CP and migrated to a similar position as wild-type TCV CP in denaturing polyacrylamide gels (see Figures 3 and 4C). Attempts to sequence the N-terminal region of the TCV-CPm CP were unsuccessful because of in vivo modification of the N terminus (data not shown), as was reported for the wild-type TCV CP (Carrington et al., 1987). It is possible that CP translation initiation occurs at an alternative AUG triplet within the 1.45-kb subgenomic RNA (the CP mRNA) of TCV-CPm. There are five in-frame AUG codons in the TCV CP ORF; the nearest downstream AUG is located 40 codons from the normal initiation site. The one upstream "in-frame" AUG triplet is followed by two stop codons. There are also two out-of-frame AUG triplets just downstream of the wild-type initiator AUG codon. None of these AUG triplets is a reasonable candidate for initiation of protein synthesis because usage either would result in a substantially truncated CP or would require ribosomal read-through of stop codons or frameshifting without the necessary sequence and structural context to perform such events (Rohde et al., 1994).

A second possibility for translation initiation of the TCV-CPm CP is ribosomal usage of a non-AUG triplet. Noncanonical translational initiation has been confirmed or suggested for several cellular mRNAs (Beames et al., 1991; Saris et al., 1991) and viral RNAs with plant (Fütterer et al., 1996; Schmitz et al., 1996) and animal (Mehdi et al., 1990; Reynolds et al., 1995) hosts (reviewed in Gallie, 1993; Rohde



et al., 1994). Because the TCV-CPm CP accumulated to ~10 to 20% of the level of wild-type TCV CP in infected plants and protoplasts, translation of the TCV-CPm CP ORF may initiate at the introduced ACG codon. This would result in a reduced amount of wild-type CP (if initiation occurred using the methionine initiator tRNA), which might be present at a level that is below a threshold for virion production. Alternatively, TCV-CPm may produce an altered CP incapable of virion formation by initiating translation at a non-AUG triplet in the vicinity of the mutated initiation codon. Because the packaging signal for TCV is within a 186-base region at the 3' end of the CP ORF (Qu and Morris, 1997), it is unlikely that the single base change in TCV-CPm RNA is itself responsible for the lack of recovered virions from infected cells.

### The Mechanism of Symptom Modulation by TCV-Associated Subviral RNAs

Unlike TCV-induced resistance in Di-0, sat-RNA C-mediated resistance of Arabidopsis to TCV-CPm only moderately affected the level of genomic RNA in inoculated leaves. However, the sat-RNA was found to substantially or completely restrict the movement of TCV-CPm into uninoculated leaves of Col-0 and Di-0. It is unlikely that a decrease of less than twofold in TCV-CPm levels in inoculated leaves is sufficient by itself to account for the complete or nearly complete lack of virus accumulation in uninoculated leaves; the level of TCV-CPm remaining in inoculated Col-0 leaves in the presence of sat-RNA C is still greater than is the level of TCV-CPm in inoculated Di-0 leaves where the virus establishes a systemic infection. In addition, TCV-CPm accumulation in inoculated leaves of Col-0 and Di-0 in the presence of sat-RNA C was similar to or greater than the level of TCV-CP<sub>CCFV</sub> in the absence of sat-RNA C (Figure 6D), although in the former case, most plants are resistant, and in the latter case, the virus moves systemically. Taken together, these results strongly suggest that sat-RNA C affects the long-distance movement of TCV-CPm in Arabidopsis. Sat-RNA C also reduced the accumulation of TCV-CPm in protoplasts less than in inoculated leaves of plants (23% in Col-0 protoplasts; an average of 35 and 46% in Col-0 and Di-0 leaves, respectively, over the course of the experiment). This result suggests the possibility that sat-RNA C may also affect cell-to-cell movement of TCV-CPm.

