Turnip crinkle virus defective interfering RNAs intensify viral symptoms and are generated de novo

(plant virus/satellite RNAs)

XIAO HUA Li*, LOUIS A. HEATONT‡, T. JACK MORRIS†, and ANNE E. SIMON*§

*Department of Plant Pathology, University of Massachusetts, Amherst, MA 01003; and ‡Department of Plant Pathology, University of California, Berkeley, CA 94720

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ABSTRACT Defective interfering (DI) RNAs have been isolated from a broad spectrum of animal viruses and have recently been identified in plant virus infections. Because of their ubiquitous nature, DIs are thought to play an important role in virus replication and yields. DI RNAs have now been found in association with a natural isolate of turnip crinkle virus (TCV-B) and are generated de novo after inoculation of turnip with virus derived from cloned transcripts. DI RNA G, naturally found in the TCV-B isolate, is a mosaic molecule with 5′ and 3′ viral segments and a repeat of 36 nucleotides at the beginning of the 3′ segment. The 5′-terminal 21 nucleotides of DI RNA G were not similar to genomic TCV sequences but did resemble sequences found at the 5′ end of other small RNAs associated with TCV (satellite RNAs). DI RNA G interferes with the accumulation of TCV genomic RNA and, unlike other DI RNAs, intensifies the symptoms of its helper virus. Infection of turnip with virus derived from cloned transcripts of TCV-B resulted in de novo generation of a DI RNA, DII RNA. DII RNA differed from DI RNA G by containing exact 5′ and 3′ ends of TCV as well as an internal virus segment.

Defective interfering (DI) RNAs have been found associated with a wide variety of animal viruses. Delineated by Huang and Baltimore (1), DI RNAs are defective versions of viral RNAs that have lost essential coding sequences required for independent replication, maturation, or packaging. DI RNAs alone are not infectious; to be infectious DI RNAs require a helper virus to restore the deleted functions (2, 3). DI RNAs often compete with the nondefective virus for limited replication components, resulting in a decrease in the accumulation of helper virus. This interference with viral replication results in protection against viral-induced cytopathic effects in cell culture and in some cases has been implicated in virus disease modulation in whole animals (4). DI RNAs of animal viruses have also proved to be valuable subjects of study for many other important virological phenomena including the identification of cis-acting sequences important in replication and in encapsidation, fundamental studies on RNA replication and recombination, and use as transient expression vectors in animal cells (3).

Although DI RNAs are generally considered as ubiquitous components of animal virus infections, they have not been common in plant virus infections (5–9). The only DI RNAs associated with a RNA plant virus that have been characterized at the molecular level are the small symptom-attenuating RNAs associated with the cherry strain of tomato bushy stunt virus (TBSV) (6). This 396-base RNA, which is packaged along with TBSV and requires the virus for infectivity, is a mosaic molecule derived from the 5′ and 3′ ends of the virus and internal viral sequences. Like many animal virus DI RNAs, TBSV DI RNA competes with the helper virus resulting in a reduced level of virus accumulation and a marked attenuation of viral symptoms in infected plants (6).

Evidence supporting the origin of these DI RNAs from the TBSV genome has been demonstrated by high multiplicity passage of a DI RNA-free isolate (ref. 7 and D. A. Knorr and T.J.M., unpublished data). These experiments are consistent with the origin of animal virus DI RNAs studied by high multiplicity passage in animal cell culture. Definitive proof of DI origin from a parental RNA virus genome, however, has not been demonstrated.

Plant RNA viruses are frequently associated with other types of small RNAs that modulate disease symptoms. Satellite RNAs (sat-RNAs) share little or no sequence homology with their helper virus thus differing from DI RNAs (10). Although many sat-RNAs attenuate symptoms, several examples of sat-RNAs that intensify symptoms have been reported (11, 12). Turnip crinkle virus (TCV), a single-stranded RNA carmovirus, supports several sat-RNAs, one of which (sat-RNA C) intensifies viral symptoms on a number of hosts (11, 13, 14). Sat-RNA C (356 bases) is an unusual chimeric molecule composed of two domains: the 5′ domain is similar to the full-length sequence of the smaller sat-RNAs and the 3′ domain is composed of sequences related to two segments in the 3′ region of genomic TCV RNA (13). We now report the identification of DI RNAs in association with an isolate of the virus that does not contain sat-RNA C. The availability of inocula derived from infectious transcripts of a complete clone of the viral genome (15) has permitted definitive demonstration of de novo generation of DI RNAs. Curiously, the DI RNAs also have the unusual ability to intensify symptoms caused by the helper virus.

