Red Clover Mottle Comovirus B-RNA Spreads between Cells in Tobamovirus-infected Tissues

By S. I. MALYSHENKO,1 L. G. LAPCHIC,2 O. A. KONDAKOVA,1 L. L. KUZNETZOVA,2 M. E. TALIANSKY1 AND J. G. ATABEKOV1*

1Department of Virology and A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899 and 2D. K. Zabolotny Institute of Microbiology and Virology of the Ukrainian Academy of Sciences, Zabolotny Street 26, Kiev-143, U.S.S.R.

(Accepted 8 October 1987)

SUMMARY

B component RNA (B-RNA) of red clover mottle comovirus (RCMV) was not transported between cells of inoculated leaf tissue unless it was co-inoculated with the M component. However when host plants were infected with sunn-hemp mosaic tobamovirus (SHMV) or mutant Nil18 of tobacco mosaic virus (TMV) before superinoculation, RCMV B-RNA was transported readily between cells. Plants were infected with SHMV or Nil18 and, 5 days after superinoculation with RCMV B component, protoplasts were isolated from the infected leaves and inoculated with RCMV M component. About 30% of such protoplasts multiplied RCMV (identified by immunofluorescence microscopy) whereas only 3 to 5% of protoplasts from similarly treated plants not initially infected with SHMV or Nil18 multiplied RCMV. Thus B-RNA spread from mixedly infected (SHMV + B-RNA or Nil18 + B-RNA) cells in the absence of M-RNA to the neighbouring cells. RCMV B-RNA spread in leaves infected with the temperature-sensitive coat protein TMV mutant Nil18 and grown at non-permissive temperature. Thus TMV coat protein is not involved in enabling RCMV RNA to be transported.

INTRODUCTION

After a plant is inoculated, virus replicates in the relatively few primarily infected cells and thereafter moves into neighbouring healthy ones. The systemic spread (transport) of infection from cell to cell is not a passive process that depends only on the concentration of virus in the primarily infected cells but is controlled by a specific function encoded by the viral genome, termed the transport function (for review, see Atabekov & Dorokhov, 1984). The mechanism of expression of the transport function is obscure, but certain virus-coded protein(s) (transport protein) seem to be responsible for this function.

Transport function is one of the factors that control virus host range (Taliansky et al., 1982b). A plant which prevents transport of a given virus from cell to cell should be regarded as resistant to, or not a host of, this virus even if the virus replicates in the initially infected cells (Taliansky et al., 1982b). In several cases this type of resistance can be overcome and non-host plants then become infected with a virus because another virus that can normally spread in this plant (a helper) is present (Taliansky et al., 1982b). Moreover some phloem-limited viruses have been reported to move into parenchyma cells of doubly infected plants as a result of complementation by a virus-specific transport function of the helper virus. For example, tobacco mosaic virus (TMV) promotes the penetration of bean golden mosaic geminivirus into mesophyll cells (Carr & Kim, 1983) and potato potexvirus X (Atabekov et al., 1984) and potyvirus Y (Barker, 1987) stimulate transport into parenchyma cells of potato leafroll luteovirus.

In the present work, red clover mottle comovirus (RCMV) which has a bipartite genome (B-RNA and M-RNA) has been used (Lapchic et al., 1976a, b). The nucleotide sequence of RCMV
M-RNA is known (Shanks et al., 1986) and the genome organization of RCMV is very like that of cowpea mosaic virus (CPMV). Thus the basic principles of genome replication and expression of the well-studied CPMV may also apply to RCMV. The coat protein genes are located in the M-RNA of comoviruses (Franssen et al., 1982; Van Wezenbeek et al., 1983) but this RNA is not capable of independent replication; it replicates only in the presence of B-RNA (Goldbach et al., 1980). On the other hand, comovirus B-RNA is capable of independent replication in isolated protoplasts, but does not spread from cell to cell in leaves of inoculated plants (Rezelman et al., 1982). The restriction of B-RNA multiplication to inoculated cells may be because the 'transport'-controlling gene is in M-RNA. A test for the spread of RCMV B-RNA in superinoculated leaves of infected plants is therefore a good test for the ability of unrelated viruses to enable the RCMV RNA to spread. We have found that two tobamoviruses can act as helper viruses in the transport of RCMV B-RNA from cell to cell.

