MPMI Vol. 8, No. 6, 1995, pp. 979–987. © 1995 The American Phytopathological Society

Open Reading Frames of Turnip Crinkle Virus Involved in Satellite Symptom Expression and Incompatibility with *Arabidopsis thaliana* Ecotype Dijon

Jong-Won Oh[1], Qingzhong Kong[2], Chuanzheng Song[2], Clifford D. Carpenter[2], and Anne E. Simon[2]

[1]Department of Microbiology, [2]Department of Biochemistry and Molecular Biology and Program in Molecular and Cellular Biology, University of Massachusetts, Amherst 01003, U.S.A.

Received 9 January 1995. Accepted 1 August 1995.

Carmoviruses are single-stranded, single component RNA viruses that include turnip crinkle virus (TCV) and the recently discovered cardamine chlorotic fleck virus (CCFV). Full-length, biologically active cDNAs were constructed for the TCV-M isolate and the Blue Lake isolate of CCFV. Using chimeric viruses constructed between isolates of TCV that produce mild or severe symptoms when coinoculated with a virulent satellite RNA, a Glu residue at position 1,144 in the polymerase open reading frame was identified as being involved in satellite-mediated symptom expression. To analyze viral determinants involved in resistance, chimeric viruses with precisely exchanged open reading frames were produced between TCV, which does not infect the *Arabidopsis thaliana* ecotype Dijon (Di-0), and CCFV, which can infect Di-0. TCV with the coat protein of CCFV was able to systemically infect Di-0 although whole plant hybridizations revealed that the hybrid virus spread more slowly than either of the two parental viruses. These results indicate that the coat protein is an important viral determinant in the resistance of Di-0 to TCV.

Additional keyword: virus movement.

Carmoviruses are the smallest and simplest of the single component, single-stranded plant RNA viruses. At slightly over 4,000 bases, all carmoviruses analyzed to date have similar genomic organizations. Commencing with the 5′ end, the carmovirus genomic RNA contains open reading frames (ORFs) that specify the following products: 28 to 29 kDa (p28); 86 to 89 kDa (p88, a readthrough product that also contains p28); 8 kDa (p8); 9 to 10 kDa (p9); and 38 kDa (p38) (Guilley et al. 1985; Carrington et al. 1989; Rivière and Rochon 1990; Skotnicki et al. 1993). The functions of the encoded products have been studied using mutagenized genomic RNA transcripts of turnip crinkle virus (TCV; Fig. 1A). p88 (and possibly p28) are subunits of the RNA-dependent RNA polymerase, while p8 and p9 are required for virus movement (Hacker et al. 1992). p38, the viral coat protein, is required for TCV cell-to-cell movement in *Nicotiana benthamiana* (Heaton et al. 1991) and long-distance movement in *Brassica campestris* (Hacker et al. 1992). The TCV coat protein also contains pathogenesis determinants, as several amino acid alterations resulted in milder symptoms than wild-type on *N. benthamiana* (Heaton et al. 1991). Besides the genomic RNA, TCV is associated with dispensable subviral RNAs such as satellite (sat-) RNAs, which require replicative and encapsidative functions supplied by products of the genomic RNA for infectivity.

TCV has an extensive host range including *Arabidopsis thaliana,* a plant that is increasingly used as a model genetic system for the study of host-pathogen interactions (Dangl 1993). With one exception, all *Arabidopsis* ecotypes tested display moderate symptoms (stunting, bolt curling) when infected with the genomic RNA alone, and severe systemic necrosis when infected with the genomic RNA from the TCV isolate TCV-M and an associated virulent sat-RNA, sat-RNA C (Li and Simon 1990; Simon et al. 1992). The exception, ecotype Dijon (Di-0), displays substantial resistance to viral infection either in the presence or absence of sat-RNA C (Simon et al. 1992). Resistance was determined to involve an inhibition of long-distance virus movement, as viral RNA did not accumulate in younger, uninoculated leaves, and the replication of viral genomic RNA occurred at similar rates and reached similar levels in protoplasts of susceptible and resistant ecotypes (Simon et al. 1992). The resistance response of Di-0 to TCV is also characterized by a localized necrosis in inoculated leaves (Simon et al. 1992; Uknes et al. 1993) and the induction of systemic acquired resistance that is effective against superinfection by bacterial and viral pathogens (Uknes et al. 1993).

To help in identifying TCV genomic RNA determinant(s) involved in host range, an analysis has been initiated on a second carmovirus, the Blue Lake isolate of cardamine chlorotic fleck virus (CCFV-BL). CCFV was recently discovered in the Mount Kosciusko alpine region of Australia in infected *Cardamine lilacina,* a wild perennial brassica (Skotnicki et al. 1993). The Club Lake isolate of CCFV (CCFV-CL), which shares 63% similarity with TCV, was re-

The first two authors contributed equally to this work and should be considered co-first authors.

Corresponding author: Anne E. Simon, Department of Biochemistry and Molecular Biology, LGRT, University of Massachusetts, Amherst 01003, U.S.A.; telephone: 413-545-0170; fax: 413-545-4529; E-mail: simon@biochem.umass.edu

ported to replicate well in *A. thaliana* (Skotnicki et al. 1993). For the current study, biologically active cDNAs for our laboratory isolate of TCV (TCV-M) and the Blue Lake isolate of CCFV (CCFV-BL) were prepared and sequenced. CCFV-BL genomic RNA transcripts produced moderate symptoms similar to those of TCV genomic RNA on all strains of *Arabidopsis* tested including the TCV-resistant ecotype Di-0. When Di-0 plants were inoculated with a chimeric genomic RNA transcript containing mainly TCV sequence but where the TCV coat protein ORF had been precisely excised and replaced with the CCFV-BL coat protein ORF, plants exhibited the moderate symptoms of CCFV. This result suggests that the avirulence determinant(s) on the TCV genomic RNA is likely in the coat protein or coat protein ORF. In addition, chimeric viruses constructed between the TCV-M cDNA, whose transcripts produced severe symptoms in the presence of sat-RNA C and a second cDNA, TCV-B (Heaton et al. 1989), which produced mild symptoms when associated with the sat-RNA, were used to identify a single amino acid in the p88 ORF that was responsible for the satellite-associated differential symptom expression.

RESULTS

The carmovirus CCFV-BL has a host range that differs from TCV.

Since the newly discovered carmovirus CCFV shares sequence similarity and at least one host *(Arabidopsis thaliana;* Skotnicki et al. 1993) with TCV, we were interested in the possible use of TCV and CCFV for the study of host range determinants in carmoviruses. Since additional hosts for CCFV had not been reported, total RNA was isolated from *C. lilacina* plants infected with the Blue Lake isolate (CCFV-BL) and mechanically inoculated onto different cruciferous plants. Total RNA was extracted 2 weeks postinoculation and assayed for viral genomic RNA on ethidium bromide–stained gels. The results, summarized in Table 1, indicated that various subspecies of *Brassica rapa* that are hosts of TCV are not hosts of CCFV-BL. All *A. thaliana* ecotypes tested, however, were susceptible to CCFV-BL, including ecotype Di-0 that is resistant to TCV.