MATERIALS AND METHODS

Virus Strains and Plant Inoculations. Two well-characterized isolates of TCV used in this study have been maintained as distinct laboratory isolates. One of the isolates, designated TCV-B, was originally acquired from R. J. Shepherd (University of Kentucky) and has been propagated at University of California, Berkeley, for many years in turnip. The complete sequence of this isolate has been determined (16) and infectious transcripts have been produced from complete cDNA clones of the genome (15). Additional isolates, derived from infectious transcripts of the clones pTCV-P1 (λ promoter) and pTCV-T1d1 (T7 promoter), have been designated TCV-B-P1 and TCV-B-T1d1, respectively. The second laboratory isolate, which has been used predominantly in the study of the TCV sat-RNAs (11, 13, 14, 17, 18), has been newly

Abbreviations: TCV, turnip crinkle virus; DI, defective interfering; TBSV, tomato bushy stunt virus; sat-RNA, satellite RNA.

*Present address: Department of Plant Pathology, Throckmorton Hall, Kansas State University, Manhattan, KS 66506.
†To whom reprint requests should be addressed.
‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29290).
designated TCV-Massachusetts (TCV-M). It was originally obtained from R. Hull (John Innes Institute, UK) and normally contains three sat-RNAs (C, D, F). Isolates that have been modified by the curing of sat-RNAs from the inocula are indicated by the lower case initial of the original isolate as well as by designating the remaining sat-RNA(s) (for example TCV-m+D is the M isolate with only sat-RNA D). Since the viral genomic RNA from TCV-M has not been sequenced, it is not clear what nucleotide differences, if any, exist between the two TCV isolates.

Inoculations were carried out using total RNA or virions isolated from infected plants. Five micrograms of total RNA or 70 ng of virions were diluted in 70 »l of RNA infection buffer (0.05 M glycine/0.03 M K2HPO4/0.02% bentonite, pH 9.2) or virion infection buffer [0.05 M Na2HPO4 (pH 7.0)] and then mechanically inoculated on leaves of 14-day-old turnip cv. Just Right or Chinese cabbage cv. Pak Choi. Choi seedlings that had been dusted with Celite. Inoculated plants were grown in a growth chamber with a 16-hr day, 21°C/13°C cycle.

To test for possible interference with viral replication, DI-RNA G was isolated from viral RNA of TCV-B by fractionation on sucrose density gradients and the quantity of the RNA was estimated spectrophotometrically. The DI RNA G fraction was mixed with in vitro TCV transcripts derived from pTCV-P1 (final concentration, 10 »l/ml) in approximate molar ratios of 1:1 and 10:1 and diluted with 3 vol of 0.05 M glycine/0.03 M K2HPO4, pH 9.2, containing 1% bentonite and 1% Celite prior to inoculation of alternate half leaves of the local lesion host, Chenopodium amaranticolor, and the systemic hosts Brassica campestris, Arabidopsis thaliana cv. Columbia, turnip cv. Just Right, and Nicotiana benthamiana. Virus content was monitored in inoculated and systemic leaves of plants 7 and 12 days following inoculation after extraction in 0.2 M sodium acetate (pH 5.2) and concentration by PEG precipitation. Virus quantitation was estimated spectrophotometrically and by electrophoretic analysis of virus particles.