METHODS

Viruses. Sunn-hemp mosaic tobamovirus (SHMV) was supplied by Dr R. D. Woods and propagated in Phaseolus vulgaris L. var. Triumph; TMV mutant Nil18, temperature-sensitive (ts) in the coat protein, was provided by Dr H. Jockush; TMV mutant Lsl ts in transport function was supplied by Dr N. Oshima; RCMV (Ukrainian strain) was that described by Lapchic et al. (1976a,b). The viruses were purified as described previously (Atabekov et al., 1970; Lapchic et al., 1976a).

B and M components of RCMV were separated by two cycles of centrifugation in CsCl density gradients (30 to 50% w/w). The RCMV sample in 0.06 M-phosphate buffer pH 7.0 was layered on gradients and centrifuged in an SW41 rotor (Beckman) for 4 h at 35000 r.p.m. Contamination of B-RNA with M-RNA was less than 0.5% (judged by the number of local lesions induced per leaf of P. vulgaris var. Top Crop by B component or unfractionated virus).

Complementation experiments. Leaves of Vigna unguiculata L. or Nicotiana tabacum var. Samsun were mechanically inoculated with a helper virus (200 μg/ml). Two days later the leaves were superinoculated with RCMV B component (500 μg/ml). These doubly inoculated leaves were harvested after 5 days and used for the isolation of protoplasts. Three to four plants were used for each test.

Isolation and inoculation of the protoplasts were performed according to Hibi et al. (1975). Suspensions of 5 x 10⁴ cowpea or tobacco mesophyll protoplasts per 1 ml were inoculated with 5 μg/ml of RCMV M or B component or a mixture of both components, each at 5 μg/ml. Forty-eight h after superinoculation the proportion of RCMV-infected protoplasts was determined by immunofluorescent 'staining' with fluorescent antibodies to RCMV (Huber et al., 1977).

RCMV content in the protoplast samples was determined by ELISA as described earlier (Malyshenko et al., 1985) using serial dilutions of purified RCMV as concentration standards.

RESULTS

RCMV B-RNA transport in the presence of SHMV

V. unguiculata plants (a systemic host for both RCMV and SHMV) were preinoculated with SHMV and superinoculated with RCMV B component (plants not preinfected with SHMV were used as a control). Protoplasts were isolated from infected leaves at different intervals after superinoculation with RCMV B component and inoculated with RCMV M component.

RCMV spread in leaves inoculated with M and B components; 3 days after the inoculation about 40% of the cells contained virus particles (Fig. 1). In contrast, in leaves inoculated only with B component B-RNA did not spread. Only 3 to 5% of protoplasts from these leaves made RCMV particles when inoculated with M component (Fig. 1, Table 1). These data resemble those of Rezelman et al. (1982) who showed that B-RNA of CPMV did not spread in inoculated leaves unless M-RNA was also present.

On the other hand, the amount of B-RNA-containing protoplasts significantly increased within 3 to 5 days in leaves infected with SHMV (helper virus) before inoculation with B component (Fig. 1, Table 1). These data show that RCMV B-RNA spread efficiently in SHMV-infected tissue. Almost as many of these cells contained B-RNA as did cells in leaves inoculated with both components (Fig. 1, Table 1). The direct ELISA testing of RCMV in protoplast samples correlates well with the immunofluorescent assay of percentage of RCMV-infected protoplasts (Table 1). Both lines of evidence support the conclusion that in B-RNA-inoculated leaves the effectiveness of transport depends totally on the presence of SHMV helper.
Systemic spreading of comovirus B-RNA

Fig. 1. RCMV B-RNA transport in the presence of the helper virus (SHMV). (●) Protoplasts isolated at intervals after inoculation of V. unguiculata leaves with M and B components; (▲) protoplasts isolated at intervals after inoculation of V. unguiculata leaves with B component, and then infected with M component and cultured for 2 days; (■) protoplasts isolated at intervals after inoculation of SHMV-infected V. unguiculata leaves with B component, and then infected with M component and cultured for 2 days. RCMV was detected by staining with fluorescent antibodies.

