Genetic Determinants Distributed in Two Genomic RNAs of Sweet Clover Necrotic Mosaic, Red Clover Necrotic Mosaic and Clover Primary Leaf Necrosis Viruses

By T. OKUNO, C. HIRUKI,* D. V. RAO AND G. C. FIGUEIREDO
Department of Plant Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

(Accepted 20 April 1983)

SUMMARY

Three serologically distinct polyhedral viruses, sweet clover necrotic mosaic virus (SCNMV), red clover necrotic mosaic virus (RCNMV) and clover primary leaf necrosis virus (CPLNV), contain bipartite RNAs with approximate mol. wt. of 1.35 × 10^6 to 1.55 × 10^6 (RNA 1) and 0.5 × 10^6 to 0.6 × 10^6 (RNA 2). The homologous and heterologous combinations of RNA 1 and RNA 2 of the three viruses were highly infectious, while individual RNA species were not. Electrophoretic mobility of genomic RNA from the pseudorecombinants showed that the progeny viruses maintained the heterologous RNA combinations identically with those in the original inocula. Serological specificity of the progeny viruses was determined by RNA 1. A mixture of isolated coat protein and RNA 1 was not infectious. RNA 1 of SCNMV was essential for systemic infection of sweet clover at 26 °C, while RNA 2 of SCNMV complemented RNA 1 of RCNMV in causing local infection in sweet clover at 26 °C. The heterologous combinations of CPLNV RNA 1 and RNA 2 of SCNMV or of RCNMV acquired the ability to infect white clover at 26 °C and also caused symptoms characteristically different from those induced by their parental viruses on Phaseolus vulgaris L. cv. Red Kidney.

INTRODUCTION

Carnation ringspot virus (CRSV) (Dodds et al., 1977) and several strains of red clover necrotic mosaic virus (RCNMV) (Gould et al., 1981) have bipartite genomes of approximately 1.3 × 10^6 to 1.5 × 10^6 (RNA 1) and 0.5 × 10^6 to 0.6 × 10^6 (RNA 2) mol. wt. which are encapsidated in polyhedral particles, 31 to 35 nm in diameter. Sweet clover necrotic mosaic virus (SCNMV) (Hiruki et al., 1981) and clover primary leaf necrosis virus (CPLNV) (Ragetli & Elder, 1977) have particles of a similar size and we show in this paper that they have bipartite genomes similar to that of RCNMV. SCNMV is serologically distinct from RCNMV and CPLNV (C. Hiruki et al., unpublished results), which are related to each other (Hollings & Stone, 1979). To elucidate the possible relationship among these viruses, the two RNA species of each were separated, in vitro genomic recombination attempted, and genetic determinants for serological specificity, host range and symptomatology were studied.

METHODS

Viruses. SCNMV (isolated from sweet clover), CPLNV (a gift from Dr H. W. J. Ragetli, Agriculture Canada Research Station, Vancouver) and RCNMV (a Swedish strain from Dr B. Gerhardson) were maintained and propagated on Phaseolus vulgaris L. cv. Red Kidney. Leaves of 9- to 10-day-old plants were sap-inoculated in the presence of 0.2 N-Na_2HPO_4-KH_2PO_4 buffer, pH 7.1. Inoculated leaves were harvested 5 to 7 days after inoculation and kept frozen at −60 °C until required.

Virus purification. After sap from infected leaves was clarified by chloroform–butanol (1:1) treatment, the virus was precipitated with 8% polyethylene glycol 6000 and 0.4% NaCl, and then subjected to two cycles of differential centrifugation (high speed 108,000 g for 90 min; low speed 16,000 g for 15 min). Except when virus antigen was used for enzyme-linked immunosorbent assay (ELISA), the virus was further purified by centrifugation in a 10 to 40% sucrose density gradient prepared in 0.025 M-Na_2HPO_4-KH_2PO_4 buffer, pH 7.0.
RNA extraction. A solution of purified virus (2 mg/ml) was mixed with an equal volume of 0.05 M-Tris–HCl pH 7.7 containing 0.005 M-sodium EDTA, 0.05 M-NaCl, 1% SDS, 0.1% bentonite. The mixture was shaken vigorously with an equal volume of water-saturated phenol at room temperature for 15 min. The aqueous phase was extracted three times with ether. RNA was precipitated by adding 5 vol. ethanol and left overnight at −20 °C. The pellet was dissolved in distilled water containing 16% glycerol or electrophoresis buffer containing SDS and urea (Okuno & Furusawa, 1979).