Northern Hybridizations. Northern hybridizations were performed as described (13) except that 0.1- »l charged, modified nylon (Nytran; Schleicher & Schuell) replaced nitrocellulose. An oligonucleotide probe, complementary to the 3' 20 terminal nucleotides of TCV-B genomic RNA (oligo 8), was labeled by incorporation with 100 »lCi of [y-32P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq; NEN) and 10 units of polynucleotide kinase (Bethesda Research Laboratories) for 30 min at 37°C by using buffer conditions suggested by the manufacturer. Full-length sat-RNA F probe was labeled using [e-32P]dATP (3000 Ci/mmol; ICN) and Escherichia coli DNA polymerase I (Bethesda Research Laboratories) by nick-translation.

Cloning and Sequencing of DI RNA G and DI1 RNA. Oligo 8 (1 »l) was hybridized to 0.5 »l of gel-purified DI RNA G or DI1 RNA in 10 »l of hybridization buffer (0.4 M NaCl/10 mM PIPES, pH 6.4) at 80°C for 1 hr then 75°C for 10 min followed by slow cooling to 60°C. The synthesis of first- and second-strand cDNAs was performed as described (13). Kpn I linkers were ligated to the double-stranded cDNAs that were then digested with Kpn I and inserted into the Kpn I site of pUC19. cDNA clones corresponding to DI RNA G or DI1 RNA were sequenced by dideoxynucleotide chain-termination (Sequenase; United States Biochemical) using standard sequencing primers (Synthetic Genetics).

Primer-Extension Sequencing. One microgram of an oligonucleotide complementary to nucleotides 90–103 of DI RNA G (oligo 12) was labeled with [y-32P]ATP and polynucleotide kinase as described above. Approximately 0.5 »l of gel-purified DI RNA G or DI1 RNA was hybridized to oligo 12 in 10 »l of hybridization buffer for 10 min at 60°C and slowly cooled to 34°C. The primer was extended using 2 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) under conditions suggested by the manufacturer. Full-length cDNA was isolated from 5% polyacrylamide/50% (wt/vol) urea gels and sequenced by the chemical modification method (19, 20).

RESULTS

Identification of Low Molecular Weight RNAs Associated With the Berkeley Isolate of TCV. Collaborative studies were initiated in an effort to compare the sat-RNAs of Massachusetts and Berkeley isolates of TCV (TCV-M and TCV-B, respectively). Preliminary indications suggested that there were interesting biological differences between the isolates; TCV-B routinely produced less severe symptoms than TCV-M on cruciferous hosts (data not shown). Since the presence of sat-RNA C is correlated with the severe symptoms produced by TCV-M (11, 14, 17), the possibility existed that the low molecular weight RNAs associated with the isolates might be different. To determine whether or not sat-RNAs are also associated with TCV-B, RNA was isolated from turnip infected with TCV-B and subjected to electrophoresis on denaturing polyacrylamide gels (Fig. 1A). Two low molecular weight RNAs not found in mock-infected turnip were identified: one RNA comigrated with sat-RNA D and a second RNA had a slightly faster mobility than sat-RNA C. To determine whether or not these two RNAs were related to the similarly sized RNAs from TCV-M, RNAs from TCV-M-
and TCV-B-infected plants were transferred to Nytran membranes after electrophoresis on denaturing polyacrylamide gels and then hybridized with either of two probes: a full-length cDNA of sat-RNA F, which shares substantial sequence similarity with both sat-RNA D and the 5' 200 nucleotides of sat-RNA C (13), or oligo 8, which is only weakly complementary to the corresponding portion of sat-RNA C (Fig. 1 B and C). The results of the hybridization analysis indicated that TCV-B contained a sat-RNA D-like RNA and a unique associated RNA that migrated slightly faster than sat-RNA C but did not contain any of the characteristic 5' sat-RNA sequences. Furthermore, the slightly smaller RNA from TCV-B hybridized strongly to oligo 8, implying a 3' sequence more similar to TCV genomic RNA than to sat-RNA C. This RNA was termed RNA G.

