The Distribution of Some Genetic Determinants in the Two Nucleoprotein Particles of Cherry Leaf Roll Virus

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(Accepted 6 May 1980)

SUMMARY

Purified preparations of the two sedimenting nucleoprotein components (M and B) of tomato ringspot virus (TomRSV) and of strains of cherry leaf roll virus (CLRV) from elderberry (G) and from rhubarb (R) had little infectivity alone whereas mixtures of homologous components were very infective. However, whereas heterologous mixtures of the components of CLRV strains G and R were also very infective, heterologous mixtures of the components of CLRV-R and TomRSV were not. These results and serological evidence indicate that CLRV and TomRSV are not closely related to each other or to other nepoviruses with similar properties.

Pseudo-recombinants produced by exchanging the nucleoprotein components of CLRV strains R and G were less stable than the parent isolates on storage in sap at 18 °C. Component M determined serological specificity whereas component B determined ability to infect Gomphrena globosa, and the lesion type and severity of systemic symptoms in Chenopodium and Nicotiana spp. Virulence also depended on the compatibility of M and B components and neither pseudo-recombinant was as virulent as the parent donating component B. Systemic infection and symptom production in Petunia hybrida required M and B components from strain R. In plant protection tests, the parent strains of CLRV protected tobacco plants against either parent and against the two pseudo-recombinants, whereas the pseudo-recombinants protected plants against either pseudo-recombinant but not against the parent strains. This suggests that determinants in both nucleoprotein components of either the protecting or challenging virus are involved.

INTRODUCTION

Cherry leaf roll virus (CLRV) occurs naturally in a wide range of plants and is found in Europe, North America and New Zealand (Cropley & Tomlinson, 1971; Jones & Wood, 1979). It has many of the properties of nepoviruses and, like many members of this group, it has two RNA species which are contained in two corresponding nucleoprotein components having different sedimentation coefficients (Jones & Mayo, 1972; Walkey et al. 1973; Harrison & Murant, 1977). Although the mol. wt. of the larger RNA species (RNA-1) of different nepoviruses is similar (c. 2.4 x 10^6), the mol. wt. of the smaller one (RNA-2) ranges from 1.4 to 2.2 x 10^6 (Harrison & Murant, 1977). Quacquarelli et al. (1978) have classified nepoviruses into three sub-groups based on the mol. wt. differences of RNA-2. This classification places CLRV together with grapevine Bulgarian latent,
peach rosette mosaic and tomato ringspot (TomRSV) viruses in sub-group 3, all members of which have an RNA-2 of more than $2.0 \times 10^8$ mol. wt. To this sub-group may be added the recently described Australian lucerne latent virus (Jones et al. 1979).

Fulton & Fulton (1970) and Jones (1973, 1976) reported that tobacco plants infected with TomRSV failed to develop symptoms when subsequently inoculated with strains of CLRV, suggesting some affinity between these two viruses. However, in gel diffusion and microprecipitin serological tests no relationship was detected between strains of CLRV and TomRSV or between these and other viruses of the proposed sub-group 3 of nepoviruses (Jones & Murant, 1971; Dias, 1975; Martelli et al. 1978; Jones et al. 1979). In this paper we confirm the lack of serological relationship and describe an unsuccessful attempt to produce pseudo-recombinant isolates by exchanging the nucleoprotein components of CLRV and TomRSV. However, we have successfully produced pseudo-recombinants of two CLRV strains and report here on some of the characters determined by each component of this virus.

**METHODS**

**Virus isolates.** The three strains of CLRV (with the abbreviations used previously; Jones, 1973, 1976) and the two strains of TomRSV studied were: CLRV-D, from American dogwood (*Cornus florida*, Waterworth & Lawson, 1973); CLRV-G, isolate S₄ from *Sambucus canadensis* (Jones & Murant, 1971; Jones & Mayo, 1972); CLRV-R, from rhubarb (*Rheum rhaponticum*, Tomlinson & Walkey, 1967); TomRSV-W, Wisconsin isolate (Stace-Smith, 1962); and TomRSV-G, grape yellow vein isolate (Gooding, 1963). All viruses were maintained in *Chenopodium quinoa* and *Nicotiana clevelandii* in glass-houses kept at between 15 and 25 °C.