Separation of RNA species. Conditions of electrophoresis were as described by Okuno & Furusawa (1979). About 300 μg of virus RNA was loaded on a 2.25 mm polyacrylamide gel slab (10 × 12 cm and 3 mm thick) and electrophoresed at 30 V for 4 h at room temperature. The RNA bands stained with 0.05% toluidine blue were cut out and the gel homogenate containing RNA was suspended in a small volume of 0.05 M-Tris–HCl pH 7.7 containing 0.005 M-sodium EDTA, 0.1 M-NaCl, 0.5% SDS and 0.1% bentonite. RNA species, extracted from the suspension by the phenol method, were further purified by linear sucrose density centrifugation (10 to 40%) in a Beckman SW28 rotor at 130000 g for 18 h and recovered by means of an Isco fractionator with an absorbance monitor. Tobacco mosaic virus (TMV) RNA and brome mosaic virus (BMV) RNA served as size markers.

Electrophoresis of coat protein. Virus coat protein was dissociated from purified virus (1 to 3 mg/ml) by adding an equal volume of 0.055 M-Tris–HCl pH 6.8, containing 3.5% SDS, 3.5% 2-mercaptoethanol, 16% glycerol and 0.005% bromophenol blue, and then heating for 2 min in boiling water. The samples were frozen at −20 °C until required. Conditions of electrophoresis were as previously described (Okuno & Furusawa, 1979). Five to 10 μl of sample was loaded into each well of the slab gel (10 × 12 cm and 1.5 mm thick). Mol. wt. markers used were bovine plasma albumin (66000), ovalbumin (45000), bovine pancreas trypsinogen (24000) and lactoglobulin (18400).

Serology. Antisera were prepared by injecting purified virus preparations into rabbits. Three, weekly intramuscular injections in Freund's complete adjuvant were followed by two intravenous injections, each of 1 ml containing 2 mg of virus. The rabbits were bled 1 week after the final injection when antiserum titres were 1/512 to 1/1024 as determined by the ring-interface test. ELISA (Clark & Adams, 1977) was used to determine the serological specificity of viruses in both the purified preparation and crude sap. For antigen preparation the final concentration of purified virus preparations was adjusted to 1 μg/ml with PBSTO buffer (0.2 M-phosphate buffer containing 0.15 M NaCl, 0.05% Tween 20 and 0.1% ovalbumin, pH 7.4). For extraction of sap samples, 1 g of each inoculated leaf was ground in the presence of 9 ml of PBSTO buffer and the resulting sap was used for ELISA. Gamma-globulin was used at 1 μg/ml and enzyme conjugate at 1 : 3000 dilution. Crude sap was used at 1/10 dilution (w/v) in extraction buffer and purified virus at 1 μg/ml. The plate was washed at least five times with phosphate-buffered saline–Tween between the reaction steps. The absorbance at 405 nm was determined with a multiple-channel photometer (Flow Laboratories) after 60 min incubation with the enzyme substrate at room temperature.

Preparation of coat protein of RCNMV. RCNMV was degraded by dialysing the virus against 1 M-CaCl2, 0.01 M-Tris–HCl, pH 7.5 and the coat protein was isolated as reported previously (Shepherd et al., 1968).

Plants. Leguminous assay plants were grown in pots containing U.C. mix (Matkin & Chandler, 1957) at 26 ± 2 °C. Chenopodium amaranticolor Coste & Reyn. plants were grown in a soil mix (vol. ratio: 3 loam, 2 peat, 1 sand).

 Infectivity assay of pseudorecombinants. The following plants were used: sweet clover [Melilotus officinalis (L.) Lam.], 20 to 30 days old; red clover (Trifolium pratense L. cv. Hungaropoly), 20 to 25 days old; white clover (T. repens L.), 20 to 30 days old; cowpea [Vigna unguiculata (L.) Walp cv. Early Ramshorn], 6 to 8 days old; C. amaranticolor, 35 to 45 days old; Red Kidney bean (Phaseolus vulgaris L. cv. Red Kidney), 7 to 10 days old; broad bean (Vicia faba L. cv. Broad Windsor), 10 to 15 days old. For pathogenicity and symptomatology tests at 17 °C and 26 °C, infection at 7 days after inoculation was determined by ELISA as well as by duplicated infectivity assays on C. amaranticolor and Red Kidney bean leaves.