**RNA G Is Composed of Sequence Derived From Genomic TCV RNA.** To determine the sequence of RNA G, cDNA clones were generated using oligo 8 as a primer for first-strand cDNA synthesis. After treatment with RNase H and E. coli DNA polymerase to produce the second DNA strand, linkers were added and the cDNA was cloned into the Kpn I site of the plasmid vector. The sequences of five nearly full-length clones of RNA G cDNA are presented in Fig. 2. All five cDNA clones varied in sequence, with the majority of nucleotide differences occurring in two hypervariable regions between bases 71 and 74 and between bases 108 and 132. To determine the sequence at the 5' end of RNA G, oligo 12 complementary to bases 90–103 was hybridized to gel-purified RNA G and then extended with reverse transcriptase. Fully extended cDNA was gel-purified and the nucleotide sequence was obtained by chemical modification methods (19, 20). The sequences of RNA G cDNA clones were compared with the sequence of genomic TCV (16) and the 3' domain of sat-RNA C, which shares sequence similarity with TCV (Fig. 2). All of RNA G, with the exception of the 5' 21 nucleotides, was derived from genomic TCV. RNA G was contiguous with TCV from TCV base 43 to base 140, then resuming at base 3863 and continuing to the 3' terminal nucleotide of TCV. The RNA G clones also contained a direct repeat of TCV bases 3863–3898. Since RNA G is composed almost exclusively of helper virus segments and contains the helper virus for infectivity (data not shown), RNA G meets the definition of a DI RNA. We will henceforth refer to RNA G as DI RNA G.

**DI RNA G Interferes With the Helper Virus Replication.** Although the sequence of RNA G clearly establishes its origin from the helper virus genome, it was also necessary to test for competitive interference to be consistent with the definition of a DI RNA. To accomplish this, inocula containing *in vitro* transcripts of the viral genome (pTCV-T1dli) alone and in the presence of various quantities of DI RNA G (approximate molar ratios of DI RNA G/helper genomic RNA of 1:1 and 10:1) were inoculated on plants to evaluate the possible interference effects of the DI RNA. Several lines of evidence suggested that interference at the level of helper virus replication could be attributed to the presence of DI RNA G in the inoculum. (i) There was a reduction by a factor of about 5 in the number of local lesions in the DI RNA-containing inocula. (ii) The systemic hosts *N. benthamiana* and *B. campestris* showed a delay of several days in systemic symptom development for the inoculum containing the highest proportion of DI RNA G. (iii) Inocula containing DI RNA G generally resulted in measurably reduced virus accumulation in both inoculated and systemic leaves of all host plants 7 and 12 days after inoculation. This was most apparent in the systemically infected leaves of *N. benthamiana* and *B. campestris* in which there was a reduction by a factor of 5 in virus accumulation in plants inoculated with the highest

**FIG. 2.** Nucleotide sequence of RNA G. cDNAs were generated by extension of oligo 8 on gel-purified RNA G by reverse transcriptase. Nucleotides following the brackets are the 5' end points of the individual cDNAs. The 5' sequence of RNA G was determined by chemical-modification sequencing of primer-extended cDNA. Dots indicate a missing nucleotide. Only bases that differ from RNA G1 are shown. The nucleotide at position 10 in the terminal 5' 21 bases was heterogeneous in the population of RNA G molecules. The 5' terminal two nucleotides could not be determined. The sequence of TCV is from the Berkeley isolate (TCV-B) (16), and the sequence of sat-RNA C is from the cDNA clone 2-47 (13). An asterisk (*) indicates the point of divergence between sat-RNA C and RNA G, and only that portion of the sat-RNA C sequence that is similar to RNA G is shown. Base numbering in italics corresponds to TCV (16).

**DI RNA G Intensifies the Symptoms of TCV.** The interference experiments also confirmed earlier observations that clonally pure inoculum resulted in milder infections. We conducted a more extensive series of experiments to confirm that DI RNA G was responsible for the symptom intensification when inoculated in the presence of helper virus. DI RNA G was gel-purified after electrophoresis on denaturing polyacrylamide gels, added to an inoculum containing TCV-m^-d^, then inoculated on turnip or Chinese cabbage. This isolate of TCV was used because of the extensive investigations on the symptom-producing properties of TCV-M (11, 14, 17). Sat-RNA D was present with the helper virus since it has not been possible to maintain a stock of TCV-M free of this satellite (18). The results (Fig. 3) showed that addition of DI RNA G correlated with greater symptom production. Young leaves of plants accumulating DI RNA G had a darker green coloration and older leaves were chlorotic. Furthermore, presence of DI RNA G was associated with greater stunting and leaf crinkling. The symptoms attributed to the accumulation of DI RNA G resembled those reported for sat-RNA C, which accumulates in TCV-M-infected plants. These experiments confirmed that DI RNA G was capable of intensifying symptom severity in the absence of sat-RNA C.