Table 1. Partial host range of Blue Lake isolate of cardamine chlorotic fleck virus (CCFV-BL) and turnip crinkle virus isolate TCV-M

Plant host	CCFV-BL	TCV-M
Brassica rapa[a]		
sp. chinnensis 'Pak Choi Joi Choi'	N[b]	Y[b]
sp. pekinesis 'Green Rocket'	N	N
sp. rapifera 'Tokyo Cross'	N	Y
sp. rapifera 'Just Right'	N	Y
Cardamine lilacina	Y	N
Arabidopsis thaliana ecotype[a]		
Col-0	Y	Y
Di-0	Y	N
La-0	Y	Y
Ag-0	Y	Y
No-0	Y	Y
Raphanus sativus	N	N

[a] Data for infectivity of TCV-M on *Brassica rapa* and *Arabidopsis thaliana* are from Li and Simon (1990).
[b] N = no; Y = yes.

Cloning the genomic RNA of TCV-M and preparation of infectious transcripts.

The use of hybrid viruses to analyze specific virus-host interactions requires biologically active cDNA clones of viruses that retain viability after the exchange of ORFs. The finding that CCFV was more related to TCV than to other carmoviruses (Skotnicki et al. 1993), coupled with the ability of CCFV-BL to systemically infect Di-0, suggested that chimeric viruses between TCV and CCFV might be useful in identifying the viral determinants involved in carmovirus host range.

To construct chimeras between TCV and CCFV, we first needed to prepare full-length, biologically active cDNAs for both viruses. Although a cDNA capable of producing infectious transcripts was available for the isolate TCV-B (Carrington et al. 1989), these transcripts did not induce the same severe symptoms on turnip in the presence of sat-RNA C as did our TCV isolate, TCV-M (data not shown). To obtain a clonal virus population of TCV-M, turnip seedlings were inoculated with low levels of TCV-M, and six individual infection sites (identified as small, round, cleared regions on the abaxial surface of inoculated leaves) were excised and used to inoculate additional seedlings along with sat-RNA C. Three of the newly inoculated plants developed severe symptoms in the presence of sat-RNA C, while the remaining plants had no additional symptoms (data not shown). Viral RNA isolated from a plant with severe symptoms was used to prepare TCV cDNA, TCVms, which was generated from three cDNA fragments (Fig. 1B). Transcripts of TCVms produced infection on 100% of inoculated plants and symptoms were intensified in the presence of sat-RNA C.

Comparison between TCVms and TCV-B.

The nucleotide sequence of TCVms was compared with the previously published TCV-B genomic RNA sequence (Carrington et al. 1989; Collmer et al. 1992; Fig. 1B). TCV-B differed from TCVms at 16 positions (14 base changes, 1 deletion, and 1 insertion) resulting in four amino acid changes. Since TCV-B produced mild symptoms in the presence of sat-RNA C, chimeric viruses were constructed between the TCV-B cDNA (TCV-T1d1) and TCVms to determine the region involved in differential symptom modulation by the sat-RNA (Fig. 1C). Between 15 and 27 turnip plants were inoculated with and without transcripts of sat-RNA C and total RNA from plants inoculated 2 weeks previously with chimeric RNA transcripts. Symptoms, analyzed 2 weeks postinoculation, were scored as "mild" if the addition of sat-RNA C to the inoculum did not affect the slight stunting and vein clearing associated with turnip infected with just the viral genomic RNA. Plants were labeled "severe" if the addition of sat-RNA C resulted in very stunted, crinkled, and dark green plants. All wild-type, chimeric, and modified viruses used for this study accumulated in turnip at approximately equivalent levels as assayed by electrophoresis of total RNA extracted from plants at 2 weeks postinoculation, and produced identical mild symptoms in the absence of sat-RNA C (data not shown). Symptom modulation results from plants infected with reciprocal chimeric constructs in the presence of satRNA C indicated that the region between bases 451 and 1,406, located in the p88 ORF, was responsible for the differential symptom modulation (Fig. 1C). This region of TCV con-

tained a single amino acid difference between TCV-B and TCVms, a Lys or Glu, respectively, at position 1,144.

To determine if the amino acid at position 1,144 influences viral symptom expression in the presence of sat-RNA C, site-directed mutagenesis was used to change the Lys at position 1,144 to a Glu in TCV-B (TCV-B-Glu). Inoculation of six plants with transcripts of sat-RNA C and total RNA isolated from plants infected with transcripts of TCV-B-Glu resulted in all plants with severe symptoms when analyzed 2 weeks postinoculation (data not shown). However, these plants gradually appeared less symptomatic at later stages of the infection, unlike plants infected with sat-RNA C and TCVms, which maintained severe symptoms for many weeks. These results suggest that while a Glu at position 1,144 is involved in severe symptoms in the presence of sat-RNA C, additional differences between TCVms and TCV-B also influence symptom expression at later stages of the infection.

Cloning the genomic RNA of CCFV-BL and preparation of infectious transcripts.

A full-length cDNA of the Blue Lake isolate of CCFV (CCFV-BL) was generated from four partial cDNA clones (Fig. 2A). Sequencing the full-length cDNA of CCFV-BL revealed a genome of 4,072 bases, 27 bases longer than CCFV-CL (Skotnicki et al. 1993). Five-percent differences in the nucleotide sequence between CCFV-CL and CCFV-BL resulted in 10 amino acid changes in p88, including an insertion of nine amino acids at position 31 (Table 2), and 2, 1, and 16 amino acid differences in p8, p9, and the coat protein, respectively (Fig. 3). The products of CCFV-BL ORFs share substantial similarity with TCVms, ranging from 89% for the C-terminal (polymerase) domain of p88 to 61% for p28 (Fig. 2A). Transcripts of CCFV-BL synthesized in vitro were infectious on Col-0 and Di-0 plants, and plants exhibited symptoms identical to plants inoculated with total RNA iso-

