Clover Primary Leaf Necrosis Virus, a Strain of Red Clover Necrotic Mosaic Virus

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ABSTRACT

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Clover primary leaf necrosis virus was identified as a strain of red clover necrotic mosaic virus (RCNMV-C) on the basis of host range, serology, nucleic acid, and coat protein analyses. RCNMV-C was serologically identical to RCNMV-48 but not to other isolates of virus examined. Under denaturing conditions, RCNMV-C RNA separated into two components whose electrophoretic mobilities were indistinguishable from those of RCNMV-A. The molecular weight of the major polypeptide of RCNMV-C was about 41,000, whereas that of the minor one was about 39,000, which is believed to be a degradation product of the coat protein.

Additional key word: dianthoviruses

Clover primary leaf necrosis virus (CPLNV) was first described as a new virus isolated from field-infected red clover plants (*Trifolium pratense* L.) in the Vancouver area of British Columbia, Canada (19). Purified preparations of CPLNV contained polyhedral particles about 36 nm in diameter sedimenting in analytical ultracentrifuge as a single component of about 136 S (19). Although some of the in vitro properties of CPLNV were similar to those of carnation ringspot virus (CRSV) (10), the two viruses were reported to be serologically unrelated (19). More recently, however, a study has shown that CPLNV is genetically related to red clover necrotic mosaic virus (RCNMV) and sweet clover necrotic mosaic virus (SCNMV) (18). In this paper, we report the results of studies on host range, serology, nucleic acid, and capsid protein mobilities of CPLNV and conclude that the virus is a strain of RCNMV that we will refer to as RCNMV-C.

MATERIALS AND METHODS

Growth conditions. All plants were grown in 12-cm-diameter plastic pots containing an autoclaved mixture of loam, sand, and peat (3:2:1, v/v) in a glasshouse at 25 ± 2 C.

Virus isolates and purification. RCNMV-C (19), RCNMV-A (Australian isolate) (4), RCNMV-SW (Swedish isolate) (2), RCNMV-34 and -48 (16,17), RCNMV-E (1), SCNMV (8), and CRSV (22) were maintained and propagated in *Phaseolus vulgaris* L. 'Red Kidney.' The procedure used for the purification of all the RCNMV isolates was the same as described by Gould et al (4), of SCNMV as described by Hiruki et al (8), and of CRSV as described by Lommel et al (13). Virus preparations used for immunization of rabbits were subjected to at least two cycles of sucrose density-gradient centrifugation as a final purification step.

Preparation of antisera. Antiserum to RCNMV-C was prepared in rabbits. Each adult rabbit received two intramuscular injections containing 1 mg each of virus emulsified with an equal volume of Freund's complete adjuvant at weekly intervals. A third injection containing 2 mg of virus was administered intravenously 2 wk later, and the animals were bled at least 1 wk after the final injection.

Serology. Immunodiffusion tests were performed as previously described (20).

Polyacrylamide gel electrophoresis of viral RNA. The procedures used for isolating viral RNA and determining their molecular weights in 3% polyacrylamide gels containing 7 M urea were essentially the same as those previously described (3).

SDS-polyacrylamide gel electrophoresis of viral protein. Purified virus preparations (1 mg/ml) were mixed with an equal volume of buffer (0.12 M Tris-HCl, pH 6.8; 4% [w/v] sodium dodecyl sulfate [SDS]; 10% [v/v] 2-mercaptoethanol; 10% [w/v] sucrose) and heated for 2 min in a boiling water bath to denature the proteins. The samples (10–25 μl) were then electrophoresed in 9% discontinuous SDS-polyacrylamide gels (12) at 10 mA for 8 hr in slab gels (10 × 12 cm and 1.5 mm thick). After electrophoresis, the gels were stained with 0.25% Coomassie blue and destained in methanol/water/acetic acid (5:5:1, v/v). Molecular weights of the viral proteins were estimated by comparing their mobilities with those of proteins of known molecular weights: bovine plasma albumin (mol wt 66,000), ovalbumin (mol wt 45,000), trypsinogen (mol wt 24,000), and tobacco mosaic virus coat protein (mol wt 17,500).

RESULTS

Biological properties of RCNMV-C. RCNMV-C infected a number of plant species including *N. tabacum* L. 'White Burley,' *Vigna unguiculata* (L.) Walp. 'Blackeye,' *P. vulgaris* L. 'Red Kidney,' *Cucumis sativus* L. 'National Pickling,' and *Chenopodium quinoa* and produced symptoms characteristic of RCNMV (9). In comparative studies of symptomatology, RCNMV-C was readily distinguishable from CRSV, SCNMV, and RCNMV-A by its reaction on the following selected host plants: *N. clevelandii, P. vulgaris, V. unguiculata, C. amaranticolor* Coste & Reyn., *C. sativus, Tetragonia expansa* Thunb., and *Gomphrena globosa* L. (Table 1).