**Virus propagation, purification and separation of components.** All virus isolates were propagated in *N. clevelandii* and purified using method 2 of Jones & Mayo (1972) for CLRV-G. Virus components were further purified and separated by sedimenting at least three times through sucrose density gradients as described by Jones & Mayo (1972).

**Local lesion assays.** These were made in *N. tabacum* cv. Samsun NN and Xanthi-nc, and in *C. amaranticolor*. Inoculum was applied with a muslin pad to half-leaves previously dusted with Carborundum (600 mesh/inch).

**Serological tests.** Gel diffusion serological tests were made as described by Jones & Murant (1971) using sap from systemically infected leaves of *C. quinoa* as antigen. For electron microscope serology, sap from virus-infected *C. quinoa* or *N. clevelandii* leaves was diluted in 0.06 M-phosphate buffer, pH 6.5, to give about 3 virus particles per field of view at $\times 20000$ magnification in the electron microscope. Samples (10 μl) of this diluted extract were added to each 10 μl drop of a dilution series of antiserum (1/8 to 1/4096) and thoroughly mixed. The resulting antiserum/virus mixtures were incubated at 4 °C for 3 h before being placed on carbon-coated grids and stained with ammonium molybdate (pH 6.5) or uranyl formate/sodium hydroxide (pH 4.8). Grids were examined in a Siemens Elmiskop I or Philips EM 301G electron microscope to detect attachment of antibody to virus particles. The titration endpoint of the antiserum was determined as the highest dilution at which all virus particles seen had some antibodies attached.

**Plant protection tests.** Tests were made in *N. tabacum* cv. Samsun NN and Xanthi-nc using infective sap of *N. clevelandii* as inoculum and following the procedure described by Jones (1973). Two to three weeks after applying the challenge inoculum, sap from areas of the half-leaves receiving the challenge inoculum or the protecting inoculum, and also from young uninoculated leaves, was inoculated to *C. quinoa*. Gel diffusion serological tests were then made with sap from systemically infected *C. quinoa* plants.
Table 1. Infectivity* of the nucleoprotein components of CLRV isolates and TomRSV

<table>
<thead>
<tr>
<th>Component mixtures†</th>
<th>Infectivity in C. amaranthicolor</th>
<th>Component mixtures†</th>
<th>Infectivity in C. amaranthicolor</th>
<th>Infectivity in Samsun NN tobacco</th>
<th>Infectivity in Xanthi NIC tobacco</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td>Expt. 2</td>
<td></td>
<td>Expt. 3</td>
<td></td>
</tr>
<tr>
<td>M_R</td>
<td>1</td>
<td>M_G</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B_R</td>
<td>13</td>
<td>B_G</td>
<td>2</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>M_R B_R</td>
<td>74</td>
<td>M_G B_G</td>
<td>10</td>
<td>68</td>
<td>49</td>
</tr>
<tr>
<td>M_W</td>
<td>9</td>
<td>M_G B_R</td>
<td>14</td>
<td>44</td>
<td>35</td>
</tr>
<tr>
<td>M_W</td>
<td>56</td>
<td>M_R</td>
<td>0</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>B_W</td>
<td>78</td>
<td>B_R</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>M_R B_W</td>
<td>299</td>
<td>M_R B_R</td>
<td>7</td>
<td>31</td>
<td>74</td>
</tr>
<tr>
<td>M_R B_W</td>
<td>11</td>
<td>M_R B_G</td>
<td>35</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

* Total number of lesions in four half leaves.
† All inocula had final A_260 values of 0.001. M and B are middle and bottom components, respectively, of CLRV isolates G and R and TomRSV isolate W.

RESULTS

Serological tests on CLRV, TomRSV and similar viruses

Two serotypes of CLRV (CLRV-G and CLRV-D) and two serotypes of TomRSV (TomRSV-W and TomRSV-G) were each examined in the electron microscope for reaction with antisera to the following viruses (homologous titres determined in gel diffusion tests in parentheses): Australian lucerne latent (1/256), CLRV strains G (1/256) and R (1/64), grapevine Bulgarian latent (1/512), peach rosette mosaic, strains from grape (1/512) and peach (1/256), and TomRSV strains W (1/256) and G (1/1024). In these tests, the CLRV isolates reacted only with the CLRV antisera and the TomRSV isolates reacted only with the TomRSV antisera. The highest dilution of antiserum reacting to CLRV isolates G and D was 1/2048 and 1/1024 for CLRV-G and 1/128 and 1/1024 for CLRV-R, respectively; that reacting to TomRSV isolates W and G was 1/2048 and 1/1024 for TomRSV-W and 1/1024 and 1/8192 for TomRSV-G, respectively. These differences in heterologous titres possibly reflect the immunization procedures of the workers supplying the antisera.