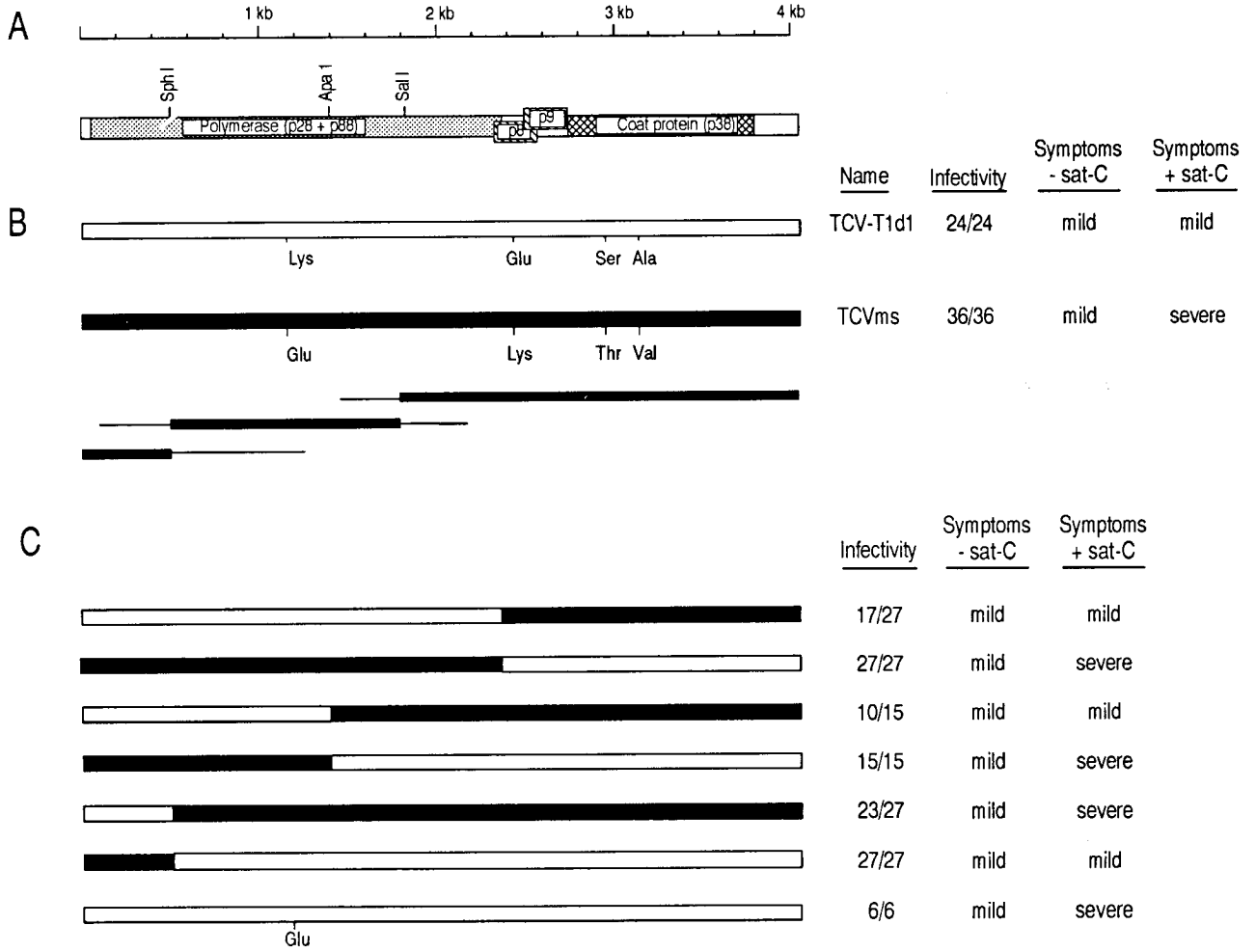

Fig. 1. Cloning full-length cDNA of TCV-M and analysis of determinants involved in infectivity and symptom production in the presence of sat-RNA C. **A,** Open reading frames of TCV. Relevant restriction enzyme sites involved in the construction of full-length cDNA clones are shown. **B,** Comparison of two full-length TCV cDNA clones used in this study. TCV-T1d1 was cloned from TCV isolate TCV-B (Carrington et al. 1989; Heaton et al. 1989). TCVms was generated in this study from the partial clones indicated below the full-length cDNA clone. The thick portions of the partial clones were used in the final full-length construct. All amino acid differences between the two full-length cDNAs are indicated. Infectivity of the transcripts was assayed using turnip plants. Symptoms produced in the presence or absence of sat-RNA C are indicated. **C,** Chimeric viruses used to determine the region involved in the mild versus severe symptom phenotype of TCV-T1d1 and TCVms transcripts in the presence of sat-RNA C. Open and filled bars denote cDNA from TCV-T1d1 and TCVms, respectively. Infectivity of the chimeric transcripts, and symptom production in the presence or absence of sat-RNA C are indicated. Infectivity refers to the number of plants displaying systemic symptoms over the total number of plants inoculated.

lated from CCFV-BL-infected *C. lilacina* plants (data not shown).

TCVms with the coat protein ORF of CCFV-BL (TCV-CP_{CCFV}) is able to systemically infect Di-0.

The TCV coat protein ORF was replaced with the CCFV coat protein ORF using a recombinant polymerase chain reaction (PCR) strategy, generating the chimeric virus TCV-CP_{CCFV} (Fig. 2B, Fig. 4). Col-0 and Di-0 seedlings were inoculated with RNA derived from plants previously inoculated with TCVms, CCFV-BL or TCV-CP_{CCFV} transcripts, and plants photographed 3 weeks later (Fig. 5). As described previously, Col-0 plants inoculated with TCVms contain bolts that are stunted and twisted while, on average, 95% of inoculated Di-0 plants are symptomless and the remainder display only very mild bolt curling (Simon et al. 1992). Col-0 inoculated with CCFV-BL or TCV-CP_{CCFV} also contained stunted and twisted bolts, with symptoms appearing somewhat less severe than those produced by TCV infection. Di-0 plants inoculated with CCFV-BL displayed moderate stunting and bolt curling, similar to symptoms found for CCFV-BL–infected Col-0 plants. Di-0 plants inoculated with TCV-CP_{CCFV} exhibited the moderate symptoms of CCFV-BL–infected plants.

RNA gel blot analysis was performed to quantitate the levels of TCVms, CCFV-BL, and TCV-CP_{CCFV} in inoculated and uninoculated leaves of Col-0 and Di-0 plants at 10 days postinoculation (DPI) (Fig. 6). In inoculated leaves of Col-0,

