Properties of Three Spontaneous Mutants of Red Clover Necrotic Mosaic Virus

By T. A. M. Osman and K. W. Buck*

Department of Pure and Applied Biology, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BB, U.K.

(Accepted 26 October 1988)

SUMMARY

Three spontaneous mutants of strain TpM-34 of red clover necrotic mosaic virus, designated M-A, M-B and M-C, were isolated. In gel diffusion tests the three mutants were serologically indistinguishable from the parent but when inoculated onto Vigna unguiculata each induced characteristic symptoms which differed from those induced by TpM-34. TpM-34 induced similar numbers of lesions at 17 °C and 26 °C. However, although inoculation with RNA of the mutants induced 70 to 80% as many lesions as inoculation with TpM-34 RNA at 17 °C, very few lesions were induced by any of the mutants at 26 °C. The symptoms induced by heterologous mixtures of RNA 1 or RNA 2 of TpM-34 with RNA 2 or RNA 1 of each mutant indicated that M-A, M-B and M-C each has mutations in both RNA components, but that symptom expression was determined predominantly by RNA 1.

Red clover necrotic mosaic virus (RCNMV), a member of the Dianthovirus group (Matthews, 1982), has a genome of two single-stranded RNA components, RNA 1 (approx. 4500 nucleotides) and RNA 2 (approx. 1500 nucleotides) (Gould et al., 1981). RNA 1 encodes the single capsid polypeptide species, M, approx. 40000 (Okuno et al., 1983; Morris-Krsinich et al., 1983) and can replicate and give rise to virus particles in protoplasts in the absence of RNA 2 (Osman & Buck, 1987). However, both RNA species are required to infect plants (Gould et al., 1981; Okuno et al., 1983; Osman et al., 1986), implying a role for RNA 2 in cell-to-cell movement of the virus.

Pseudorecombinants formed by infecting plants with heterologous mixtures of RNA 1 and RNA 2 from different isolates of RCNMV, or from RCNMV and other dianthoviruses, indicate that the type of symptoms induced in a range of plants can be determined by RNA 1, RNA 2 or both RNAs (Okuno et al., 1983; Osman et al., 1986; Rao & Hiruki, 1987). Furthermore, the symptoms induced by some pseudorecombinants are different from those induced by either of the parental viruses (Okuno et al., 1983). However, only pseudorecombinants between serologically distinct viruses have been studied. We now report on the properties of three spontaneous mutants of an isolate of RCNMV.

Czechoslovakian isolate TpM-34 of RCNMV (Musil, 1969; Hollings & Stone, 1977) which had been subjected to three cycles of single lesion isolation in Chenopodium quinoa (Osman et al., 1986) was propagated in Phaseolus vulgaris (French bean) cv. The Prince as described previously (Osman et al., 1986). In search of spontaneous mutants of TpM-34, 100 leaves on 50 cowpea plants (Vigna unguiculata cv. California Blackeye No. 5) were inoculated with sap from infected bean plants and incubated at 17 °C in a Fisons Fitotron 600H growth cabinet with an 18 h photoperiod and a light intensity of 5000 lux. After 3 to 4 days local lesions 1 to 2 mm in diameter were produced. Most of these lesions continued to expand, developing reddish-brown necrotic margins, and often becoming confluent with adjacent lesions. After 15 days the plants, which had become stunted and chlorotic as observed previously (Hollings & Stone, 1977; Osman et al.,
Fig. 1. Detection of RCNMV RNA in non-inoculated leaves of cowpea infected with mixtures of RNA 1 and RNA 2 of TpM-34 and its mutants by spot hybridization: 1(a), 2(a), 3(a), RNA 1 (TpM-34) plus RNA 2 (TpM-34); 1(b), RNA 1 (M-A) plus RNA 2 (M-A); 2(b), RNA 1 (M-B) plus RNA 2 (M-B); 3(b), RNA 1 (M-C) plus RNA 2 (M-C); 1(c), RNA 1 (TpM-34) plus RNA 2 (M-A); 2(c), RNA 1 (TpM-34) plus RNA 2 (M-B); 3(c), RNA 1 (TpM-34) plus RNA 2 (M-C); 1(d), RNA 2 (TpM-34) plus RNA 1 (M-A); 2(d), RNA 2 (TpM-34) plus RNA 1 (M-B); 3(d), RNA 2 (TpM-34) plus RNA 1 (M-C). Spot hybridizations were carried out as described by Osman & Buck (1987) using extracts of 0.5 g leaves and a 32P-labelled cDNA probe to RNAs 1 and 2 of TpM-34.

