Carrot Red Leaf and Carrot Mottle Viruses: Observations on the Composition of the Particles in Single and Mixed Infections

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SUMMARY

Particles of carrot red leaf virus (CRLV; luteovirus group) purified from chervil (Anthriscus cerefolium) contain a single ssRNA species of mol. wt. about $1.8 \times 10^6$ and a major protein of mol. wt. about 25000. CRLV acts as a helper for aphid transmission of carrot mottle virus (CMotV; ungrouped) from mixedly infected plants. Virus preparations purified from such plants possess the infectivity of both viruses but contain particles indistinguishable from those of CRLV; some of the particles are therefore thought to consist of CMotV RNA packaged in CRLV coat protein. When RNA from such preparations was electrophoresed in agarose/polyacrylamide gels, CMotV infectivity was associated with an RNA band that migrated ahead of the CRLV RNA band and had an estimated mol. wt. of about $1.5 \times 10^6$, similar to that previously found for the infective ssRNA extracted directly from Nicotiana clevelandii leaves infected with CMotV alone. Preparations of dsRNA from CMotV-infected N. clevelandii leaves contained two species: one of mol. wt. about $3.2 \times 10^6$, presumably the replicative form of the infective ssRNA, and the other, mol. wt. about $0.9 \times 10^6$, of unknown origin and function. The infective agent in buffer extracts of CMotV-infected N. clevelandii was resistant to RNase (although the enzyme acted as a reversible inhibitor of infection at high concentrations) and is therefore not unprotected RNA. It may be protected within the approximately 52 nm enveloped structures previously reported.

Carrot red leaf virus (CRLV; Watson et al., 1964) was purified by Waterhouse & Murant (1981) and shown to be a luteovirus. A second virus, carrot mottle (CMotV), which is ungrouped, commonly occurs together with CRLV in infected carrot (Daucus carota) plants and depends on it for transmission by the aphid Cavariella aegopodii (Watson et al., 1964). However, CMotV is transmissible by manual inoculation whereas CRLV is not. Data from serological neutralization and other types of experiment (Waterhouse & Murant, 1983) indicated that, in mixed infections of the two viruses in chervil (Anthriscus cerefolium), some particles are produced that consist of CMotV RNA in a coat composed partially or entirely of CRLV protein; presumably these are the particles that enable CMotV to be transmitted by C. aegopodii. In thin sections of Nicotiana clevelandii leaves infected with CMotV alone, Murant et al. (1969) found approximately 52 nm enveloped structures which they suggested might be the particles of the virus; partially purified preparations containing them, along with much cell membrane material, were infective. However, the possibility was not excluded that the structures were cytopathic vesicles involved in early stages of virus replication. Halk et al. (1979) showed that infective CMotV ssRNA, which is readily extracted from infected N. clevelandii leaves, migrated in agarose/polyacrylamide gels as a molecule of mol. wt. about $1.5 \times 10^6$ to $1.6 \times 10^6$. In this paper we describe some additional observations on the composition of particles purified from chervil infected with CRLV alone, or with CRLV + CMotV, and on the nature of the infective particles in N. clevelandii infected with CMotV alone.

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Particles of the CRLV isolate of Murant et al. (1969) and Waterhouse & Murant (1981, 1983) were purified by extraction with Driselase (Kyowa Hakko Kogyo Co., Tokyo, Japan) followed by rate zonal centrifugation in sucrose gradients and then by isopycnic banding in CsCl (Waterhouse & Murant, 1981). For determination of coat protein mol. wt., the virus particles were dissociated and the product electrophoresed in gels of 3.5, 5, 7.5 or 10% polyacrylamide, as described by Bern & Murant (1979). The preparations yielded a major component whose mol. wt. was estimated at about 25,000 regardless of gel concentration. In addition, there were three minor components of mol. wt. approx. 40,000, 50,000 and 60,000. The 50,000 mol. wt. component possibly represented subunit dimers but the nature of the other components is not clear. Despite the extensive purification of the CRLV preparation, the possibility cannot be excluded that one or more of these minor proteins were of plant origin but firmly bound to the virus particles.

For determination of RNA mol. wt., the virus particles were dissociated by mixing the virus preparation with an equal volume of 0.02 M-Tris–HCl, 0.001 M-EDTA, 4% SDS, pH 9.0, and heating the mixture at 60 °C for 15 min. Electrophoresis in 2.4% polyacrylamide gels was done under non-denaturing conditions as described by Bern & Murant (1979). Marker RNA species, nucleotide numbers (from sequence data) and mol. wt. values (calculated for the sodium salts from the sequence data) were: tobacco mosaic virus (TMV) RNA (6395 nucleotides, mol. wt. 2.21 × 10^6; Goelet et al., 1982), and the two ribosomal RNA species of Escherichia coli (2904 and 1541 nucleotides, mol. wt. 1.01 × 10^6 and 0.53 × 10^6; Brosius et al., 1978, 1980; Carbon et al., 1978). The CRLV preparation produced a single nucleic acid band; in three experiments, its mol. wt. was estimated to be 1.78 × 10^6 (5–14 kb), not significantly different from that found for the RNA of potato leafroll virus (1.82 × 10^6; 5–25 kb) which was included for comparison. The stained band disappeared from gels that were soaked for 2 h at 30 °C in a solution containing 10 μg/ml RNase A (Sigma) in 0.05 M-Tris-HCl pH 7.5, with or without 0.3 M-NaCl. The band was unaffected when the gels were incubated in a solution of 10 μg/ml DNase (Sigma) in 0.05 M-Tris–HCl, 0.003 M-MgCl₂, pH 7.5. These observations indicate that CRLV nucleic acid is ssRNA.