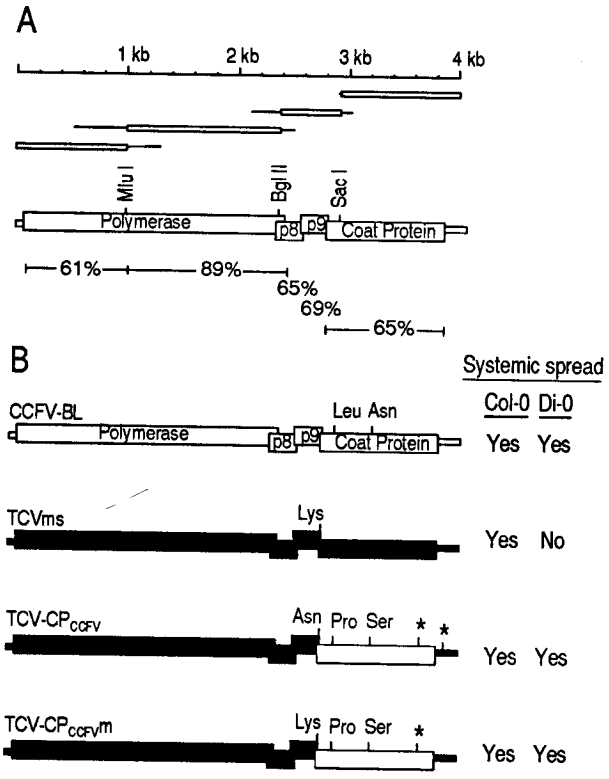

Fig. 2. Cloning full-length cDNA of CCFV-BL and analysis of chimeric viruses derived from CCFV-BL and TCVms. **A,** Partial cDNA clones and relevant restriction enzyme sites used in the generation of a full-length cDNA for CCFV-BL. The thick portions of the partial clones were used in the final full-length construct. Percent similarities between CCFV-BL and TCVms open reading frames (ORFs) are indicated. **B,** Chimeric constructs with precisely exchanged ORFs that were generated between CCFV-BL and TCVms. Open and filled regions denote cDNA from CCFV-BL and TCVms, respectively. Generation of TCV-CP_{CCFV} required the conversion of the C-terminal residue of p9 from Lys to Asn. Other amino acid alterations and two additional nucleotide changes (denoted by asterisks) resulting from the cloning of TCV-CP_{CCFV} are indicated. Infectivity of transcripts derived from the constructs on *Arabidopsis thaliana* ecotypes Col-0 and Di-0 is shown.

Table 2. Differences between cardamine chlorotic fleck virus isolates CCFV-CL and CCFV-BL in the p88 open reading frame[a]

Position	CCFV -CL	CCFV-BL
23	I	L
31	Deletion	QQICDEEAG
37	E	A
72	R	S
98	V	I
153	V	W
386	G	R
387	G	D
388	S	Deletion
689	L	I

[a] One letter amino acid code is used.

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P8                                                              49
CL     MDNQSESDNS VKRRSGKKVN KQVRGSEKSA TKRSVASHAA RS-FSQRPSG
BL     .......... ..........S .....I.... .......... ..........
TCVms  ..PERIPY.. LS--DSDATG .RKK.GK... K..L...... S.VLNKKRNE
                                71
CL     DSA--GGNWV LVADKIEVSI SFNF
BL     .......... .......... ....
TCVms  G..SH..T.. .....V.... N...

P9                                                              50
CL     MQLARSVNAH LAILLGVIGF WLLIRLKFQY PSISDQLPVP SPWVKYLILS
BL     .......... .......... .......... .......... ....E.....
TCVms  --------MK VLLVT..L.L L...KW.S.S T.T.N.-KCQ C.TSPWV.YA
                                                            94
CL     SFNSLSLVLL LCHLIPDIKP AVTYYNTTDN TKSQHISIST ANGN
BL     .......... .......... .......... .......... ....
TCVms  FY........ ......E... IH.S...H.S S.Q.....N. G..K