Sat-RNA C was able to attenuate symptoms of all plants inoculated with TCV-CP<sub>CCFV</sub> (Kong et al., 1995). The percentage of decrease in TCV-CP<sub>CCFV</sub> accumulation in inoculated leaves and in protoplasts due to the presence of sat-RNA C was not substantially different (70% inhibition in protoplasts, 80% inhibition in Col-0, and 64% inhibition in Di-0 inoculated leaves over the course of the experiment). This suggests that sat-RNA-mediated repression of TCV-CP<sub>CCFV</sub> synthesis may be more important than is restriction in movement in limiting TCV-CP<sub>CCFV</sub> to the inoculated leaf. However, it is possible that an additional effect of sat-RNA C on virus

long-distance movement is responsible for the restriction of the remaining 20% of the virus to the inoculated leaf.

Plants can have several defense response pathways that are distinguishable by their dependence or independence on the induction of salicylic acid (Malamy et al., 1996). To determine whether the salicylic acid-dependent pathway is responsible for sat-RNA C-mediated resistance, we tested a number of Col-0-derived Arabidopsis plants containing mutations in this pathway. Our results showed that these mutant plants behaved like the wild type with respect to symptoms, and by analogy, systemic movement of TCV, TCV-CP<sub>CCFV</sub>, and TCV-CPm in the presence of sat-RNA C. In addition, there was no PR protein mRNA accumulation above basal levels in inoculated leaves of Col-0 infected with TCV-CPm and sat-RNA C and no SAR induction.

If the salicylic acid-dependent pathway is not involved in sat-RNA C-mediated symptom attenuation, how might sat-RNA C restrict the movement of at least TCV-CPm to the inoculated leaf? We recently showed that a second TCV subviral RNA, DI RNA G, which shares a similar but not identical 3' terminal segment with sat-RNA C, is able to intensify symptoms of TCV and to attenuate symptoms of TCV-CP<sub>CCFV</sub> but cannot attenuate symptoms of TCV-CPm (Kong et al., 1997). By making chimeric subviral RNAs between sat-RNA C and DI RNA G, the terminal 3' 53 bases of sat-RNA C were shown to be responsible for symptom attenuation (Kong et al., 1997).

One possible model for symptom attenuation by DI RNA G and sat-RNA C is if the wild-type CP outcompetes a putative host factor (X) for binding to the similar 3'-terminal 53 bases of sat-RNA C and DI RNA G. Binding of X to the 3' ends of these subviral RNAs in the absence of CP may induce a host defense response, leading to restriction of virus movement and resulting in symptom attenuation. When TCV-CP<sub>CCFV</sub> is the helper virus, the CCFV CP (which shares only 50% identical residues with the TCV CP; Oh et al., 1995) does not recognize the 3' end of sat-RNA C and DI RNA G, thus allowing X to bind and resistance to be induced. When TCV-CPm is the helper virus, reduced levels of either wild-type CP or mutant CP (depending on the identity of the TCV-CPm CP) affects binding to sat-RNA C but not DI RNA G. As a consequence, X has more access to the 3' end of sat-RNA C than does DI RNA G, resulting in symptom attenuation for sat-RNA C but not for DI RNA G.

Recent studies have led to proposals that host factors are involved in trafficking viruses from phloem parenchyma cells into phloem sieve elements and back out. These steps are required for long-distance virus movement (Gilbertson and Lucas, 1996; Schaad and Carrington, 1996). One possibility is that X is a host factor involved in long-distance movement and that sequestration by binding to sat-RNA C prohibits its normal trafficking functions. Alternatively, sat-RNA C in the absence of the wild-type CP may induce an active form of resistance that is independent of salicylic acid and PR protein synthesis. Efforts to differentiate between these possibilities are currently under way.