1986), were shown to have developed a systemic infection by local lesion assay and spot hybridization (Fig. 1). On many of the leaves a few necrotic lesions were observed which did not expand further. Sixty such lesions were individually cut out and ground with 100 μl of sodium phosphate buffer pH 7.0. Each homogenate was used to inoculate a leaf on a cowpea plant. After 5 days, 10 non-expanding lesions which had developed on the leaves of the plants were selected and virus from each was subjected to five cycles of single lesion isolation in C. quinoa followed by a further cycle in cowpea. The 10 mutants were grouped into three classes based on their symptoms and three, designated M-A, M-B and M-C, were selected as representatives of each class.

**Mutant M-A** induced reddish-brown necrotic local lesions about 1 mm in diameter 4 to 5 days after inoculation and these did not expand further (Fig. 2a). No symptoms of systemic infection were observed in the plants 15 days after inoculation. Furthermore no viral RNA could be detected in sap from uninoculated leaves by spot hybridization (Fig. 1).

**Mutant M-B** induced reddish-brown necrotic local lesions slightly larger than those induced by M-A (about 2 mm in diameter, 4 to 5 days after inoculation) (Fig. 2b). Although further expansion of these lesions could not be detected visually, evidence of a low level of systemic infection was obtained by spot hybridization (Fig. 1) 15 days after inoculation.

**Mutant M-C** also induced reddish-brown necrotic local lesions about 2 mm in diameter 4 to 5 days after inoculation, but these were accompanied by some veinal necrosis (Fig. 2c) not observed in infections with the other two mutants or with the wild-type virus. A low level of systemic infection was detected by spot hybridization (Fig. 1) 15 days after inoculation.

Local lesion assays on cowpea confirmed the spot hybridization data; whereas sap samples from uninoculated leaves of cowpea plants inoculated with TpM-34 gave 27 lesions in two experiments, analogous samples from plants inoculated with M-A, M-B or M-C gave zero, two or five lesions respectively. This suggests that the mutants were impaired in their ability to induce a systemic infection. However, since no measurements were made more than 15 days after inoculation, the results could reflect a delay in spread of the mutant viruses.

All three mutants were shown to be temperature-sensitive by measurement of the number of local lesions produced on inoculated leaves of cowpea plants maintained at 17 °C or 26 °C. At 17 °C the three mutants induced 70 to 80% as many lesions as the wild-type virus. However, although wild-type virus induced similar numbers of lesions at 17 °C and 26 °C, very few (<1%) of control) lesions were induced by any of the mutants at 26 °C.
Fig. 2. Symptoms induced on cowpea leaves inoculated with the three types of spontaneous mutants of TpM-34 and incubated at 17 °C: (a) M-A, (b) M-B, (c) M-C.
Fig. 3. Symptoms induced on cowpea leaves inoculated with heterologous mixtures of RNA 1 and RNA 2 of TpM-34 and its mutants: (a) RNA 1 (TpM-34) plus RNA 2 (M-A); (b) RNA 2 (TpM-34) plus RNA 1 (M-A); (c) RNA 1 (TpM 34) plus RNA 2 (M-B); (d) RNA 2 (TpM-34) plus RNA 1 (M-B); (facing page) (e) RNA 1 (TpM-34) plus RNA 2 (M-C); (f) RNA 2 (TpM-34) plus RNA 1 (M-C).
In serological gel diffusion tests using the antiserum to RCNMV described previously (Osman & Buck, 1987), all three mutants gave single precipitin lines which coalesced with the line produced by wild-type virus (not shown). Hence the epitopes on the virus coat protein appeared to be unaffected by any of the mutations.

RNA was prepared from each of the mutant viruses and analysed by agarose gel electrophoresis as described by Osman & Buck (1987). Each mutant had two RNA components with electrophoretic mobilities indistinguishable from the corresponding RNA components of TpM-34. Therefore there were no substantial deletions in the RNA components of any of the three mutants.

Pseudorecombinant analysis was used to determined which RNA of each mutant determined the symptoms. Virus particles were extracted and purified from bean plants infected by each mutant or wild-type TpM-34 and virus RNA was prepared as described previously (Osman & Buck, 1987). The two RNA components were separated by electrophoresis in a 1% low melting point agarose gel and extracted as described by Maniatis et al. (1982). The purity of each separated RNA component was shown by gel electrophoresis and Northern hybridization with a 32P-labelled cDNA probe to TpM-34 RNA 1 and RNA 2 as described by Osman & Buck (1987) and by the absence of lesions when inoculated onto cowpea. In contrast numerous lesions were formed when cowpea plants were inoculated with homologous and heterologous mixtures of the RNAs of mutants and wild-type virus (Table 1).