Attempts to produce pseudo-recombinants using components of CLRV-R and TomRSV-W

Table 1, experiment 1, shows that the infectivity of the individual components of each virus, although much less than that of mixtures of the homologous M and B components, was greater than the infectivity of heterologous mixtures of these components. Isolates were cultured from nine of the local lesions produced by heterologous mixtures of components M and B and all were found to resemble the parent strain donating component B in serotype, host range and symptomatology (data not shown).

Infectivity of components of CLRV strains G and R and production of pseudo-recombinants

Purified preparations of the two components of each CLRV strain were assayed for infectivity separately and in combination. Table 1 (experiments 2 and 3) shows that the separated components had little or no infectivity, whereas homologous and heterologous mixtures of components M and B were much more infective. In subsequent experiments it was found that the inoculum containing M_R plus B_G did not induce necrotic local lesions in Samsun tobacco (see Table 3; Fig. 3) but did so readily in C. amaranthicolor. The number of necrotic lesions induced by this inoculum in Samsun tobacco (Table 1) is therefore an indication of the amount of contamination with the parent strains.
Table 2. Serological behaviour of single lesion isolates obtained from inocula of mixtures of the components of CLRV strains G and R

<table>
<thead>
<tr>
<th>Component mixtures</th>
<th>No. isolates with serological specificity of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain R</td>
</tr>
<tr>
<td>M_R B_R</td>
<td>10</td>
</tr>
<tr>
<td>M_G B_G</td>
<td>0</td>
</tr>
<tr>
<td>M_R B_G</td>
<td>10</td>
</tr>
<tr>
<td>M_G B_R</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 1. Double diffusion test in 1% agarose gel. Centre well contains antiserum to CLRV-G diluted 1/16. Wells 1 to 4 contain sap from *C. quinoa* plants infected with CLRV isolates: 1, M_R B_R; 2, M_R B_G; 3, M_G B_G; 4, M_G B_R.

Pseudo-recombinants were obtained by culturing virus from single lesions produced by the inoculum M_R B_G in *C. amaranticolor* and by that of M_G B_R in Samsun tobacco. All isolates were maintained in *C. quinoa* and *N. clevelandii*.

**Properties of CLRV pseudo-recombinants**

**Serological specificity**

Table 2 and Fig. 1 show that serological specificity was determined by the strain donating component M. The two exceptions to this in the combination M_G B_R are attributed to contamination of component B_R with M_R.

**Host range and symptomatology**

The parent isolates and single lesion isolates of each of the combinations M_R B_G and M_G B_R were inoculated to *Gomphrena globosa* and *Petunia hybrida*, species previously found to react differently to CLRV strains R and G (Jones, 1973) and to five other hosts. Table 3 summarizes some of the differences observed between the parent viruses and the pseudo-recombinants and shows that infection and symptom production in *G. globosa* was determined by component B of strain R. However, reliable infection and symptom production in *Petunia hybrida* seem to require both component M and component B of strain R.
Table 3. **Host range comparison of CLRV strains G and R, and isolates of their pseudo-recombinants**

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Reactions in inoculated (I) and uninoculated (S) leaves</th>
<th>Reactions* induced by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CLRV-G (M_G B_G)</td>
</tr>
<tr>
<td><strong>C. quinoa</strong></td>
<td>I</td>
<td>CLL</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>SN, recovery</td>
</tr>
<tr>
<td><strong>C. amaranticolor</strong></td>
<td>I</td>
<td>NLL</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>SN, recovery</td>
</tr>
<tr>
<td><strong>G. globosa</strong></td>
<td>I</td>
<td>O</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>O</td>
</tr>
<tr>
<td><strong>P. hybrida</strong></td>
<td>I</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>O</td>
</tr>
<tr>
<td><strong>P. vulgaris</strong></td>
<td>I</td>
<td>C/NLL</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>SN</td>
</tr>
<tr>
<td><strong>N. clevelandii</strong></td>
<td>I</td>
<td>NLL</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>SM/N</td>
</tr>
<tr>
<td><strong>N. tabacum cv.</strong></td>
<td>I</td>
<td>NLL</td>
</tr>
<tr>
<td>Samsun NN</td>
<td>S</td>
<td>SM, recovery</td>
</tr>
</tbody>
</table>

