Coat protein of cucumber necrosis virus is not required for efficient generation or accumulation of defective interfering RNAs

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It is generally assumed that defective interfering (DI) forms of viruses are encapsidated in structural proteins encoded by the helper virus. Virion RNA extracts from cucumber necrosis virus (CNV) infections showing high levels of cellular DI RNAs contain barely detectable levels of DI RNAs, suggesting that DI RNAs are encapsidated very inefficiently. In addition, accumulation of CNV DI RNAs occurs at equal efficiency in co-inoculated plants using either synthetic wild-type CNV genomic RNA as helper or a mutant of CNV which lacks the coat protein-coding sequence. Together this shows that the CNV coat protein is not required for efficient accumulation of CNV DI RNA in plants. Factors that could account for the high level of CNV DI RNAs in plants are discussed.

Defective interfering (DI) forms of viruses are deletion mutants of the viral genome that are generated by errors made during replication. DI genomes have lost the ability for independent replication but have retained, at a minimum, the cis-acting sequences required for efficient replication. DI genomes are generally assumed to be packaged in the coat protein of the helper virus and it is believed that the possession of the cis-acting sequences for replication and encapsidation contribute to the ability of DI genomes to interfere with accumulation of the helper virus (for reviews see Perrault, 1981; Holland, 1991; Roux et al., 1991). DI RNAs are ubiquitous among animal viruses (Holland, 1991) and are also found in association with several plant viruses (Romero et al., 1993).

We have recently reported that DI RNAs are present in plants infected with laboratory cultures of cucumber necrosis tombusvirus (CNV), a small spherical virus containing a monopartite, positive-sense RNA genome approximately 4.7 kb in length (Finnen & Rochon, 1993). In addition, CNV DI RNAs can be formed spontaneously during infection from the helper virus genome after several passages of virus inoculum or much more rapidly (a single passage) from a CNV mutant that does not express the p20 non-structural protein (Rochon, 1991). De novo generation of DI RNAs has also been described for two other tombusviruses, the cherry strain of tomato bushy stunt virus (TBSV-Ch; Knorr et al., 1991; Law & Morris, 1994) and cymbidium ringspot virus (CyRSV; Burgyan et al., 1991). It has been suggested that DI RNAs are formed de novo from the genome via template switching by the replicase during synthesis of viral RNA (Holland, 1991). A non-homologous RNA recombination model for the generation and evolution of tombusvirus DI RNAs involving stepwise deletions has recently been proposed (White & Morris, 1994). Sequence analysis of cDNA clones of CNV DI RNAs revealed that they range in size from 433 to 622 nucleotides and retain sequences from the CNV 5' untranslated and 3'-terminal regions as well as from an internal portion of the genome (Finnen & Rochon, 1993). Plants infected with our laboratory culture of CNV contain very high levels of DI RNA (Rochon & Johnston, 1991; Finnen & Rochon, 1993). However we have noted that RNA extracts from virus purified from such plants contain barely detectable levels of DI RNA species. The apparent low efficiency of encapsidation, in conjunction with our recent finding that a CNV mutant lacking the coat protein-coding sequence accumulates and spreads efficiently in plants in the absence of coat protein (McLean et al., 1993), prompted us to investigate the role of the CNV coat protein in the accumulation and generation of CNV DI RNAs.

The efficiency of encapsidation of CNV DI RNAs in infected Nicotiana clevelandii, a systemic host of CNV, was assessed by comparing the ratio of DI RNA to genomic length RNA in virions with that found in infected plants. Leaves from several infected plants were collected and total RNA was extracted from a small proportion selected randomly (Rochon & Johnston, 1991). Virus was purified from the remaining leaves using a modification of the pH 5.0 method (Tremaine et al., 1993).
can be seen that high levels of DI RNA are found in leaves or from virus at the two stages of purification. It
then extracted from equal volumes of each portion of the gradient. Fig. 1 shows an ethidium bromide-stained non-denaturing agarose gel of RNA extracted from infected gradient. Lanes 1 and 6 contain total RNA extracted from infected leaves. Lanes 2 and 7 contain RNA extracted from the crude PEG virus pellet obtained prior to caesium chloride purification. Lanes 3 and 8 contain RNA extracted from material present in the lower portion of the caesium chloride gradients, lanes 4 and 9 from the middle portion of the gradients, and lanes 5 and 10 from the upper portion of the gradients. Equal volumes of RNA extracted from equal volumes of each portion of the gradient were used in lanes 3 to 5 and 8 to 10. The gel was stained in 0.5 μg/ml ethidium bromide and photographed under u.v. illumination. Positions of CNV genomic and DI RNAs are shown on the right.