RESULTS

RNA species and the infectivity of pseudorecombinants

Virions of SCNMV, RCNMV and CPLNV each contained two species of RNA (Fig. 1). The approximate molecular weights of RNA 1 and RNA 2 of SCNMV, RCNMV and CPLNV under partially denaturing conditions were 1.35 × 106 and 0.55 × 106, 1.45 × 106 and 0.59 × 106, and 1.55 × 106 and 0.61 × 106 respectively. Any combination of RNA 1 and RNA 2 from these viruses was highly infectious whereas either RNA 1 or RNA 2, when tested alone, was not (Table 1). However, RNA 1 from SCNMV and CPLNV sometimes produced one local lesion per three to five half-leaves of C. amaranticolor, probably because of low-level
Genetic determinants of dianthoviruses

Fig. 1. Analysis on a 2.25% polyacrylamide gel of RNA extracted from (a) CPLNV, (b) RCNMV, (c) SCNMV, (d) BMV and (e) TMV. Two to five µg of RNA was loaded onto each well.

contamination by RNA 2. Single lesion isolates were obtained by repeated inoculations with individual lesions produced on cowpea and V. faba for the propagation of pseudorecombinants.

Physical properties of pseudorecombinants

The progeny virus derived from heterologous combinations of RNA 1 and RNA 2 among SCNMV, RCNMV and CPLNV maintained their RNA components without change in the original electrophoretic mobility (Fig. 2a).

The electrophoretic mobility on SDS-polyacrylamide gels of the coat protein of the progeny viruses derived from single lesions was similar to that of the virus providing RNA 1 (Fig. 2b). Approximate mol. wt. of coat proteins of RCNMV, SCNMV and CPLNV were 37500, 38000 and 38500 respectively.

Serological specificity of the progeny viruses originating from heterologous combinations of RNA 1 and RNA 2 species was identical to that of the donor virus of RNA 1 (Table 1).

Biological properties of pseudorecombinants

In an attempt to characterize genetic determinants for biological properties distributed on the two genomic RNAs of SCNMV, RCNMV and CPLNV, several plant species known to respond differently to the parental viruses were selected. RCNMV did not infect sweet clover plants at 26 °C, while local and rarely systemic infection with necrotic spots developed at 17 °C (Table 2). Abbreviations used to describe pseudorecombinants are given in the legend to Fig. 2. SCNMV and a pseudorecombinant (S1, R2) caused a severe systemic mosaic symptom in sweet clover at both 26 °C and 17 °C. On the other hand, another pseudorecombinant, (R1, S2), caused
Table 1. Infectivity of homologous and heterologous combinations of RNA 1 and RNA 2 among SCNMV, RCNMV, and CPLNV and serological specificity of their progeny viruses

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Virus supplying RNA species*</th>
<th>C. amaranticolor</th>
<th>Cowpea</th>
<th>V. faba</th>
<th>SCNMV</th>
<th>RCNMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>RNA 1 SCNMV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>RNA 2 RCNMV</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>RCNMV SCNMV</td>
<td>1016</td>
<td>49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>SCNMV SCNMV</td>
<td>901</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>SCNMV RCNMV</td>
<td>812</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>RCNMV RCNMV</td>
<td>843</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>RNA 1 CPLNV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>RNA 2 CPLNV</td>
<td>84</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>SCNMV SCNMV</td>
<td>317</td>
<td>57</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>RCNMV SCNMV</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>RCNMV RCNMV</td>
<td>225</td>
<td>31</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>CPLNV SCNMV</td>
<td>167</td>
<td>15</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>CPLNV RCNMV</td>
<td>191</td>
<td>13</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>SCNMV CPLNV</td>
<td>306</td>
<td>22</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>RCNMV CPLNV</td>
<td>175</td>
<td>18</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Concentrations of each RNA component in inocula were 1 μg/ml for SCNMV and RCNMV RNA and 0.05 μg/ml for CPLNV RNA respectively. The concentration of RNA 1 or RNA 2 when used alone was 4 μg/ml.

† Total lesion number on 12 (expt. A) or 6 (expt. B) half-leaves of C. amaranticolor, 6 whole leaves of cowpea or 5 whole leaves of V. faba cv. Broad Windsor was determined.