Table 1. RCMV B-RNA transport in SHMV-infected and healthy leaves of V. unguiculata

<table>
<thead>
<tr>
<th>1st inoculation (helper virus)</th>
<th>Inoculum</th>
<th>2nd inoculation (RCMV component used for superinoculation)</th>
<th>3rd inoculation* (RCMV component used for protoplast infection)</th>
<th>Percentage of protoplasts infected with RCMV†</th>
<th>Amount of RCMV in protoplasts (ng per 10^5 protoplasts)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>B + M (control)</td>
<td>−</td>
<td>−</td>
<td>39</td>
<td>290</td>
</tr>
<tr>
<td>−</td>
<td>B</td>
<td>−</td>
<td>0</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>−</td>
<td>B</td>
<td>M</td>
<td>5</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SHMV</td>
<td>B</td>
<td>−</td>
<td>3</td>
<td>5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SHMV</td>
<td>B</td>
<td>M</td>
<td>30</td>
<td>24</td>
<td>140</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>B + M</td>
<td>35</td>
<td>26</td>
<td>130</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Protoplasts were isolated and inoculated 5 days after the second inoculation.
† Determined by staining with fluorescent antibodies to RCMV.
‡ Detected by ELISA.

RCMV B-RNA transport in leaves infected by TMV ts mutants

TMV ts mutant Ni118 produces a defective coat protein that is denatured at the non-permissive temperature (33°C) (Bosch & Jockush, 1972).

Mixed infection experiments with Ni118 were carried out in tobacco N. tabacum var. Samsun, since V. unguiculata is not a systemic host for Ni118. Table 2 shows that the numbers of cells containing B-RNA were much greater in mixedly inoculated (Ni118 + B) leaves at the temperature non-permissive for Ni118 (33°C) than in leaves inoculated only with B component (control). These results agree with the ELISA data and suggest that the transport function does not depend on the helper virus coat protein.

In addition a series of similar experiments was done with TMV mutant Ls1, which is temperature-sensitive for virus transport (Nishiguchi et al., 1978). Ls1 did not help the transport of RCMV B-RNA at the non-permissive temperature (Table 3).
Table 2. Transport of RCMV B-RNA in the presence of TMV ts mutant Ni118 in N. tabacum

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>1st inoculation (helper virus used for preinfection of N. tabacum plants)</th>
<th>2nd inoculation (RCMV component used for superinoculation)</th>
<th>3rd inoculation* (RCMV component used for protoplast infection)</th>
<th>Percentage of protoplasts infected with RCMV†</th>
<th>Amount of RCMV protoplasts (ng per 10⁵ protoplasts)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni118§</td>
<td>B</td>
<td>M</td>
<td>28</td>
<td>120</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Ni118§</td>
<td>–</td>
<td>M</td>
<td>0</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>B + M</td>
<td>37</td>
<td>180</td>
<td>&lt;10</td>
</tr>
<tr>
<td>–</td>
<td>B</td>
<td>M</td>
<td>5</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Protoplasts were isolated and inoculated 5 days after the second inoculation.
† Determined by staining with fluorescent antibodies to RCMV.
‡ Detected by ELISA.
§ Plants were grown at 33 °C (non-permissive temperature) and ELISA tests detected no TMV coat protein in them.