## METHODS

### Virus Strains and *Arabidopsis thaliana* Lines

Plasmids containing full-length cDNAs of satellite RNA C (sat-RNA C) (Song and Simon, 1994), turnip crinkle virus (TCV) (TCVms; Oh et al., 1995), and TCV with the coat protein (CP) of cardamine chlorotic fleck virus (TCV-CP<sub>CCFV</sub>) (Oh et al., 1995) downstream from T7 RNA polymerase promoters have been described previously. TCV with a single base alteration of the initiation codon of the CP open reading frame (ORF) (TCV-CPm) was generated by site-directed mutagenesis (Kunkel, 1985) using the oligonucleotide primer 5'-AACACTGGA-AACGGAAATGATC-3' (the U-to-C alteration is underlined). The mutation changed the initiation codon of the CP ORF to ACG and maintained the amino acid sequence of the slightly overlapping p9 ORF. Seeds for nahG (transgenic Columbia [Col-0] plants expressing the bacterial *nahG* gene), BGL2GUS (transgenic Col-0 plants expressing the  $\beta$ -glucuronidase gene under the control of the pathogenesis-related PR-2 gene promoter), and BGL2GUS-derived mutants *npr1* (nonexpresser of pathogenesis-related [PR] proteins) and *cpr1* and *cpr6* (constitutive expressers of PR proteins) plants were kindly provided by X. Dong (Duke University, Durham, NC).

### Plant Growth and Inoculations

*Arabidopsis* plants were grown in growth chambers as described by Simon et al. (1992). Transcripts synthesized in vitro from cloned cDNAs using T7 RNA polymerase were used to inoculate plants (Simon et al., 1992). For experiments examining the effect of sat-RNA C on virus accumulation and movement, Col-0 and Dijon (Di-0) seedlings at the six- to eight-leaf stage were inoculated on the oldest leaf pair with 300 ng per leaf of TCV, TCV-CP<sub>CCFV</sub>, or TCV-CPm transcripts in the presence or absence of 30 ng of sat-RNA C transcripts. For superinoculation experiments, Col-0 and Di-0 seedlings at the five- to six-leaf stage were inoculated on the first leaf pair with sat-RNA C and either TCV-CPm or TCV-CP<sub>CCFV</sub> or with inoculation buffer alone; 4 days later, the same plants were inoculated on the second leaf pair with TCV-CPm if TCV-CP<sub>CCFV</sub> was used for the first inoculation, and vice versa.

### Reverse Transcription-Polymerase Chain Reaction

Three micrograms of total RNA isolated from TCV-CPm-infected Col-0 or Di-0 plants was mixed with 50 pmol of an oligonucleotide complementary to positions 3033 to 3050 of the TCV genomic RNA, and the reaction was performed as previously described using Moloney murine leukemia virus reverse transcriptase (Carpenter et al., 1995), except that Pyrostate buffer (Molecular Genetic Resources, Tampa, FL) replaced the Moloney murine leukemia virus reverse transcriptase buffer. For polymerase chain reaction, half of the precipitated cDNA reaction was subjected to 27 cycles of polymerase chain reaction by using the thermal stable enzyme Pyrostate (Molecular Genetic Resources) and primers complementary to positions 3033 to 3050 and homologous to positions 2546 to 2562. The cDNA was sequenced after cloning into the SmaI site of plasmid pBluescript KS- (Stratagene, La Jolla, CA).

### Protein Gel Blot Analysis

Total proteins were extracted from *Arabidopsis* plants or protoplasts by grinding the plant material or 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 proteins or isolated virions 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 proteins were transferred to NitroPlus membranes (Micron Separations Inc., Westborough, MA), as previously described by Ausubel et al. (1987), and virions were transferred to the same membrane, according to the methods of Laakso and Heaton (1993). Protein gel blot analysis was performed as described by Ausubel et al. (1987), with modifications. Antibodies against the TCV CP were generated in rabbits injected with gel-purified CP prepared from purified TCV virions (Zhang, 1995) and used in a 1:1000 dilution. The second antibody (anti-rabbit IgG horseradish peroxidase; Gibco BRL) was used in a 1:4000 dilution. Chemiluminescent staining was performed with the Chemiluminescent LuminoGLO substrate kit (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). Membranes incubated with the substrate were covered with Saran wrap and exposed to x-ray film for several seconds to minutes before developing the film.