Serology. In double-diffusion tests, antiserum to RCNMV-C with a homologous titer of 1:256 reacted at dilutions of 1:256 against RCNMV-48; 1:128 against RCNMV-A; and 1:8 against RCNMV-34, RCNMV-SW, and RCNMV-E. The absence of crossed precipitin lines or spurs between RCNMV-C and RCNMV-48 indicates that the two isolates are identical (Fig. 1). The serological relationship between RCNMV-C and two other dianthoviruses, SCNMV and CRSV, was also investigated in immunodiffusion tests. Purified preparations of SCNMV reacted distantly to RCNMV-C antiserum with a heterologous titer of 1:4, whereas no positive reaction was observed with CRSV.

Polyacrylamide gel electrophoresis of viral RNA. RCNMV-C RNA separated into two major components when electrophoresed in 3% polyacrylamide gels under denaturing conditions (Fig. 2A). The components, designated RNA-1 and RNA-2, were estimated to have molecular weights of about 1.5×10^6 and 0.5×10^6, respectively, when their electrophoretic mobilities were compared with those of brome mosaic virus (RNA-1, 1.1×10^6; RNA-2, 1.0×10^6; RNA-3, 0.7×10^6; and RNA-4, 0.3×10^6). The electrophoretic mobilities of the two RNA components of RCNMV-C were indistinguishable from those of RCNMV-A when coelectrophoresed in polyacrylamide gels under denaturing conditions.

SDS-polyacrylamide gel electrophoresis of viral coat protein. Protein from highly purified preparations of RCNMV-C migrated as one major polypeptide and a minor one present in very low amounts as

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judged by visual inspection of the stained gels (Fig. 2B). The molecular weight of the major polypeptide was about 41,000 when compared with a series of marker proteins, whereas that of the minor one was about 39,000. Under similar conditions, the proteins from RCNMV-A also migrated as one major band (mol wt 41,000) and one minor band (mol wt 39,000).

DISCUSSION

As an aid in deciding whether a newly described virus should be considered a new virus or a strain of an already described virus, a set of guidelines has been developed (5). By following these guidelines, we consider that CPLNV is a typical dianthovirus (14). The results of our investigations on its host range, biochemical properties, and serological relationship to other members of the dianthovirus group suggest that the virus could be considered a strain of RCNMV (RCNMV-C).

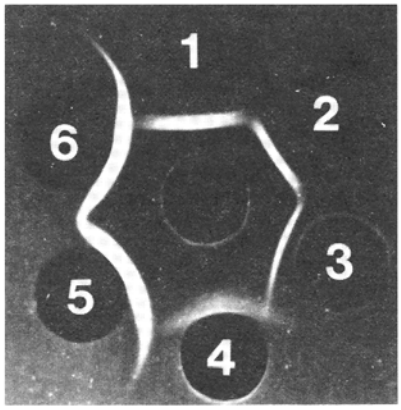

Fig. 1. Serological reaction in immunodiffusion plate. Central well contained antiserum to RCNMV-C; peripheral wells were charged with antigens (1) RCNMV-E (England), (2) RCNMV-34 (Czechoslovakia), (3) RCNMV-A (Australia), (4) RCNMV-SW (Sweden), (5) RCNMV-C (Canadian), and (6) RCNMV-48 (Czechoslovakia).

Symptomatology of RCNMV-C differed somewhat from that reported previously (19). For example, in this investigation, *C. amaranticolor* plants were locally infected with small lesions (Table 1), whereas the same test plant was reportedly resistant to the virus (19). Likewise cowpea, infected only locally in the previous investigation (19), was found to be systemically susceptible to RCNMV-C. Negative results with *G. globosa* (Table 1) may be due to a tendency of this test plant to react inconsistently to RCNMV-C (19).

Although RCNMV-C is serologically related to other isolates of the virus, considerable antigenic variation appears to exist between them. It is interesting to note that although RCNMV-48 and RCNMV-C come from two different geographical regions, remarkably they are identical. The reaction of RCNMV-C antiserum to SCNMV was very weak, reaching a heterologous titer of only 1/4, whereas no reaction was observed with CRSV. These results are in agreement with a previous report (8). Recently, however, a distant antigenic relationship between RCNMV-C and CRSV was demonstrated in indirect enzyme-linked immunosorbent assays (7). Such a relationship is expected since it is possible to construct viable pseudorecombinants between the two viruses (T. Okuno and C. Hiruki, *unpublished*).