**De Novo Generation of a TCV DI RNA.** The TCV-B isolate has been maintained for many years and through many molar ratio of DI RNA G (0.2–0.4 mg of virus per g of tissue) relative to the control (2–4 mg of virus per g of tissue).
inoculated cabbage and mustard plants accumulated new RNAs of 340–390 nucleotides, four representatives of which are shown in Fig. 4. All of the new RNAs resembled DI RNA G by hybridizing strongly to the TCV-specific probe (oligo 8) and by not hybridizing to the sat-RNA-specific probe (sat-RNA F cDNA) (data not shown). The new RNA species from Fig. 4, lane 1, was cloned, and the sequence of three cDNAs indicated that the new RNA (DI1 RNA) was a colinear deletion mutant of genomic TCV, containing both the 5′ and 3′ ends of TCV as well as an interior segment (Fig. 5). Unlike DI RNA G, DI1 RNA began with the 5′ terminal nucleotide of TCV. The sequence of DI1 RNA was more similar to genomic sequence than DI RNA G, which has been maintained with the virus after prolonged passage, lending support to the de novo origin of DI1 RNA.

Further confirmation of the de novo origin of DI RNAs from cloned inocula was obtained by analyzing RNA present in virus purified from turnip plants several months after inoculation with transcripts derived from pTCV-T1d1. In this experiment, individual virus isolates were examined from turnip plants. In all cases, new RNA species of various sizes (300–440 bases) that strongly hybridized to genomic RNA probes could be identified in the virions (data not shown). These were assumed to be novel DI RNA species similar to DI1 RNA in origin, although confirmatory cloning and sequence analysis has not been performed. These results strongly support the contention that TCV generates DI RNAs de novo from genomic sequence upon replication in cruciferous hosts.

**DISCUSSION**

Thus far, turnip crinkle virus is unique among plant viruses in its association with three types of small RNAs: (i) sat-RNAs (sat-RNA D, sat-RNA F) that share no sequence with TCV, (ii) sat-RNAs (sat-RNA E, sat-RNA F) that hybridize to TCV-specific oligos (oligos 1–6), and (iii) sat-RNAs (sat-RNA G) that hybridize to TCV-specific probes (oligos 7–8).

**Fig. 3.** Symptoms produced by DI RNA G. Turnip cv. Just Right (A) or Chinese cabbage cv. Pak Choi. Injury (B) were photographed 3 weeks after the following inoculations. Plants: 1, buffer; 2, TCV-m + sat-RNA D; 3, TCV-m + sat-RNA D + gel-purified DI RNA G; 4, TCV-M.

**Fig. 4.** Accumulation of new RNAs in plants infected with virus derived from in vitro-synthesized TCV. Total RNA was extracted from plants that had been inoculated 2 weeks previously with buffer (mock), genomic RNA from TCV-M + sat-RNA D (TCV-m + D), TCV-M, TCV-B, or TCV-B-P1 and subjected to electrophoresis on 5% polyacrylamide/30% urea gels, which were then stained with ethidium bromide. TCV-B-P1 were virions isolated from turnip inoculated with in vitro-synthesized transcripts of genomic TCV (TCV-B isolate). Square bracket indicates RNA species of 340–390 nucleotides including sat-RNA C in lane TCV-M and DI RNA G in lane TCV-B. Lanes 1–4 contain RNA from four individual plants inoculated with TCV-B-P1. DI1 RNA was derived from the new RNA species in lane 1.