* CLL = chlorotic local lesions, NLL = necrotic local lesions, SN = systemic necrosis, SM = systemic mottle, S = symptomless infection, O = no infection. Numbers in parentheses indicate number of isolates tested when more than one.
Lesion type in *C. quinoa* (Fig. 2) and the general severity of symptoms in *C. quinoa* (Fig. 2) and *C. amaranticolor* were largely determined by the strain donating component B. However, in these two species and in the two *Nicotiana* spp. tested neither pseudo-recombinant was as virulent as the parent donating component B (Fig. 2) suggesting some interaction between components M and B. The isolate with the combination M\(_B\) B\(_G\) was the least virulent in all hosts and, unlike either parent, it induced very faint chlorotic local lesions in *N. tabacum* (Fig. 3) and no lesions in either *N. clevelandii* or *P. vulgaris*. Systemic infection in *P. vulgaris* was determined by the strain donating component B.
Table 4. Results of plant protection tests in Nicotiana tabacum cv. Samsun NN and Xanthi-nc involving CLRV strains G and R, and single isolates of their pseudo-recombinants

<table>
<thead>
<tr>
<th>Protecting virus</th>
<th>Challenging virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>G (M&lt;sub&gt;G&lt;/sub&gt; B&lt;sub&gt;G&lt;/sub&gt;)</td>
<td>G (M&lt;sub&gt;G&lt;/sub&gt;B&lt;sub&gt;G&lt;/sub&gt;)</td>
</tr>
<tr>
<td>G (M&lt;sub&gt;G&lt;/sub&gt; B&lt;sub&gt;G&lt;/sub&gt;)</td>
<td>(9) G</td>
</tr>
<tr>
<td>R (M&lt;sub&gt;R&lt;/sub&gt;B&lt;sub&gt;R&lt;/sub&gt;)</td>
<td>(4) R</td>
</tr>
<tr>
<td>M&lt;sub&gt;G&lt;/sub&gt;B&lt;sub&gt;R&lt;/sub&gt;</td>
<td>(4) G</td>
</tr>
<tr>
<td>M&lt;sub&gt;R&lt;/sub&gt;B&lt;sub&gt;G&lt;/sub&gt;</td>
<td>(2) G, R</td>
</tr>
</tbody>
</table>

* = No necrotic lesions produced by challenge virus; + = necrotic lesions produced by challenge virus. Values in parentheses indicate number of plants tested. G or R = serotype detected in assays from uninoculated leaves of plants.

Properties in vitro

Single isolates of each pseudo-recombinant in sap of C. quinoa plants infected 7 days earlier had similar dilution end-points (10<sup>-4</sup> to 10<sup>-5</sup>) and thermal inactivation points (55 to 60°C) as the parents. However, whereas sap containing the parent isolates was infective for more than 16 days at 18°C, that containing the pseudo-recombinants lost infectivity after 4 to 8 days.

Behaviour in plant protection tests

CLRV strains G and R and their pseudo-recombinants were inoculated in all combinations to N. tabacum cv. Samsun NN and Xanthi-nc. The reaction of both cultivars was the same. Table 4 shows that, in contrast to earlier findings (Jones, 1973), strain G protected plants from the effects of strain R. The reasons for this difference are not known - possibly either or both of these strains have changed in culture, or their behaviour depends on the experimental conditions. Each parent strain protected plants against the effects of both parents and both pseudo-recombinants. By contrast, neither pseudo-recombinant protected plants against either parent strain, although both pseudo-recombinants protected against each other. This suggests either that the determinants involved in the phenomenon of plant protection in the protecting or challenging viruses are carried both in component M and in component B of CLRV, or that the expression of determinant(s) in either component M or component B depends upon the compatibility of these two components. Although inocula of M<sub>R</sub>B<sub>G</sub> do not induce necrotic local lesions in tobacco plants (Table 3), its serotype was detected in challenge-inoculated plants protected with M<sub>G</sub>B<sub>R</sub> but not in those protected with strain G.