‡ Serological specificity was determined by ELISA by using both cowpea leaf sap and purified viruses (1 μg/ml) from single lesion isolates (+, A405 above 2.00; -, A405 below 0.05).

only local infection with necrotic spots in sweet clover at 26°C and mostly local and rarely systemic infection with necrotic spots at 17°C (Table 2).

Although SCNMV, RCNMV and CPLNV did not infect white clover at 26°C, pseudorecombinants (C1, S2) and (C1, R2) caused local infection in this host. No infection occurred in white clover at either 26°C or 17°C with pseudorecombinants containing SCNMV RNA 1 (Table 2). While CPLNV did not infect V. faba at 26°C, all pseudorecombinants as well as SCNMV and RCNMV caused necrotic local infection on this host at 17°C and mostly localized symptomless infection at 26°C.

On C. amaranticolor, cowpea and Red Kidney bean plants, the local and systemic symptoms were usually similar to those induced by the donor viruses that supplied RNA 1. However, in a few cases such as the pseudorecombinant (C1, S2) large concentric necrotic lesions developed on Red Kidney bean leaves, while their RNA 1 donor CPLNV caused only small necrotic lesions (Fig. 3). On cowpea leaves at 26°C, CPLNV caused small whitish necrotic spots while the pseudorecombinant (C1, S2) like SCNMV and the pseudorecombinant (S1, C2) caused brownish ring spots. Only SCNMV caused acute death of the inoculated leaves due to rapid desiccation associated with necrotic ring spots and vein necrosis.

Effect of coat protein on infectivity of genomic RNA

When a mixture of 5 μg/ml of protein from RCNMV and 5 μg/ml RNA 1 or RNA 2 from the same virus was tested on C. amaranticolor no activation by coat protein of the infectivity of RNA 1 or RNA 2 was observed.
Genetic determinants of dianthoviruses

**Fig. 2.** Analyses of RNA species and protein coat of the progeny viruses originally from the homologous and the heterologous combinations. (a) Analysis on a 2.25% polyacrylamide gel of RNA species originating from: lane 2, RNA 1 and RNA 2 of SCNMV; lane 3, RNA 1 and RNA 2 of RCNMV; lane 4, RNA 1 of RCNMV and RNA 2 of SCNMV; lane 5, RNA 1 of SCNMV and RNA 2 of RCNMV. BMV RNA was included as a marker (lane 1). (b) Analysis on a 10% SDS–polyacrylamide gel of coat protein in the progeny viruses originally from the combinations of RNA 1 and RNA 2 among SCNMV, RCNMV and CPLNV. Pseudorecombinants are coded as follows: S, SCNMV; R, RCNMV; C, CPLNV; 1, RNA 1; 2, RNA 2. Lane 1, mol. wt. markers; lane 2, (R1, C2); lane 3, (R1, R2); lane 4, (C1, R2); lane 5, (C1, C2); lane 6, (S1, C2); lane 7, (S1, S2); lane 8, (C1, R2); lane 9, CPLNV; lane 10, RCNMV; lane 11, SCNMV.

**Table 2. Pathogenicity and symptomatology of SCNMV, RCNMV and CPLNV and their pseudorecombinants**

<table>
<thead>
<tr>
<th>Virus supplying RNA species</th>
<th>Infection patterns* induced in V. faba at (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sweet clover</td>
</tr>
<tr>
<td>RNA 1</td>
<td>RNA 2</td>
</tr>
<tr>
<td>SCNMV</td>
<td>SCNMV</td>
</tr>
<tr>
<td>RCNMV</td>
<td>RCNMV</td>
</tr>
<tr>
<td>SCNMV</td>
<td>SCNMV</td>
</tr>
<tr>
<td>RCNMV</td>
<td>RCNMV</td>
</tr>
<tr>
<td>CPLNV</td>
<td>CPLNV</td>
</tr>
<tr>
<td>CPLNV</td>
<td>SCNMV</td>
</tr>
<tr>
<td>CPLNV</td>
<td>RCNMV</td>
</tr>
<tr>
<td>SCNMV</td>
<td>CPLNV</td>
</tr>
<tr>
<td>RCNMV</td>
<td>CPLNV</td>
</tr>
</tbody>
</table>

* L, Local; M, mosaic; N, necrotic; T, tolerant (symptomless infection); R, resistant (no infection); lt, small amount of infection; lt', negligible amount of infection (no recovery of virus to Red Kidney bean plant, but positive with ELISA); -, not tested.