**Fig. 5.** Nucleotide sequence of DI1 RNA. DI1 RNA was isolated from turnip inoculated with virus derived from pTCV-P1 (TCV-B-P1) and hybridized to oligo 8 (see Fig. 1), and then cDNA was generated by extension of the oligonucleotide with reverse transcriptase. Nucleotides following the brackets are the 5′ end points of the cDNA. The sequence on the 5′ side of the brackets was determined using primer-extended cDNA as described for DI RNA G. Dots indicate a missing nucleotide. Only nucleotides that differ from DI1-1 RNA are shown. Numbering of TCV genomic RNA is shown in italics.
homology with the helper virus (10); (ii) chimeric molecules composed of sat-RNA sequences at the 5' end and virus-related sequence at the 3' end (sat-RNA C); and (iii) DI RNAs, composed entirely or nearly entirely of virus segments (DI RNA G and DI1 RNA). A schematic representation of the relationship among TCV, its DI RNAs, and sat-RNA C is presented in Fig. 6. Although there is no single consensus sequence at the various junction points, DI1 RNA and sat-RNA C contain the same 3' end segment of TCV, from nucleotide 3898 to nucleotide 4051. The end of the repeated sequence in DI RNA G is also at the same junction sequence (nucleotide 3898).

The origin of the terminal 5' 21 nucleotides of DI RNA G is obscure. A search of the EMBL data bank (May 1989) did not reveal any sequences that closely match the 21 nucleotides. This sequence differs from the corresponding sequence of TCV by at least 11 bases. However, of 13 unambiguous nucleotides at the 5' end of DI RNA G, 70% are found in the same position at the 5' end of sat-RNA D. The origin of these 21 nucleotides may be due to (i) divergence from TCV as a result of prolonged passage in association with the helper virus; (ii) the result of a recombination event between sat-RNA D and TCV that might have occurred during the original formation of DI RNA G; and (iii) the presence of a nonviral segment. Several alphavirus DI RNAs also have 5' sequences that have diverged from original viral sequences. DI RNAs derived from Sindbis virus have been found with 5' end sequences nearly identical to rat tRNA^Ap (21), and Semliki forest virus DI RNAs also contain a heterogeneous 5' end (22).

DI RNAs, although commonly associated with animal viruses, have only recently been identified in plant virus infections (5–9). The best characterized plant virus DI RNA is the symptom modulating DI RNA associated with the tombusvirus TBSV (6). This 0.4-kilobase DI RNA is a mosaic composed of the 5' noncoding region, an internal segment from the RNA replicase gene, and a 3' proximal domain composed of the last 130 bases of the extreme 3' noncoding sequence. The features of the TBSV DI RNAs are similar to those of DI RNA G and DI1 RNA of TCV in that they have retained similar lengths of sequence from both ends of the helper genome.

The discovery of DI1 RNA in a TCV isolate derived from cloned inoculum is a definitive demonstration of de novo generation of a viral DI RNA from an RNA viral genome. The demonstrated origin of other animal and plant viral DI RNAs is not as rigorous. The identification of distinct size classes of DI RNAs upon high multiplicity passage of DI RNA-free isolates of animal RNA viruses is generally interpreted as strong evidence for de novo generation. Such evidence has been presented in support of de novo generation of DI RNAs for plant tombusviruses as well (ref. 7 and T.J.M., unpublished data). The tombusviruses are thought to be closely related to the carmoviruses (23), the group to which TCV belongs. It may be that the ability to generate DI RNAs is a common feature of their member virus replicases.

DI RNA G is responsible for intensified symptoms when inoculated with TCV on mustard and other cruciferous hosts. Plants in which the DI RNAs are replicating exhibit increased stunting and leaf crinkling. The TCV-similar domain of sat-RNA C was found to be at least partially responsible for the dark-green severely stunted and crinkled leaves associated with the sat-RNA (17). DI RNA G also contains this 3' end domain that further suggests that this region is involved in symptom production. The role of DI1 RNA in symptom production has not been extensively investigated. Although DI RNA G is a plant DI RNA that intensifies viral symptoms, defective forms of animal retroviruses are also thought to have a role in pathogenesis (24, 25). However, defective retroviruses are not simply deleted versions of their helper virus; many have acquired new cellular genes that are implicated in disease production.

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