Experiments were next done to investigate the RNA composition of particles purified from chervil plants mixedly infected with CRLV and CMotV. When such plants are grown in the high light intensity conditions best suited for CRLV (18 °C, 22 h daylength, and a photosynthetic photon flux density (PPFD) of 325 μmol (400 to 700 nm) m⁻² s⁻¹ the CMotV component gradually diminishes in amount and after several passages may be eliminated from the culture. In contrast, at low light intensities it is the CRLV component that multiplies poorly (Elnagar & Murant, 1978). As a compromise, the mixed virus culture was propagated in chervil at 20 °C, 16 h daylength, and a PPFD of 250 μmol (400 to 700 nm) m⁻² s⁻¹, in which conditions both components were maintained indefinitely. The yields of virus particles in preparations made from mixedly infected plants [calculated by taking the A1 cm260 = 7, as assumed (Waterhouse & Murant, 1981) for particles of CRLV] ranged from 0.1 to 1.1 mg/kg leaf, comparable at best with those of CRLV alone. All six preparations tested were infective for CMotV when rubbed on the leaves of herbaceous test plants, as reported previously (Waterhouse & Murant, 1983). The most infective preparation induced 83 lesions per inoculated leaf of Phaseolus vulgaris cv. The Prince when inoculated at 8 μg virus particles per ml. This infectivity is low compared with that of other mechanically transmissible viruses, suggesting that even in this preparation only a small proportion of the particles contained CMotV RNA.

The RNA from each of the six preparations from mixedly infected plants was analysed by electrophoresis in 0.5% agarose, 2.4% polyacrylamide gels, the RNA from 100 to 200 μg virus particles being placed on each gel. After electrophoresis the gels were scanned and sliced, and the RNA eluted from the slices and inoculated to P. vulgaris, as described by Halk et al. (1979). Only with one of the six preparations were any of the eluted RNA samples infective (Fig. 1); the infective material co-migrated with a minor RNA band that travelled slightly ahead of the more prominent CRLV RNA band. In the five experiments in which no infectivity was recovered from the gels, only the CRLV band was observed.

The mol. wt. of CMotV RNA estimated from the position of the minor band in the single successful experiment was about 1.54 × 10^6 (4.44 kb). This estimate agrees with that of about 1.5 × 10^6 to 1.6 × 10^6 obtained by Halk et al. (1979) for the infective RNA in extracts from leaves.
Fig. 1. Microdensitometer trace (arbitrary units) of a photograph of a gel stained with toluidine blue O (---) and infectivity (tested on half-leaves of *P. vulgaris*) from batches of five consecutive 1 mm slices of gel (histogram) after electrophoresis in a gel of 0-5% agarose + 2-4% polyacrylamide of RNA from 100 μg virus purified from chervil infected with CRLV + CMotV. Arrows indicate the positions of CRLV RNA and of the markers: 1, TMV RNA; 2 and 3, *E. coli* ribosomal RNA.

Fig. 2. Electrophoresis in 7% polyacrylamide slab gels of dsRNA preparations from (a) MRDV, (b) CMotV and (c) RDV.

of *N. clevelandii* infected with CMotV alone. That estimate too was obtained by determining the position of the CMotV infectivity after electrophoresis of the RNA preparations in agarose/polyacrylamide gels; the position of the CMotV RNA could not be determined by staining because it was obscured by the host 1.3 × 10⁶ mol. wt. ribosomal RNA band.

The dsRNA in extracts of CMotV-infected *N. clevelandii* was analysed in further experiments. Systemically infected leaves were ground with a pestle and mortar in liquid nitrogen and the dsRNA recovered by phenol extraction and chromatography on CF-11 cellulose (Whatman), essentially as described by Dodds & Bar-Joseph (1983), except that the phenol reagent consisted of 9 vol. water-saturated phenol + 1 vol. *m*-cresol + 1 g/l 8-hydroxyquinoline, and the ethanol:buffer ratio used for binding the dsRNA to the CF-11 column was 20:80. The nucleic acid eluted from the column with buffer alone was recovered by ethanol precipitation, resuspended in 0.05 M-Tris–HCl, 0.05 M-NaCl, 0.01 M-MgCl₂, pH 7.6, and incubated for 30 min at 30 °C in the presence of 10 μg/ml DNase I, then adjusted to 0.3 M with NaCl and treated with 10 ng/ml RNase A for 1 h at 30 °C. The digest was then re-extracted with phenol reagent. The resulting dsRNA preparations were analysed by electrophoresis for 21 h at 3.75 V/cm in 7% polyacrylamide slab gels in the presence of 0.015 M-Tris–HCl, 0.04 M-phosphate, 0.002 M-EDTA, pH 7.6 (Reddy & Black, 1973). The gels were stained with silver (Igloi, 1983). Preparations of dsRNA from maize rough dwarf virus (MRDV) and rice dwarf virus (RDV), kindly provided by Dr G. Boccardo, were used as markers. The mol. wt. of the dsRNA species of MRDV range from 2·88 × 10⁶ to 1·08 × 10⁶ (Reddy *et al.*, 1975) and those of RDV from 3·1 × 10⁶ to 0·48 × 10⁶ (Reddy *et al.*, 1974).