Fig. 1. Analysis of CNV DI RNAs present in leaf and virion RNA extracts. RNA was extracted from infected plants or from virus purified from infected plants as described in the text and then electrophoresed through a 1% agarose gel containing TBE buffer (0.1 M-Tris, 0.1 M-boric acid, 0.002 M-EDTA, pH 8.3). RNA in lanes 1 to 5 was extracted from plants infected with wild-type CNV synthetic transcripts whereas RNA in lanes 6 to 10 was extracted from plants containing CNV genomic and DI RNA. Lanes 1 and 6 contain total RNA extracted from infected leaves. Lanes 2 and 7 contain RNA extracted from the crude PEG virus pellet obtained prior to caesium chloride purification. Lanes 3 and 8 contain RNA extracted from material present in the lower portion of the caesium chloride gradients, lanes 4 and 9 from the middle portion of the gradients, and lanes 5 and 10 from the upper portion of the gradients. Equal volumes of RNA extracted from equal volumes of each portion of the gradient were used in lanes 3 to 5 and 8 to 10. The gel was stained in 0.5 μg/ml ethidium bromide and photographed under u.v. illumination. Positions of CNV genomic and DI RNAs are shown on the right.

1983). Briefly, leaves were homogenized using a Waring blender in two to four volumes of 0.1 M-sodium acetate pH 5.0 containing 5% 2-mercaptoethanol, filtered through four layers of cheesecloth and the filtrate was allowed to stand at 4 °C for 10 to 20 min. Insoluble cellular components were pelleted by centrifugation at 10000 g for 15 min and the supernatant was adjusted to 8% (w/v) polyethylene glycol (PEG 8000). This mixture was stirred for 2 h at 4 °C and virus pelleted by centrifugation as above but for 20 min. The partially purified virus pellet was resuspended in 10 mM-sodium phosphate buffer pH 7.0 and virion RNA was extracted from a small portion of the suspension at pH 9.0 in the presence of EDTA (Rochon & Tremaine, 1988). The remaining portion of the resuspended pellet was further purified by isopycnic centrifugation through caesium chloride (density 1.33 g/ml). Material corresponding to the lower, middle (CNV-containing) and upper portions of the gradient was removed, dialysed and RNA was then extracted from equal volumes of each portion of the gradient. Fig. 1 shows an ethidium bromide-stained non-denaturing agarose gel of RNA extracted from infected leaves or from virus at the two stages of purification. It can be seen that high levels of DI RNA are found in infected leaf RNA extracts (lane 6) but that little or no DI RNA can be seen in virion RNA extracts at either stage of purification (see lanes 7 and 9). In addition, DI RNA could be detected in neither the upper nor lower portions of the caesium chloride gradient (lanes 8 and 10). Therefore the failure to detect DI RNAs following caesium chloride purification was not because virus particles that contain DI RNAs have an altered buoyant density (i.e. because they have either more or less RNA per particle than particles containing only CNV genomic RNA). The inability to detect DI RNAs in virion RNA extracts despite their presence at very high levels in infected tissue suggests that CNV DI RNAs were not efficiently encapsidated during infection. However, it still cannot be completely ruled out that DI RNAs are encapsidated efficiently but are lost early in the virus purification procedure used.