Coat Protein                                                    50
CL     MAIKEDPRLV KMASLGVPWA VKVTTKGWGS LTTKQKIAAR AALQIPMRVT
BL     .......... .......... .......... .......... ..........
TCVms  --MEN...VR .F..D.AQ.. I.WQK...ST ..SR..QT.. ..MG.KLSPV
                                                           100
CL     KQPTPKVVRV SAPAAVSYRE VTGAPRVTTA GKVLTHTASE LITTLKKNTS
BL     ......I... .......... .......... .......... ..........
TCVms  A..VQ..T.L ...V.LA... ..TQ...S.. RDGI.RSG.. .........D
                                                           150
CL     LEPKYVEAIV NPSRVGTFNQ LPDIAASYEK YKFTSLRFRY SSTCPTSTGG
BL     ...M...... .......... .......... .......... ..........
TCVms  T....TT.VL ...EP..... .IKE.VQ... .R........ .PMS.ST...
                                                           200
CL     KVALAFERDA ANPLPDNLTA FYNLEGNTSS NPRDSFVLTV PCDNVARFVA
BL     .......... .......... .......... .......... .....V....
TCVms  ......D... .K.P.ND.AS L..I..CV.. V.WTG.I... .TDSTD....
                                                           250
CL     DGGSDPKLVD FGKLLAASYG QGEGDANGLG EIHIEYTVQL YNRLGSAQRS
BL     .......... .......... .......... .......... ..........
TCVms  ..I....... ....IM.T.. ..AN..AQ.. .VRV...... K..T..TSDA
                                                           297
CL     QIGSLDGKFL GPRLVEVTSG KSDKGTKFTF SFHGCGL--- IPILWLAKPR
BL     .......... .......AA. ..E..... ......VLHP ..VVSEG-.V
TCVms  ...DFA.VKD .....SWSKT .GTA.WEHDC HFL.T.NF-S LTLFYEKA.V
                                                           347
CL     WPLTAGSAVA CEVLGQSGDD SVQWAGIRIS EREQTVEFEH TETTPEAKWS
BL     ....V..... ......P... .......... .......... ..........
TCVms  SG.ENAD.SD FS...EAAAG ......VKVA ..G.G.KMVT ..EQ.KG..Q
         351
CL       VSRL
BL       ....
TCVms    AL.I
```

Fig. 3. Amino acid sequence comparison among CCFV-CL (CL), CCFV-BL (BL) and TCVms for open reading frames p8, p9, and the coat protein. The CCFV-CL sequence is from Skotnicki et al. (1993). Dots denote amino acid identity with the CCFV-CL sequence. Dashes denote deletions.

TCV-CP_{CCFV} accumulated 9% more than TCVms, and twofold more than CCFV-BL. Uninoculated Col-0 leaves contained 50% of the inoculated leaf level of TCV-CP_{CCFV}, which was less than the 66 and 80% levels for CCFV-BL and TCVms, respectively. In inoculated leaves of Di-0, TCVms accumulated to only 30% of the level found in inoculated Col-0 leaves, and was undetectable in uninoculated Di-0 leaves, as previously found (Simon et al. 1992). In contrast, CCFV-BL accumulated 27% more in inoculated Di-0 leaves than TCVms and 23% of this level was present in uninoculated leaves. TCV-CP_{CCFV} accumulated twofold higher in inoculated Di-0 leaves than did TCVms, and was present in high levels (41% of the inoculated leaf level) in uninoculated leaves of Di-0.

Whole plant in situ hybridizations were performed at various times postinoculation of a single oldest leaf, to monitor genomic RNA levels directly in the infected plant (Simon et al. 1992). Our current protocol for detection of RNA viruses in situ differs in two respects from the previous version: use of oligonucleotides as probes, and baking plants before prehybridization. Both changes substantially increase the signal to noise ratio. In addition, the initial ethanol and pronase treatment of the plants has been altered to minimize RNA degradation. The results, presented in Figure 7, indicate that TCVms was detectable in the inoculated leaf of Col-0 by 2 DPI and had spread to the younger leaves by 4 DPI. By 8 to 10 DPI, TCVms was detected throughout the younger leaves of Col-0, but in older leaves viral RNA was either not detected, or confined to the primary vein, or in the primary vein and sectors at the base of older leaves. In contrast, TCVms was first detected in the inoculated leaf of Di-0 by 4 DPI and was not detected outside the inoculated leaf by 10 DPI. CCFV-BL was first detectable by 3 to 4 DPI of Col-0 or Di-0 plants and moved more slowly than TCVms, requiring between 4 and 6 days to move into uninoculated leaves of both ecotypes. As with TCVms, CCFV-BL was either not detected in older leaves at 8 to 10 DPI or confined to the vascular system or clearly defined sectors at the base of the leaves.

TCV-CP_{CCFV} was also first detected in the inoculated leaf of Col-0 or Di-0 by 3 to 4 DPI. However, subsequent movement of TCV-CP_{CCFV} was slower than either TCVms or CCFV-BL, requiring between 6 and 8 days for detection in uninoculated leaves. In addition, at 8 to 10 DPI, TCV-CP_{CCFV} was only detected in the four youngest leaves (in addition to the inoculated leaf), indicating a slower or poorer ability to migrate into older leaves than that of the parental viruses. This result suggests that the susceptibility of Di-0 plants to TCV-CP_{CCFV}

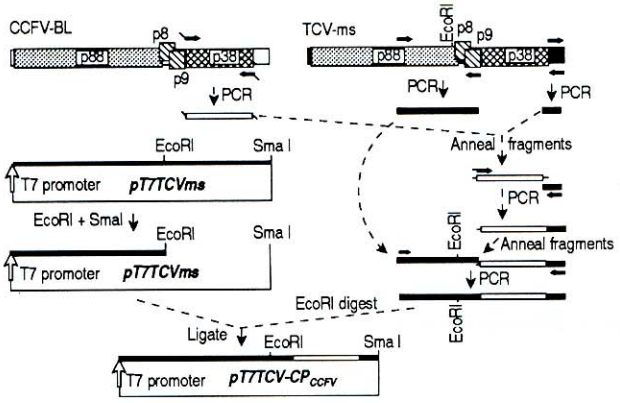

Fig. 4. Recombinant polymerase chain reaction (PCR) strategy used in the construction of TCV-CP_{CCFV}. Positions of oligonucleotides used in the construction and directions of elongation are indicated above and below the representations of the genomic RNAs at the top of the figure. Fragments were joined by annealing overlapping PCR products as described in Materials and Methods.

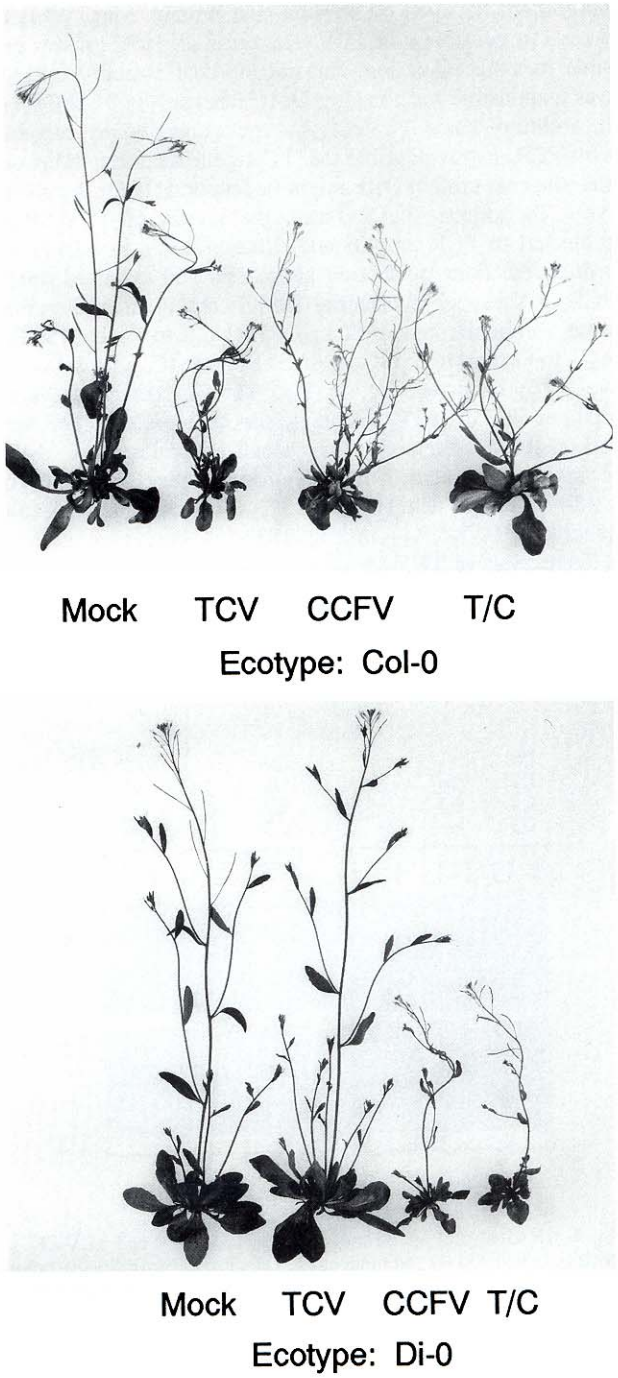

Fig. 5. Symptoms of *Arabidopsis thaliana* ecotypes Col-0 and Di-0 3 weeks postinoculation with TCVms, CCFV-BL, or TCV-CP_{CCFV} (T/C). Plants were inoculated with RNA isolated from *A. thaliana* infected with the viral RNA transcripts indicated below each plant. Plants labeled "Mock" were rubbed with the inoculation buffer alone.