Two bands of dsRNA were consistently observed in the CMotV preparations (Fig. 2), but none in preparations from healthy *N. clevelandii*. The mol. wt. of the larger dsRNA of CMotV was estimated to be approx. 3·2 × 10⁶ (4·62 kbp) and is presumably the replicative form of the infective ssRNA. The mol. wt. of the smaller dsRNA of CMotV was estimated to be approx. 0·9 × 10⁶ (1·30 kbp); its nature is unknown.
Table 1. **Effect of RNase on infectivity of CMotV in leaf extracts**

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* Numbers of local lesions in four half-leaves of *P. vulgaris.*
† In experiment 3, the RNase-treated leaf extracts were extracted with phenol reagent, and the infectivity of ethanol-precipitable nucleic acid assayed.

Taken together, these experiments confirm that the infective ssRNA of CMotV has a mol. wt. of approx. $1.5 \times 10^6$ and that it occurs within some of the approximately 52 nm virus particles that are purified from plants mixedly infected with CRLV and CMotV. There seems no doubt that it is the production of these particles that enables CMotV infection to be transmitted by the aphid vector of CRLV, *C. aegopodii*, as suggested by Waterhouse & Murant (1983).

The nature of the infective material in *N. clevelandii* plants infected with CMotV alone is less obvious. Earlier work (Murant *et al.*, 1969) indicated that the infectivity of CMotV, in buffer extracts of infected leaves, as determined by local lesion assay in *Chenopodium quinoa*, was not in the form of unprotected RNA because it was stable for several hours at room temperature and was unaffected by RNase A at 0.25 ng/ml. These observations suggested that the CMotV RNA in these leaf extracts was protected in some way, possibly within the 52 nm enveloped structures which were the putative virus particles. In contrast, Falk *et al.* (1979), working with lettuce speckles mottle virus (LSMV), an agent that shares several characteristics with CMotV including its dependence on a luteovirus (beet western yellows virus) for transmission by aphids, its moderate stability at room temperature, and the production of similar enveloped structures in infected cells, reported that infectivity in buffer extracts from *N. clevelandii* leaves is diminished by treatment with RNase at 1 ng/ml and abolished at RNase concentrations exceeding 100 ng/ml; these results too were obtained by local lesion assay in *C. quinoa*. Falk *et al.* (1979) argued from their observations that in single infections LSMV exists as a defective virus lacking a functional coat protein, and that the enveloped structures were probably sites of virus replication.

At the high concentrations used by Falk *et al.* (1979), RNase is known to inhibit infection by intact virus particles, even those of TMV and potato virus X whose RNA is well protected from nucleolysis (Loring, 1942; Bawden & Kleczkowski, 1948). Experiments were therefore done to confirm that the infectivity in crude leaf extracts of CMotV-infected *N. clevelandii* is resistant to RNase and to ascertain whether at higher concentrations RNase acts as an inhibitor of infection.

Extracts of CMotV-infected *N. clevelandii* leaves were made by grinding in 0.017 M-phosphate buffer pH 7.3, and treated for 30 min at room temperature with various concentrations of RNase A. Experiments 1 and 2 in Table 1 indicate that the number of lesions induced in *P. vulgaris* was not affected by 0.25 ng/ml RNase, but gradually decreased as the RNase concentration was increased between 1 and 500 ng/ml. However, when RNase-treated leaf extracts were extracted with phenol reagent, infective RNA was recovered by ethanol precipitation (experiment 3, Table 1). Indeed, equal levels of infectivity were found in material recovered in this way from extracts treated with 500 ng/ml RNase and from untreated extracts. Thus, it seems that the decrease of CMotV infectivity on treatment with RNase is caused not by nucleolysis but by inhibition of infection in the presence of the enzyme. These results confirm that CMotV infectivity in crude leaf extracts is in some way resistant to degradation by RNase and is therefore not unprotected RNA. Possibly the same is true for LSMV. This protection may well be provided within the enveloped structures that occur in cells infected with each of these viruses. In our opinion, it still remains an open question whether these structures are virus particles of a kind unusual among plant viruses or are merely cytopathic vesicles concerned with RNA replication.
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REFERENCES


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