Like other RCNMV isolates (9), RCNMV-C coat protein is also a single polypeptide with an approximate molecular weight of 41,000. Recently, a translation product of RCNMV RNA-1 in rabbit reticulocyte lysate has been reported to have a molecular weight of 39,000 and was shown to be the coat protein (15) that is similar to that obtained in our virus preparations. We believe that the minor component is produced by degradation of the virus coat protein during purification or in storage as occurs in several other plant viruses (6,11,21), since we observed that freshly purified virus preparations contain small amounts of the minor band, although it becomes more pronounced after prolonged storage of the preparations at 4 C.

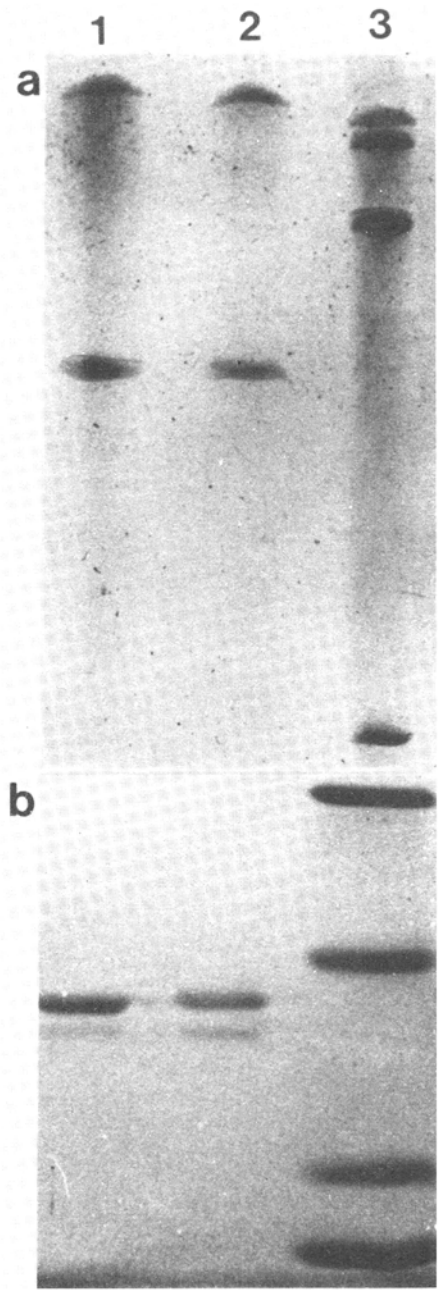

Fig. 2. (A) Analysis of RNAs on a 3% polyacrylamide gel containing 7 M urea. Lanes 1–3: RNAs from (1) RCNMV-AUS, (2) RCNMV-C, and (3) brome mosaic virus. **(B)** Analysis of viral coat proteins from RCNMV-AUS (lane 1) and from RCNMV-C (lane 2) in 9% discontinuous SDS-polyacrylamide gel system. Bands in lane 3 are molecular weight markers containing (from top) bovine plasma albumin (66,000 daltons), ovalbumin (45,000 daltons), trypsinogen (24,000 daltons), and tobacco mosaic virus coat protein (17,500 daltons).

Table 1. Comparative symptomatology of selected dianthoviruses

Plant species[a]	RCNMV-C[b]	RCNMV-AUS[c]	SCNMV[d]	CRSV[e]
Phaseolus vulgaris 'Red Kidney'	s,c,LL;sy,y,ns[f]	l,c,LL; sy,y,ns	r,b,rs; sy,vn	s,c,rs; sy,vn
Vigna unguiculata 'Blackeye'	c,LL; sy,m	r,b,LL;sy,vn	r,b,LL;sy,vn	r,b,LL; sy, vn
Chenopodium amaranticolor	s,c,LL	l,LL,c,h	LL	l,c,LL,c,h
Cucumis sativus	s,LL,c,h	l,LL,c,h	LL	l,c,LL,c,h
Tetragonia expansa	LL	LL; sy,m	LL; sy,m	LL; sy,m
Gomphrena globosa	NI	r,b,LL	r,b,LL	L,nrs; sy,m,ld
Nicotiana clevelandii	NI	ns; sy,m	ns; sy,m	ns; sy,m

[a] A minimum of six plants was inoculated for each test species, and symptoms were recorded 12–15 days after inoculation. Before inoculation, all plants were kept in the dark for at least 12 hr to increase sensitivity.
[b] Red clover necrotic mosaic virus (Canadian isolate).
[c] Red clover necrotic mosaic virus (Australian isolate).
[d] Sweet clover necrotic mosaic virus.
[e] Carnation ringspot virus.
[f] Coded symptom descriptions: b = brown, c = chlorotic, h = halo, l = large, ld = leaf distortion, l. = local, LL = local lesions, m = mosaic, nrs = necrotic ringspot, ns = necrotic spots, r = reddish, rs = ringspot, s = small, sy = systemic, vn = veinal necrosis, y = yellow, and NI = plants produced no symptoms and virus could not be recovered by back-inoculation to susceptible hosts.

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