is not due to a more rapid rate of spread of the chimeric virus in general, but must involve other interactions between virus and host.

The TCV coat protein and/or its gene is involved in resistance of Di-0 to TCV.

Replacing the TCVms coat protein ORF with the analogous ORF from CCFV also resulted in changing the C-terminal amino acid of p9 from Lys to Asn, due to overlapping ORFs between p9 and the coat protein. Since p9 is involved in movement of TCV (Hacker et al. 1992), it was possible that this alteration, and not the coat protein exchange, was responsible for breaking Di-0 resistance by TCV-CP_{CCFV}. In addition, since TCV-CP_{CCFV} was generated by recombinant-PCR, it was possible that PCR-generated mutations outside the coat protein ORF might be responsible for the phenotype. To address these issues, the region of TCV-CP_{CCFV} subjected to PCR amplification (bases 2,371 to 4,054) was sequenced. Four nucleotide alterations had occurred due to PCR or subsequent bacterial amplification: three alterations were within the coat protein ORF (U_{3039} to C, Leu to Pro; A_{3222} to G, Asn to Ser; C_{3475} to U) and one (C_{4007} to U) was in the 3′ untranslated region (Fig. 2B). To determine if the changes in TCV-CP_{CCFV} outside the coat protein ORF were responsible for the ability to systemically infect Di-0, the C-terminal amino acid of p9 was changed back to the TCVms wild-type amino acid (Lys) and U_{4007} was changed back to C, generating TCV-CP_{CCFV}m (Fig. 2B).

Transcripts of TCV-CP_{CCFV}m were able to systemically infect both Di-0 and Col-0, producing symptoms indistinguishable from TCV-CP_{CCFV} (data not shown). This result indicates that these two mutations did not contribute to the resistance-breaking property of TCV-CP_{CCFV}. Since the other three mutations in TCV-CP_{CCFV} were in the coat protein ORF, we conclude that either the TCV coat protein or its ORF is associated with the resistance of Di-0 to TCVms.

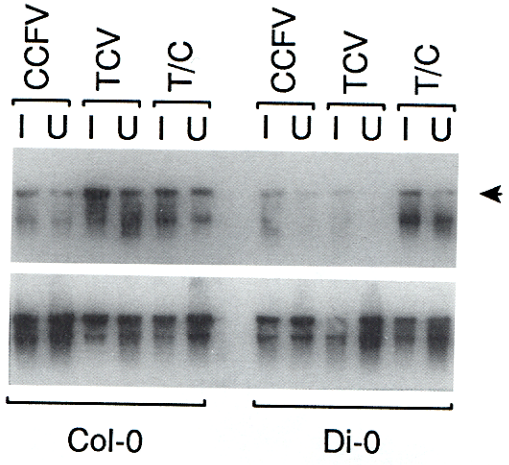

Fig. 6. RNA gel blot analysis of TCVms, CCFV-BL and TCV-CP_{CCFV} levels in inoculated (I) and uninoculated (U) leaves of *Arabidopsis thaliana* ecotype Col-0 and Di-0 plants. RNA derived from transcripts of TCVms, CCFV-BL or TCV-CP_{CCFV} (T/C) was inoculated onto the two oldest leaves of 2-week-old plants (four-expanded-leaf stage). Total RNA was extracted 10 days later, denatured in formamide, and then subjected to electrophoresis through standard nondenaturing 1.2% agarose gels. The gel blot was probed with an oligonucleotide complementary to positions 958 to 978 of TCVms (top) and then re-probed with a ribosomal DNA probe (bottom). The sequences of TCVms and CCFV-BL are identical in the region complementary to the probe. Arrow denotes viral genomic RNA.

DISCUSSION

Differential ability of TCV cDNA clones to produce intensified symptoms in the presence of the virulent TCV-M sat-RNA, sat-RNA C.

The construction of viral chimeras between TCV and CCFV required the cloning of full-length cDNAs for TCV-M and CCFV-BL. The TCV-M isolate is apparently composed of a population of viruses, some of which produce severe symptoms in the presence of sat-RNA C while others produce no additional symptoms. Transcripts synthesized from the full-length cDNA TCVms produced severe symptoms and were fully infectious on all inoculated turnip plants. Using viral chimeras between TCVms and TCV-T1d1, which produces mild symptoms in the presence of sat-RNA C, we determined that the region between nucleotides 451 and 1,406 in the putative viral RNA-dependent RNA polymerase (Hacker et al. 1992) was responsible for the differential symptom expression. In this region, TCV-T1d1 contains a Lys while TCVms contains a Glu. Conversion of the Lys at position 1,144 to a Glu in TCV-T1d1 was sufficient to convert the symptoms produced by TCV-T1d1 from mild to severe when coinoculated with sat-RNA C onto turnip plants. Amino acid identity in the TCV RNA-dependent RNA polymerase at position 1,144 may have affected the level of sat-RNA C accumulating in plants, which could affect symptom expression due to the presence of the sat-RNA. A study published earlier, which was performed contemporaneously with our study, found that TCV-T1d1 with an Asp to Gly alteration at position 1,025 in the RNA-dependent RNA polymerase could also change symptoms from mild to severe in turnip plants coinoculated with sat-RNA C. Symptom intensification was correlated with a faster rate of sat-RNA C accumulation in protoplasts (Collmer et al. 1992).

The coat protein of TCVms affects long-distance movement in *A. thaliana* ecotype Di-0.

TCV requires the coat protein for both cell-to-cell and long-distance movement (Heaton et al. 1991; Laakso and Heaton 1993), while the coat protein requirements of CCFV have not been determined. We previously demonstrated that resistance of Di-0 to TCV-M was at the level of long-distance movement (Simon et al. 1992). Our current results indicating that TCVms does not exit the inoculated leaf of Di-0 by 10 DPI are in slight contrast to previous results that suggested TCVms accumulated in the leaf opposite of the inoculated one by 7 DPI, but did not spread to any other leaves by at least 8 DPI (Simon et al. 1992). The reason for the discrepancy between these results and our current results is unknown, but the discrepancy could reflect differences in growth conditions, since environmental factors were found to influence the number of plants exhibiting complete resistance to viral infection (Simon et al. 1992).