To determine whether CNV DI RNAs can accumulate in plants in the absence of coat protein, synthetic transcripts derived by T7 RNA polymerase run-off transcription of three different previously described cloned CNV DI RNAs (DIs 42, 9 and 15) were co-inoculated on plants (1:10 molar ratio of helper to DI RNA; see Finnen & Rochon, 1993) with either synthetic wild-type RNA derived from a full-length CNV cDNA clone (pK2/M5; Rochon & Johnston, 1991) or with synthetic RNA of a mutant of CNV that lacked nearly the entire coat protein-coding sequence [CP(−); McLean et al., 1993]. Fig. 2 shows an ethidium bromide-stained non-denaturing agarose gel of total RNA extracted from leaves of N. clevelandii co-inoculated with the various combinations of DI RNA and helper virus. It can be seen that in each co-inoculation DI RNA accumulates to approximately equal levels using either wild-type or CP(−) genomic RNAs as helper (compare lanes 4 and 5, 6 and 7, and 8 and 9). Hence we conclude that CNV coat protein is not required for efficient accumulation of CNV DI RNAs in infected plants.

The results described above show that encapsidation of DI RNAs is not required for their efficient accumulation in plants. To assess a possible role for the CNV coat protein in the de novo generation of DI RNA from genomic RNA, we used a previously described mutant of CNV which generates high levels of DI RNA very rapidly after only a single passage of inoculum at high m.o.i. This mutant fails to express the CNV p20 non-structural protein owing to a single point mutation (CNV nucleotides 3832 to 4702; Rochon & Tremaine, 1989) of the p20 stop codon which contains the
entire mutated p20 coding sequence into the corresponding region of CP(−) using standard techniques (Sambrook et al., 1989). Concentrated sap from transcript-inoculated leaves was used to infect a second set of plants (i.e. first passage plants; see Rochon, 1991). RNA extracts from these leaves were analysed for the presence of DI RNAs. It can be seen in Fig. 3 that the CP(−)/p20(−) mutant generates and accumulates high levels of DI RNA in a single passage of high m.o.i. to the same efficiency as the p20 mutant containing the coat protein gene (compare lanes 3 and 4). We therefore conclude that CNV coat protein is not required for de novo generation of DI RNAs.

The above results show that the CNV coat protein is dispensable both for the generation and accumulation of CNV DI RNAs. The lack of a role for the coat protein in the stabilization of DI RNA is consistent with our previous observation that a CNV mutant, CP(−), lacking nearly the entire CNV coat protein-coding sequence, replicates and spreads both from cell to cell and systemically in infected plants (McLean et al., 1993). In addition, work by others with two different tombus-viruses, TBSV-Ch (Hillman et al., 1987) and CyRSV (Burgyan et al., 1992), has suggested that the DI RNAs of these viruses are inefficiently encapsidated. More recently, it has been shown that encapsidation does not significantly influence the relative levels of competitiveness of two different TBSV-Ch DI RNAs (White & Morris, 1994). Also, as pointed out by Burgyan et al. (1992), size does not appear to be a prerequisite for assembly of CyRSV Ds, since DI RNAs very close in size to CyRSV satellite RNA (which is encapsidated) are not found to be encapsidated. However, in the case of turnip crinkle Carmovirus, it has been suggested that size may affect the ability of its DI RNAs to become encapsidated as substitution of deleted portions of non-viable DI RNAs with non-viral sequences of the same length can restore DI RNA accumulation (Li & Simon, 1991). It remains to be determined whether small size alone affects the ability of CNV DI RNAs to become encapsidated.

Although virion-associated DI RNAs are not observed on ethidium bromide-stained agarose gels, Northern blot analysis has revealed that low levels of DI RNA species can be detected (Rochon & Johnston, 1991; data not shown). In addition, infections initiated with virus purified from DI-containing infections show attenuated symptoms and high levels of cellular DI RNAs are found in leaf RNA extracts. This indicates that DI RNAs are encapsidated during infection, but that encapsidation is very inefficient.