**DISCUSSION**

Although a new virus group (dianthovirus) has been approved recently to include carnation ringspot virus (CRSV), RCNMV and SCNMV (Matthews, 1982), it is important to obtain more information on the interrelationships as well as genetic interaction of these viruses. The results
above show that SCNMV, RCNMV and CPLNV have bipartite genomes and that the larger genomic RNA (RNA 1) of each virus contains the cistron for coat protein. Earlier studies with CRSV, the type member of the group, have also shown that the genomic information controlling two likely characteristics of the coat protein, the irreversible virion aggregation and the
dissociation of virions by SDS at pH 5, is contained on RNA 1 (Dodds et al., 1977). Our experiments also showed that infectious pseudorecombinants of RNA 1 and RNA 2 could be formed interchangeably between SCNMV and CRSV as well as between RCNMV and CRSV (T. Okuno & C. Hiruki, unpublished data).

Pea enation mosaic virus (PEMV) is another virus, though unrelated to the dianthovirus group, that has been reported to have genetic information for coat protein in its RNA 1 (Hull & Lane, 1973). These characteristics are somewhat unusual because in most of the plant viruses with divided genomes investigated thus far, except CRSV and PEMV, coat protein is encoded by the smaller or the smallest RNA of the viral genomes (Bruening, 1977; Van Vloten-Doting & Jaspers, 1977).

In analysing genetic determinants of SCNMV, RCNMV and CPLNV, none of them infected white clover at 26 °C while pseudorecombinants (C1, S2) and (C1, R2) caused local but asymptomatic infection in this host. This fact suggests that some interaction exists between C1 and S2 or R2 in overcoming the suppressive temperature effect on establishing infection in this host. Likewise, the homologous combination of CPLNV (C1, C2) as well as a pseudorecombinant (R1, C2) infected the host only locally without symptoms at 17 °C. This result suggests that both RNA 1 and RNA 2 are involved in determining a host response such as localized tolerance, presumably as a result of interaction with host genes. The results also show that host responses to these three viruses and their pseudorecombinants are influenced by greenhouse temperatures.

On sweet clover, SCNMV (S1, S2) and a pseudorecombinant (S1, R2) caused a severe systemic mosaic symptom at both 26 °C and 17 °C, whereas RCNMV (R1, R2) caused local and rarely systemic symptoms of necrotic spots at 17 °C and no infection at 26 °C. Thus, evidence was obtained that S1 is essential for systemic infection of sweet clover at 26 °C. A pseudorecombinant (R1, S2) caused symptoms similar to those caused by RCNMV (R1, R2) at 17 °C but localized infection with necrotic spots at 26 °C. This result indicates that S2 complements R1 in causing local infection at 26 °C. Evidence that the symptom expression at 26 °C results from interaction between RNA 1 and RNA 2 is provided also by several pseudorecombinants tested on V. faba.

Further analysis of the results presented in Table 2 provides evidence that certain host responses are controlled by interactions between RNA 1 and RNA 2. Firstly, pseudorecombinants (R1, C2) and (C1, R2) caused localized symptomless infections which were different from those of either parental virus. Secondly, when a pseudorecombinant (C1, S2) was tested on primary leaves of Red Kidney bean, on which CPLNV, the RNA 1 donor, caused very small necrotic lesions and SCNMV, the RNA 2 donor, relatively small concentric lesions, it produced large concentric lesions, an indication of some interacting effect determining the type of lesion (Fig. 3). Thirdly, pseudorecombinants (S1, C2) and (C1, S2) caused brownish rings, a response that was intermediate between the whitish spots caused by CPLNV and the rapid necrotic death following necrotic ring spots and vein necrosis when tested on cowpea at 26 °C.

In addition to a serological relationship reported previously between CPLNV and certain strains of RCNMV (Hollings & Stone, 1979), the bipartite nature of CPLNV RNA and the ability of its RNA components to form pseudorecombinants with RCNMV and SCNMV were revealed in this investigation. The fact that RNA 1 and RNA 2 complement each other among SCNMV, RCNMV, CPLNV and CRSV indicates that these viruses are valid members of the newly created dianthovirus group.

This work was supported in part by a research grant 78-0038 from the Agriculture Research Council of Alberta. The authors thank Dr R. T. Plumb for a gift of red clover seed and Mr T. Tribe for his technical assistance in the preparation of illustrations.

REFERENCES


*(Received 28 September 1982)*