The lack of detectable TCVms in the vasculature of inoculated Di-0 leaves (Simon et al. 1992; Fig. 7, this report) suggests that the block to long-distance movement is in loading of the phloem. One possibility is that while putative receptors

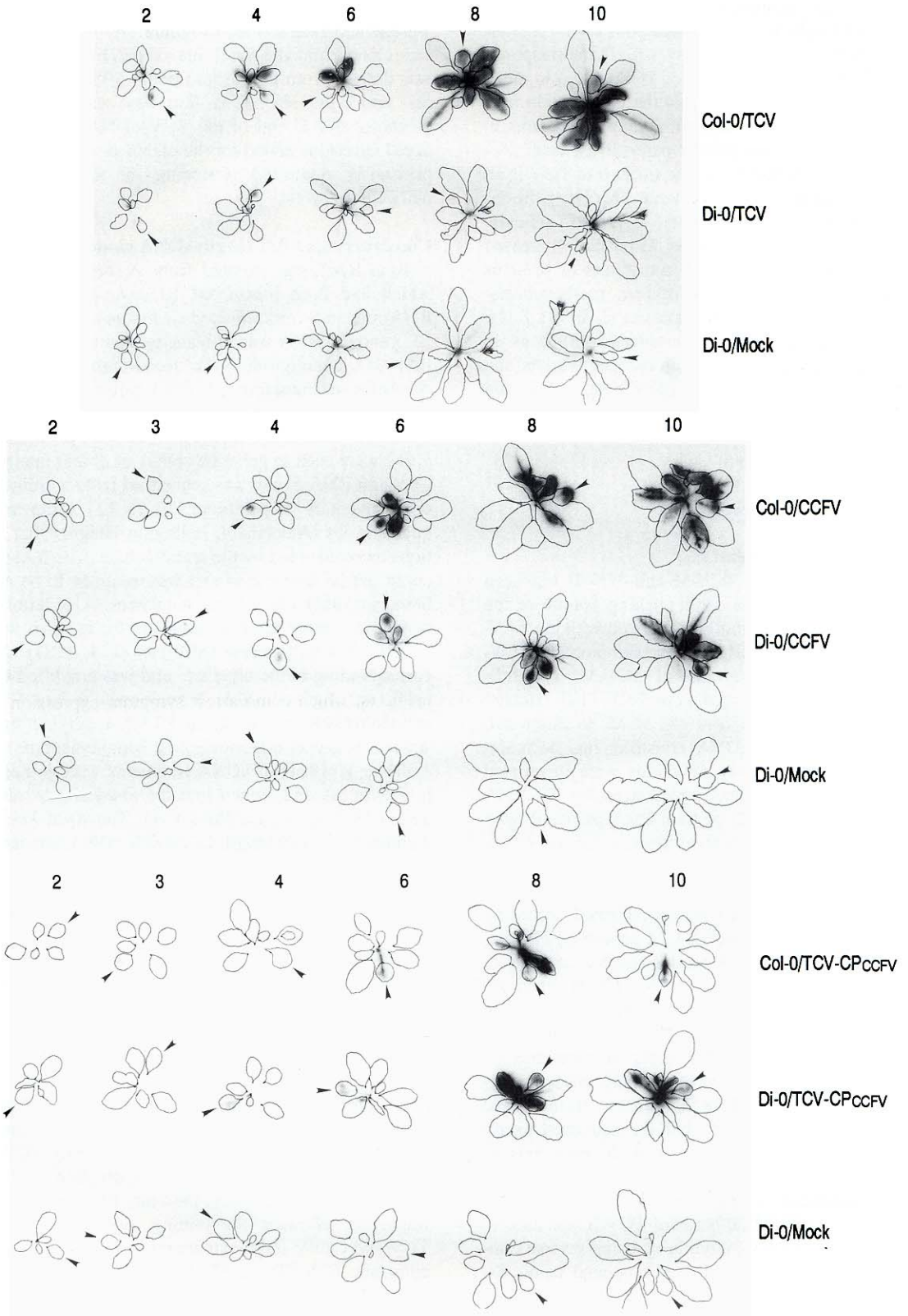

Fig. 7. Localization of viral genomic RNA using whole plant in situ hybridizations. One of the two oldest leaves of 2-week-old *Arabidopsis* Col-0 or Di-0 seedlings (indicated by arrows) was inoculated with the viral RNAs shown at the right. Numbers on top of each panel denote number of days postinoculation when plants were collected and frozen at –80°C. At the conclusion of the experiment, a ^{32}P-labeled oligonucleotide probe was hybridized directly to the genomic RNA in thawed, de-proteinated plants, as described in Materials and Methods. X-ray film was then placed on the radioactive plants and exposed. Outlines of leaves were drawn on a clear plastic sheet and photographed with the autoradiogram. Plants labeled "Mock" were rubbed with the inoculation buffer alone.

in the plasmodesmata connecting bundle sheath cells to phloem parenchyma cells in Di-0 interact poorly with a product of TCVms, they are able to interact with the corresponding product of CCFV-BL. The ability of TCV-CP_{CCFV} to move systemically through Di-0 suggests that the coat protein may be a viral determinant that interacts with putative receptors in plasmodesmata that connect to phloem parenchyma cells.

A second possibility is that resistance of Di-0 to TCV is an active response following virus inoculation involving the induction of so-called resistance factors. Levels of salicylic acid, an inducer of systemic acquired resistance, increased fivefold in TCV-infected Di-0, while transcripts of proteins associated with hypersensitive resistance (pathogenesis-related proteins) increased 20-fold (Uknes et al. 1993). Since tobacco mosaic virus coat protein has been identified as an elicitor of the hypersensitive response in tobacco (Culver and Dawson 1989), it is possible that the TCV coat protein and not the CCFV coat protein is able to induce a resistant response in Di-0, thereby accounting for resistance of Di-0 to TCV, and susceptibility of Di-0 to CCFV.

MATERIALS AND METHODS

Virus strains and plant inoculations.

The origin of TCV isolates TCV-M and TCV-B has been described (Li et al. 1989). The distinguishing feature of the isolates is that sat-RNA C (naturally associated with the TCV-M isolate) has no appreciable effect on the symptoms of TCV-B while intensifying the symptoms of TCV-M. The full-length cDNA of TCV-B is referred to as TCV-T1d1 (Heaton et al. 1989). CCFV-BL was a kind gift of M. L. Skotnicki. The sat-RNA C full-length cDNA construct has been described (Song and Simon 1994). Seedlings were inoculated with either full-length transcripts synthesized by T7 RNA polymerase (Simon et al. 1992) or total RNA prepared from plants previously inoculated with transcripts.

Construction of full-length cDNA clones of TCV-M.

Basic molecular manipulations were performed according to Ausubel et al. (1987). To clone TCV-M genomic RNA that specified a single homogeneous viral sequence capable of producing the typical severe symptoms of TCV-M when coinoculated with sat-RNA C, 2-week-old turnip seedlings were inoculated with 1 to 5 μg of total RNA isolated from TCV-M–infected turnip plants. Three DPI, single infection sites (as assayed by cleared spots visible on the abaxial surface of inoculated leaves when plants were illuminated from above) were excised, the tissue crushed, and the sap used to reinoculate individual turnip plants along with transcripts of sat-RNA C. RNA from a plant exhibiting severe symptoms was used for subsequent cloning. An oligonucleotide complementary to the 3′ terminal 20 bases of TCV-B was used to prime first-strand TCV-M cDNA synthesis using reverse transcriptase (RT). The second strand was generated using *Escherichia coli* DNA polymerase I and RNase H as previously described (Cascone et al. 1993). Fragments from two overlapping clones that represented nearly the entire sequence of TCV-M exclusive of 100 base pairs at the 5′ end were joined at the *Sal*I site at position 1,810 (Fig. 1B). cDNA corresponding to the 5′ 1,250 bases was synthesized using RT-PCR and oligonucleotides complementary to positions 1,250 to 1,265 and homologous to positions 1 to 20. Overlapping fragments were joined as described in Figure 1B. The full-length cDNA was cloned into the *Sma*I site of pT7E19(+)-loopout, which was derived from pT7E19(+) (Petty 1988) by deletion of the *Sst*I and *Kpn*I sites. The four base pairs between the T7 promoter and 5′ end of the TCV cDNA, including an additional G residue added for the cDNA cloning, were deleted as previously described (Carpenter et al. 1995), generating pT7TCVms.

Construction of full-length cDNA clones of CCFV-BL.