The genomes of many viruses are known to contain cis-acting sequences involved in the specificity of encapsidation. If such sequences do mediate encapsidation of CNV genomic RNA, these sequences appear to be absent from CNV DI RNAs since little or no DI RNA can be detected in virion RNA preparations. Furthermore, competition for encapsidation proteins by DI genomes has been suggested as a means for their interfering capability (Holland, 1991; Perrault, 1981; Roux et al., 1991). The observation that the presence of CNV DI RNAs is often associated with a reduction in

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**Fig. 2.** Analysis of DI RNA accumulation in DI RNA co-infections using CNV wild-type or CP(−) synthetic transcript as helper. Total RNA was extracted from *N. clevelandii* leaves and electrophoresed as described for Fig. 1. Lane 1 contains RNA extracted from a mock-inoculated plant, lane 2 from a plant inoculated with wild-type synthetic transcripts and lane 3 from a CP(−)-infected plant. Lanes 4, 6 and 8 contained RNA extracted from plants co-inoculated with wild-type synthetic CNV RNA and DI RNAs 42, 9 or 15, respectively. Lanes 5, 7 and 9 were co-inoculated with CP(−) synthetic RNA and DI RNAs 42, 9 or 15, respectively [see Finnen & Rochon (1993) for details of each DI RNA]. Positions of CNV, CP(−) and DI RNAs are shown on the right.

**Fig. 3.** Analysis of DI RNAs generated *de novo* from the CNV p20 mutant and from the CNV double mutant, CP(−)/p20(−). Total RNA was extracted from infected *N. clevelandii* plants and electrophoresed as described for Fig. 1. Lane 1 contains RNA from a mock-inoculated plant, and lanes 2 to 5 RNA from passage number 1 of CP(−), CP(−)/p20(−), p20 mutant or wild-type, respectively. Positions of CNV genomic, CP(−) and DI RNAs are shown on the right.
the levels of genomic RNA (see for example Fig. 1, lanes 1 and 6) suggests that competition for the coat protein does not contribute significantly to the interference effect.

DI RNAs accumulate to very high levels in CNV-infected N. clevelandii where they are often more abundant than any individual cytoplasmic or organellar ribosomal RNA (see for example Fig. 2, lane 4). The observation that DI RNA accumulation occurs efficiently in the absence of coat protein and our lack of evidence for efficient encapsidation in planta suggests that factors other than encapsidation or interaction with the coat protein contribute to stability of CNV DI RNAs. Several different factors could be involved including secondary or tertiary structure of the DI RNA, ability of DI RNAs to bind ribosomes, and/or interaction of DI RNAs with host- or virus-encoded proteins. Computer-generated secondary structure predictions of several different CNV DI RNAs reveal that they can potentially fold into highly structured molecules with free energy values ranging from −810.6 to −554.4 kJ/mol (D. M. R. Rochon & R. L. Finnen, unpublished). Also, we have noted that several different sequenced CNV DI RNAs retain the AUG codon that initiates synthesis of the 5'-proximal CNV p33 open reading frame (Finnen & Rochon, 1993), therefore increasing the likelihood that CNV DI RNAs can bind ribosomes and thereby be protected from cellular nucleases. The possibility that the process of translation can contribute to the stability of a defective RNA of clover yellow mosaic potexvirus, has been suggested previously (White et al., 1992). In previous work we have suggested that a CNV non-structural protein, p21, may be involved in CNV cell-to-cell movement (Rochon & Johnston, 1991). The tobacco mosaic virus cell-to-cell movement protein has been demonstrated to be a single-strand nucleic acid-binding protein (Citovsky et al., 1990). It is possible that the CNV p21 protein is also a nucleic acid-binding protein that could stabilize and therefore assist in the accumulation of CNV DI RNAs. It seems reasonable to suggest that factor(s) which contribute to the stability of CNV DI RNAs may also be involved in stabilizing CP(−) RNA or CNV wild-type RNA during replication and movement in plants. Studies that aim to elucidate factors involved in stabilizing DI RNAs may contribute to our understanding of those which assist in the stabilization of genomic RNA.

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References


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