### RNA Gel Blot Analysis

For analysis of viral RNA levels in whole plants, three plants per inoculum were pooled. For analysis of viral RNA levels over time in inoculated and uninoculated leaves, leaves were pooled from 15 to 30 plants collected at 2, 4, 7, and 10 days postinoculation (DPI). Total RNA was isolated (Simon et al., 1992), subjected to electrophoresis on nondenaturing agarose gels, and blotted onto nitrocellulose, as described previously (Kong et al., 1995). In addition to samples of total RNA on some gels, 0.15  $\mu$ g of TCV, TCV-CP<sub>CCFV</sub>, or TCV-CPm transcripts was used as a viral genomic RNA standard(s). An oligonucleotide complementary to positions 3892 to 3911 of TCV genomic RNA was used to detect TCV, TCV-CP<sub>CCFV</sub>, or TCV-CPm. Blots were sequentially hybridized with probes for viral genomic RNA, sat-RNA C, and rRNA, as previously described (Kong et al., 1995), and then washed with 6  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS at 30°C for 15 min and then at 4°C below the melting temperature of the oligonucleotide for 3 to 5 min. The autoradiograms were scanned with a densitometer, and viral RNA levels were adjusted according to the level of rRNA.

For analysis of PR protein gene expression, total RNA was isolated from inoculated or uninoculated leaves of Col-0 and Di-0 plants at 5 DPI, and probes were prepared using cDNAs of *Arabidopsis* PR-1 and PR-5 (gifts from J.A. Ryals, Paradigm Genetics, Inc., Cary, NC, and K. Lawton, NOVARTIS Crop Protection, Research Triangle Park, NC) and a random primer DNA labeling system (Gibco BRL). Hybridization and washings were performed as previously described (Simon et al., 1992).

### Preparation and Inoculation of *Arabidopsis* Protoplasts

Protoplasts were prepared from Col-0 callus cultures. Calluses were generated from sterilized seeds plated on 1.0% Murashige and Skoog (Gibco BRL) agar supplemented with 2 mg/mL kinetin and

2 mg/mL 2,4-D and incubated in a growth chamber at 20°C (35  $\mu\text{mol m}^{-2} \text{ sec}^{-1}$  lights under a 16-hr-light, 8-hr-dark cycle). Cultures were passaged every 3 to 4 weeks. Protoplasts were prepared from white, friable callus as described by Ishikawa et al. (1993), with the following modifications. The callus was transferred to 0.6 M mannitol and agitated at 25°C for 20 min before centrifugation at 900g for 5 min; cellulase and pectinase were obtained from Calbiochem (La Jolla, CA); incubation time in enzymes/PIM solution (Ishikawa et al., 1993) was increased to 3.5 to 4 hrs; protoplasts were filtered through 53- $\mu\text{m}$  nylon mesh (Small Parts, Miami Lakes, FL); no additional incubation was required after filtering the protoplasts; and centrifugation in the protoplast washing steps was at 250g for 5 min.

### Virion Isolation and Analysis

Virus particles were isolated from infected protoplasts as previously described (Qu and Morris, 1997), with modifications. Protoplasts ( $5 \times 10^6$ ) were collected and resuspended in 200  $\mu\text{L}$  of 0.2 M sodium acetate, pH 5.2. Sterile glass beads (30  $\mu\text{L}$ ; 0.1 mm diameter) in the same buffer were added, and the mixture was vortexed for three 15-sec intervals. The aqueous phase was recovered after brief centrifugation, and the solid phase was re-extracted twice with 200  $\mu\text{L}$  of 0.2 M sodium acetate, pH 5.2. The aqueous phases were combined and incubated at 0°C for 1 hr. Cell debris was removed by centrifugation at 13,200 rpm for 2 min in a microcentrifuge. The supernatant was combined with one-quarter volume of 40% polyethylene glycol 8000/1 M NaCl and incubated at 0°C for 12 hr. Virions were collected by centrifugation at 13,200 rpm in a microcentrifuge for 1 hr and dissolved in 30  $\mu\text{L}$  of 10 mM sodium acetate, pH 5.5. Samples (5  $\mu\text{L}$ ) 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).

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