Total RNA was isolated from *A. thaliana* ecotype Di-0, which had been inoculated 10 days previously with total RNA prepared from infected *C. lilacina*. Gel purified CCFV-BL genomic RNA was polyadenylated (Carrington and Morris 1984), and oligo-d(T) was used to prime first-strand cDNA synthesis. *Escherichia coli* DNA polymerase and RNase H were used to generate double-stranded cDNA. Oligonucleotides complementary to the 3′ end and positions 2,993 to 3,012 were used to generate cDNA of 3′ and internal regions. The viral RNA 5′ end was sequenced from an oligonucleotide complementary to positions 111 to 127 using an RNA sequencing kit (Amersham, Arlington Heights, IL) and conditions recommended by the manufacturer. The 5′-terminal base could not be determined and was assumed to be a G residue based on other carmovirus sequences. A 5′-terminal G also results in a more efficient template for in vitro transcription by T7 RNA polymerase (Milligan et al. 1987). A fragment corresponding to the virus's 5′ end was amplified by RT-PCR using an oligonucleotide homologous to the 5′ end (also containing a *Kpn*I site and a T7 RNA polymerase promoter sequence) and an oligonucleotide complementary to positions 1,302 to 1,318. Four cDNA fragments were joined as shown in Figure 2A and cloned into the *Sma*I site of pBluescriptII SK(+) (Stratagene, La Jolla, CA). The *Kpn*I-*Xba*I fragment containing the full-length CCFV-BL cDNA was recloned into the *Kpn*I-*Xba*I site of pUC19 producing plasmid pT7CCFV-BL. Run-off in vitro transcription of *Sma*I-digested pT7CCFV-BL produces virus transcripts with the natural 3′ end.

Construction of chimeric viruses between TCVms and CCFV-BL.

pT7TCVms (containing a full-length cDNA of TCV-M whose in vitro synthesized transcripts induced severe symptoms when coinoculated with sat-RNA C) and pT7CCFV-BL (containing a full-length cDNA of CCFV-BL) were the progenitor plasmids for chimeric virus construction. Oligonucleotides were designed to precisely amplify TCV or CCFV ORFs by PCR. Some oligonucleotides also contained short sequences that overlapped the ends of other PCR-generated fragments, allowing the joining together of the amplified TCV and CCFV fragments by recombinant PCR methods. To construct TCV-CP_{CCFV} (TCV with the CCFV coat protein ORF), primers homologous to positions 1,665 to 1,680 and complementary to positions 2,728 to 2,747 of TCV genomic RNA were used to PCR-amplify a TCV fragment containing the *Eco*RI site and ending at the 3′ end of the p9 ORF (Fig. 4). The coat protein ORF of CCFV (with overlapping TCV-specific ends) was amplified using one primer homologous to (from 5′ to 3′) positions 2,728 to 2,747 of TCV genomic

RNA and positions 2,710 to 2,731 of CCFV genomic RNA, and a second primer complementary to positions 3,797 to 3,817 of TCV genomic RNA and positions 3,756 to 3,776 of CCFV genomic RNA. The 3′ terminal untranslated region of TCV genomic RNA was amplified using primers homologous to TCV positions 3,797 to 3,817 and complementary to the terminal 3′ end. Thirty cycles of PCR were performed, annealing at 5 to 10°C below the calculated melting point of the shorter primer, denaturing at 94°C for 1 to 3 min (depending on the length of the double-stranded fragment) and extending at 72°C for 1 to 4 min (depending on the length of the fragment to be amplified). PCR was performed using 20 to 30 units/μl of Pyrostase (Molecular Genetic Resources, Tampa, FL) and 250 μM of each dNTP in buffer recommended by the manufacturer. After fragments were joined as illustrated in Figure 4, the final product was cleaved with *Eco*RI and used to replace the corresponding *Eco*RI-*Sma*I fragment in plasmid pT7TCVms. Recombinant plasmids were identified by restriction enzyme analysis and the junction sequences, and PCR-derived regions were sequenced by the chain termination method (Sequenase, Amersham).

Site-directed mutagenesis.

Site-directed mutagenesis was performed as described by Kunkel (1985).

Whole-plant in situ hybridization.

Col-0 and Di-0 plants at the four- to five-leaf stage were inoculated on a single, oldest leaf with 0.1 μg of total RNA isolated from *A. thaliana* plants infected with in vitro synthesized transcripts of TCVms, CCFV-BL, or TCV-CP_{CCFV}. Plants were collected at various times postinoculation and stored at −80°C until use. At the conclusion of the experiment, all plants were placed in individual 60-mm petri plates, immersed in cold 95% ethanol, and then incubated at −80°C for 12 h, −20°C for 6 h, and then 25°C overnight with gentle shaking. Plants were rinsed with 95% ethanol and then incubated in 10 ml of 0.1 mM NaN_3, 0.1% sodium dodecyl sulfate (SDS), 0.5 mg of self-digested pronase per ml (Sigma, St. Louis, MO), 10 mM EDTA (pH 8.0) at 38 to 40°C for 4 to 6 h with gentle shaking. After rinsing four times with double distilled H_20, the plants were incubated in 10 ml of 0.2 N HCl at 25°C for 20 min, and then rinsed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Leaves of each plant were carefully arranged to minimize overlapping and then air dried for at least 3 h. The plants were baked at 80°C under vacuum for 2 h, wet in 2× SSC, and then prehybridized in 5 ml of 2× SSC, 5× Denharts' solution, 0.2% SDS, 1.0 mg denatured salmon sperm DNA per ml, 25% formamide at 38 to 40°C overnight with gentle shaking. An oligonucleotide complementary to positions 958 to 977 of TCVms was labeled using γ-[^{32}P]ATP and T4 polynucleotide kinase, and 0.2 to 0.5 pmol added to each plant. Hybridization was performed at 38 to 40°C overnight with gentle shaking. Plants were rinsed twice with 2× SSC, and then washed in 2× SSC, 0.1% SDS, at 50°C for 30 min. The fragile plants were placed on a piece of acetate sheet cut to plant size, dried at room temperature, covered with plastic wrap, and then subjected to autoradiography. Exposure time was normally 12 h or less without an intensifying screen.

ACKNOWLEDGMENTS

This work was supported by NSF grants MCB-9419303 and MCB-9315948 to the fifth author and a University of Massachusetts graduate student fellowship to the third author.

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