A Defective Interfering RNA Molecule in Cymbidium Ringspot Virus Infections

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SUMMARY

A small RNA (DI RNA), approx. 400 bp in length, was found in plants inoculated with cymbidium ringspot tombusvirus (CyRSV). DI RNA contained sequences derived from at least three regions of the CyRSV genome and was encapsidated in CyRSV protein. DI RNA was formed in plants inoculated with purified genome-size RNA and a double-stranded form was detected in dsRNA extracted from infected tissue. Symptoms of CyRSV infection in Nicotiana clevelandii were less severe when DI RNA was present in the inocula used suggesting that DI RNA interferes with symptom expression.

Hillman et al. (1985) have shown that in Nicotiana clevelandii plants inoculated with petunia asteroid tombusvirus (PAMV), a small molecule is formed that can attenuate the severity of virus-induced symptoms. This RNA was later shown to contain sequences derived from several regions of the genomic RNA (Hillman et al., 1987) and to have properties similar to those of defective interfering (DI) RNAs of animal viruses (Barrett & Dimmock, 1986). A recent study on the RNA composition of cymbidium ringspot tombusvirus (CyRSV)-infected N. clevelandii tissue did not reveal any such RNA, nor was it detected in purified virions (Russo et al., 1988). However, after a few transfers by sap inoculation to N. clevelandii, an RNA molecule of this type was formed. In the present paper evidence is given that this RNA resembles that described by Hillman et al. (1985, 1987) and some of its properties are described.

Genome-length RNA prepared from CyRSV virions as described by Russo et al. (1988) is shown in Fig. 1 (lane 1). It was used to inoculate one N. clevelandii plant. Ten days later, RNA was extracted from infected tissue (Russo et al., 1988), electrophoresed in a 1-5% agarose gel cast in 90 mm-Tris, 90 mm-boric acid, 1 mm-EDTA and stained with ethidium bromide. No RNA was found in the position expected for an approx. 400 bp RNA (Fig. 1, lane 2). Total RNA from N. clevelandii inoculated with sap from the first plant (Fig. 1, lane 3) and from plants infected by a further three passages also lacked a 400 bp RNA. However, RNA from plants infected in the fifth passage contained a small RNA that was not present in previous passages or in healthy control plants (Fig. 1, lanes 4 and 5).

A Northern blot was prepared after denaturation of RNA with formaldehyde and formamide and electrophoresed in MOPS buffer (20 mm-MOPS, 5 mm-sodium acetate, 1 mm-EDTA pH 7.0) (Maniatis et al., 1982). 32P-labelled cDNA probes were made by random priming (Taylor et al., 1976) of genomic RNA or of satellite RNA (Gallitelli & Hull, 1985) purified by electrophoresis twice in low gelling temperature (LGT) agarose (Miles) (Maniatis et al., 1982). The small RNA (DI) hybridized with cDNA to genomic RNA, but not with cDNA to satellite RNA (Fig. 2).

An additional set of Northern blots was prepared and hybridized to nick-translated plasmids...
Fig. 1. Electrophoresis in agarose of purified genomic RNA (lane 1) and total RNA preparations from infected (lanes 2 to 4) and healthy (lane 5) plants. The plant from which the RNA shown in lane 2 was extracted was inoculated with the genomic RNA shown in lane 1. Lanes 3 and 4 show RNA from plants serially inoculated with sap and represent the second and fifth passage, respectively. The arrow points to DI RNA detected only in the fifth passage. The gel was stained with ethidium bromide.

Fig. 2. Northern blot of RNA preparations from plants infected with CyRSV plus satellite RNA (lane 1, 3) or CyRSV plus DI RNA (lanes 2, 4). Radioactive probe was either cDNA to genomic RNA (lanes 1, 2) or satellite RNA (lanes 3, 4). The arrow and arrowhead indicate DI RNA and satellite RNA, respectively. The small RNA visible at the position of satellite RNA in lane 1 is probably a degradation product of genomic RNA (Gallitelli & Hull, 1985).

(Rigby et al., 1977) containing CyRSV RNA sequences. Clones pCyR-1, pCyR-7, pCyR-1E and pCyR-102 were used. The construction of these plasmids and their position relative to CyRSV RNA were described by Russo et al. (1988). In addition, clone pCyR-1ES was prepared by digesting clone pCyR-1S with EcoRI, electro-eluting the resulting 800 bp fragment and inserting it in the EcoRI site of pUC13. In these hybridization trials (Fig. 3) DI RNA hybridized with all plasmids except pCyR-1ES, thus suggesting that DI RNA was a multiple deletion mutant species of CyRSV RNA.