Discussion

Our infectivity studies on the two nucleoprotein components of two distinct strains of CLRV confirm and extend data on the requirement of both components for maximum infectivity (Jones & Mayo, 1972) and our present studies indicate that TomRSV behaves similarly (Table 1). Although we were able to produce pseudo-recombinants by exchanging the M and B components of two strains of CLRV, we failed in one experiment to detect pseudo-recombinants in inocula made by exchanging the M and B components of CLRV-R and TomRSV-W. This suggests that although these two viruses are very similar in many of their characters and show some affinity to each other on the basis of plant protection tests (Fulton & Fulton, 1970; Jones, 1973, 1976), they are not closely related. This suggestion is supported by our failure to detect any serological relationship between these two viruses using the sensitive technique of electron microscope serology. Furthermore,
this test indicated that none of the known or tentative members of the proposed sub-
group 3 of nepoviruses (Quacquarelli et al. 1978) is related serologically. This is in contrast
to the serological relationships that occur among a few members in each of the other two
proposed sub-groups (Harrison & Murant, 1977; Quacquarelli et al. 1978). However,
this difference may simply reflect the small number of viruses assigned to sub-group 3.

The production of pseudo-recombinants from CLRV strains G and R allowed the
identification of characters determined by each of the two nucleoprotein components of
this virus. Previous studies have shown that components B and M of CLRV each contain
a single RNA species of mol. wt. \(2.4 \times 10^6\) (RNA-1) and \(2.1 \times 10^6\) (RNA-2) respectively
(Jones & Mayo, 1972; Walkey et al. 1973) and it seems reasonable to assume that the
determinants for each character are located on these RNA molecules. Indeed, Haber &
Hamilton (1980) have obtained pseudo-recombinants with properties somewhat similar
to those reported here by exchanging the purified RNA species of CLRV strains from
elderberry and rhubarb. Our results agree with those of Haber & Hamilton (1980) in
indicating that serological specificity is determined by RNA-2 and infection of Gomphrena
is determined by RNA-1. However, neither of our pseudo-recombinants behaved exactly
like either parent in most of the hosts listed in Table 3. Our evidence indicates that al-
though the major determinant of symptoms in these hosts is in RNA-1, RNA-2 has a
modifying influence. Thus, in combination with RNA-1 from R, RNA-2 from R favoured
virulence more than did RNA-2 from G. Similarly, in combination with RNA-1 from G,
RNA-2 from G favoured virulence more than did RNA-2 from R. This apparent com-
patibility of the RNA species for virulence in CLRV is similar to the compatibility ob-
served between the two RNA species of raspberry ringspot virus in relation to seed
transmission in Stellaria media (Hanada & Harrison, 1977). In these respects our data
differ from those of Haber & Hamilton (1980) who identified most of the determinants
for symptoms in Chenopodium and Nicotiana species on RNA-2. These differences in
results may possibly be caused by the use of different isolates of the CLRV parent strains
or different environmental conditions. Differences in host range and other properties have
previously been noted between serologically indistinguishable isolates of CLRV (Jones,
1973).

Little is known of the causes underlying the phenomenon of plant protection. However,
our data on the behaviour of the parents and pseudo-recombinants of CLRV in tobacco
plants indicate that in either the protecting or challenging virus RNA-1 and RNA-2 are
involved. Furthermore, our data are not incompatible with the hypothesis that the involve-
ment of both RNA species is due to the modifying influence of RNA-2 on the expression
of determinant(s) for virulence on RNA-1 (Table 3). Thus, if the virulence of the CLRV
isolates reflects their rate of virus multiplication, we can postulate that the pseudo-
recombinants failed to protect plants against the parent strains because their rate of
multiplication was slower than that of the parents. Because our dilution endpoint data
are not sufficiently precise to establish an association of virulence with the rate of multi-
plication, further work is necessary to explore this possibility.

We thank S. Haber and R. I. Hamilton for sending us a copy of their paper prior to pub-
llication; A. A. Brunt, H. F. Dias, G. V. Gooding, G. P. Martelli, R. Stace-Smith, J. A.
Tomlinson and H. E. Waterworth for gifts of virus isolates and/or antisera; Mrs E. Russell
and J. G. Lindsay for technical assistance, and T. Geoghegan for the photographs.
Pseudo-recombinants of cherry leaf roll virus

REFERENCES


(Received 21 December 1979)