Table 3. Lack of RCMV B-RNA transport in the presence of TMV ts mutant Ls1 in N. tabacum

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>1st inoculation (helper virus used for preinfection of N. tabacum plants)</th>
<th>2nd inoculation (RCMV component used for superinoculation)</th>
<th>3rd inoculation* (RCMV component used for protoplast infection)</th>
<th>Amount of RCMV in protoplasts (ng per 10⁵ protoplasts)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ls1 (24 °C)‡</td>
<td>B (24 °C)</td>
<td>M</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Ls1 (24 °C)</td>
<td>B (33 °C)</td>
<td>M</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>B (33 °C)</td>
<td>B + M</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>M</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>

* Protoplasts were isolated and inoculated 5 days after the second inoculation.
† Detected by ELISA.
‡ Temperatures in parentheses are those at which plants were kept after inoculation. 24 °C is permissive, 33 °C is non-permissive.

DISCUSSION

The most probable candidate for the role of TMV 'transport' protein is the M, 30000 (30K) protein encoded by the viral genome. TMV mutants temperature-sensitive for virus transport contain mutations in the gene coding for 30K protein (Leonard & Zaitlin, 1982; Ohno et al., 1983; Zimmern & Hunter, 1983) and do not assist the transport of dependent viruses (Taliansky et al., 1982a). Similarly we have now found that the TMV Ls1 mutant which is ts for virus transport failed to assist the transport of RCMV B-RNA at the non-permissive temperature. Preinfection of N. tabacum plants with Ls1 at the permissive temperature (24 °C) promotes RCMV B-RNA transport only at 24 °C, and not at 33 °C (non-permissive temperature) (Table 3). Thus Ls1 'transport' protein should be produced in N. tabacum during the 'preinfection' stage until the switch to 33 °C. Therefore failure to complement RCMV B-RNA after the switch may be accounted for by the quite rapid inactivation of Ls1 'transport' protein or its disappearance following the temperature shift. Earlier results (Rezelman et al., 1982) and our data presented here suggest that the location of the gene controlling transport of comoviruses is in M-RNA. M-RNA codes for two coat proteins and two other polypeptides (58K and 48K) with overlapping amino acid sequences (Shanks et al., 1986). One or both of these proteins may act as a 'transport' protein. In addition there is a tentative sequence similarity between part of the CPMV M-RNA polyprotein contained in the 58K region and regions in the 30K TMV 'transport' gene (Meyer et al., 1986).
In the present work, it was found that RCMV M-RNA transport function directing RCMV RNA spread from cell to cell can be substituted for by unrelated helper viruses (SHMV and TMV ts mutant N118). Possibly this RCMV B-RNA transport is carried out by the 'transport' (possibly 30K) protein of the helper. Tobamovirus coat protein is not involved in the RCMV B-RNA transport because this can occur in the absence of a functionally active helper coat protein.

It should be noted that tobamoviruses are capable of complementing RCMV B-RNA transport not only in cowpea plants (a systemic host plant for RCMV), but also in tobacco plants which are resistant to RCMV. These results are consistent with our earlier finding that transport function can be considered to be a factor controlling the host range (Taliansky et al., 1982b): the resistance of a plant to a virus due to a blocked spread of infection may be overcome in mixedly infected plants if a helper virus provides 'transport' protein(s).

The exact mechanisms by which putative viral 'transport' protein could facilitate cell-to-cell spread of the virus are unknown. However since complementation in transport function is accomplished not only by related, but also by unrelated viruses (Tables 1, 2, 3; Fig. 1; Taliansky et al., 1982a,b; Carr & Kim, 1983; Atabekov et al., 1984; Barker, 1987), then 'transport' protein of a helper virus is likely to have some general effect on cellular physiology, and this ultimately allows cell-to-cell viral movement. Putative 'transport' proteins of some viruses probably modify cells so as to allow virus transport by affecting host mRNA synthesis in the nucleus. This is supported by the nuclear location of the TMV 30K protein (Watanabe et al., 1986). It is also probable that 'transport' proteins of other viruses directly interact with the cell wall itself as in the case of the putative 'transport' 32K protein of alfalfa mosaic virus (Godefroy-Colburn et al., 1986).

REFERENCES


*(Received 19 June 1987)*