A cDNA clone of DI RNA was constructed. DI RNA was isolated from total RNA preparations by two passages in LGT agarose. cDNA was synthesized with random hexanucleotide primers using the 'cDNA Synthesis System Plus' kit (Amersham), oligo(dC)-tailed with terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) and annealed with plasmid pAM18 (Amersham) digested with PstI and tailed with oligo(dG). The annealed DNA was used to transform Escherichia coli strain JM101 made competent with CaCl₂ (Maniatis et al., 1982). Recombinant clones were identified by colony hybridization (Maniatis et
al., 1982) with cDNA prepared by random priming using CyRSV RNA as template. Positive clones were further screened by digesting purified plasmids with PstI and Southern blotting. Blots were hybridized with nick-translated plasmids pCyR-102 and pCyR-7, which represent the extremities of genomic CyRSV RNA (Russo et al., 1988). Recombinant plasmids hybridizing with both clones were chosen, since these are likely to have the hybrid composition of DI RNA. One such clone, pDI4, approx. 500 bp long, was chosen for further studies. It is longer than DI RNA because of the dC tails.

Hillman et al. (1985, 1987) did not obtain DI RNA upon inoculation of PAMV with virus devoid of small interfering RNA molecules. However, with CyRSV a limited number of passages was enough to produce DI RNA. That it was not detectable in the original inoculum (genome-size RNA) is shown in Fig. 4. No RNA of the size of DI RNA was detected in the genomic RNA preparation (lane 1), or in the extracts of plants inoculated with it (lane 2), whereas it was present in virions purified from plants sap-inoculated at the fifth passage (lane 3), and in extracts of plants inoculated with these virus preparations (lane 4). It can be concluded, therefore, that DI RNA was synthesized even though it was not in the inoculum.

To test for a specific double-stranded form of DI RNA, the 4 M-LiCl insoluble material in total RNA prepared from infected tissue was further treated with RNase A and DNase I (Sigma) (Diaz-Ruiz & Kaper, 1978). The putative ds RNA was denatured, electrophoresed, blotted as before, and hybridized with DNA probes made from plasmid pDI4. Briefly, 2 µg plasmids were denatured with alkali (Chen & Seeburg, 1985), and annealed to either SP6 (probe A) or T7 promoter (probe B) primer (Promega). A mixture of dATP, dGTP, TTP (each 25 µM), dCTP 2 µM and [α-32P]dCTP (Amersham; 3000 Ci/mmol) (25 µCi) was added and polymerization was carried out in the presence of 5 units of Klenow enzyme (Amersham). Probes were ethanol-precipitated, and denatured with alkali before hybridization. Orientation of probes (plus or minus-sense) was defined by the hybridization pattern obtained with encapsidated RNAs. Probe A, but not B, hybridized with genomic and subgenomic RNAs (Fig. 5, lanes 1, 4); therefore, probe A was considered a plus-sense probe, and probe B a negative-sense probe. Both plus- and minus-sense probes hybridized with denatured dsRNAs (genomic, subgenomic and DI RNAs) (Fig. 5, lanes 2, 5), whereas purified DI RNA hybridized strongly with plus-sense probe, and much less with minus-sense probe (Fig. 5, lanes 3, 6). The same result was obtained with differently arranged wells, thus discounting the possibility of spillage artefacts (not shown).
Short communication

The presence of minus strand DI RNA in a gel-purified single-stranded DI RNA preparation is probably due to contamination with a small amount of dsRNA. At the moment, however, we cannot exclude the presence of self-complementary sequences in DI RNA. If confirmed, the presence of DI RNA in both polarities would support the possibility of its autonomous replication.

The biological activity of DI RNA was tested by comparing the symptoms induced in plants by inoculation with CyRSV plus satellite RNA, CyRSV plus DI RNA, and genomic RNA alone. *N. clevelandii* plants were inoculated with 10 μl of the appropriate preparation on each of three leaves (six replicates). Plants inoculated with genomic RNA alone (30 ng/μl; the same preparation as that shown in Fig. 4, lane 1) showed apical top necrosis, and then died. Those inoculated with virus (0.2 mg/ml) containing satellite RNA were stunted without top necrosis, whereas those inoculated with virus (0.2 mg/ml) containing DI RNA (the same preparation as that shown in Fig. 4, lane 3) developed only limited distortion of younger leaves. The decrease in symptom severity in plants in which DI RNA is present may be due to a decreased synthesis of genomic RNA as shown in Fig. 1.

The occurrence of a defective interfering RNA associated with a member of the tombusvirus group other than PAMV suggests that generation of DI RNA may be a feature characteristic of

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Fig. 4. Northern blot of purified genomic RNA (lane 1), total RNA preparation from a plant inoculated with genomic RNA (lane 2), RNA from virions extracted from plants at the fifth passage (lane 3), total RNA preparations from a plant inoculated with virions extracted to give RNA shown in lane 3 (lane 4). Lane 5 contains purified DI RNA and lane 6 RNA from a healthy plant. Hybridization was with nick-translated plasmid pDI4.

Fig. 5. Northern blot of denatured RNA extracted from virions (lanes 1, 4), 4 M-LiCl-insoluble RNA from a plant inoculated with virus containing DI RNA (lanes 2, 5), and purified DI RNA (lanes 3, 6). Hybridization on left panel was with probe A (plus-sense), on right panel with probe B (minus-sense).
this group of plant viruses. The symptom-modulating activity of this RNA may be a useful tool to help understand the molecular basis of virus pathogenesis and for obtaining transgenic plants resistant to virus infections. Investigations are now under way to test both these possibilities.

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REFERENCES


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