The symptoms induced by mixtures of RNA 1 of TpM-34 and RNA 2 of each mutant were similar to those induced by TpM-34 (Fig. 3a, c, e) and the plants became systemically infected as judged by spot hybridization (Fig. 1). In contrast symptoms induced by mixtures of RNA 2 of TpM-34 and RNA 1 of each mutant were similar to those induced by the mutant (Fig. 3b, d, f). A low level of systemic infection was detected in infections induced by RNA 2 (TpM-34) and either RNA 1 (M-B) or RNA 1 (M-C) (Fig. 1). It is clear therefore that each mutant has an RNA-1 mutated in the primary determinant of the symptoms induced. However, it appears that each mutant also has an altered RNA 2. This is shown by the reduced numbers of lesions (Table 1) and the reduced levels of viral RNA in systemically infected leaves (Fig. 1) induced by mixtures of RNA 1 (TpM-34) and RNA 2 of each mutant, compared to those induced by the homologous mixture of TpM-34 RNAs.
Table 1. Local lesions produced in cowpea inoculated with combinations of RNA species from TpM-34 and mutants

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Number of lesions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA 1 (TpM-34) + RNA 2 (M-A)</td>
<td>49</td>
</tr>
<tr>
<td>RNA 2 (TpM-34) + RNA 1 (M-A)</td>
<td>86</td>
</tr>
<tr>
<td>RNA 1 (TpM-34) + RNA 2 (M-B)</td>
<td>52</td>
</tr>
<tr>
<td>RNA 2 (TpM-34) + RNA 1 (M-B)</td>
<td>83</td>
</tr>
<tr>
<td>RNA 1 (TpM-34) + RNA 2 (M-C)</td>
<td>50</td>
</tr>
<tr>
<td>RNA 2 (TpM-34) + RNA 1 (M-C)</td>
<td>83</td>
</tr>
<tr>
<td>RNA 1 (TpM-34) + RNA 2 (TpM-34)</td>
<td>92</td>
</tr>
<tr>
<td>RNA 1 (M-A) + RNA 2 (M-A)</td>
<td>86</td>
</tr>
<tr>
<td>RNA 1 (M-B) + RNA 2 (M-B)</td>
<td>83</td>
</tr>
<tr>
<td>RNA 1 (M-C) + RNA 2 (M-C)</td>
<td>79</td>
</tr>
</tbody>
</table>

* The final concentration of each RNA species was 5 μg/ml in 0.05 M-sodium phosphate buffer pH 7.0, containing 200 μg/ml bentonite and 10 mg/ml celite.
† Total number of lesions on two leaves 5 days after inoculation. I and II refer to separate experiments.

The symptoms induced by mutant M-A of TpM-34 were similar to those induced by English isolate H (Osman et al., 1986). However, the symptoms induced by pseudorecombinants formed between isolates TpM-34 and H were determined by RNA 2 of the parent isolate, whereas those induced by pseudorecombinants formed between TpM-34 and M-A were determined primarily by RNA 1.

The symptoms induced may depend inter alia on interactions between three processes: the amount of virus replication, the efficiency of cell-to-cell movement of the virus and the strength of the hypersensitive response. Since RNA 1 encodes the virus coat protein and can replicate alone in protoplasts, the extent of virus replication in single cells will be governed by RNA 1. The efficiency of virus cell-to-cell transport will be determined not only by the intrinsic properties of the transport protein, encoded by RNA 2, but also by its amount and will therefore also depend on RNA 1. Cell-to-cell transport of the virus is also likely to be reduced by the hypersensitive response as shown recently for tobacco mosaic virus (Moser et al., 1988). Depending on the relative contributions of the above three processes, the symptoms could therefore be determined predominantly by either RNA 1 or RNA 2, or may depend equally on both RNAs.

With a strong hypersensitive response and a weak cell-to-cell transport function, systemic spread of the virus could be prevented, as with isolate H. With a strong cell-to-cell transport function the hypersensitive response may be overcome. This could explain the ability of the necrotic lesions induced by isolate TpM-34 to expand and the dominant effect of RNA 2 in pseudorecombinants formed between isolates TpM-34 and H. If the ability of the virus to replicate was impaired, and/or a stronger hypersensitive response were induced, the hypersensitive response could again become the dominant effect, leading to absence of, or a much reduced, systemic infection. In this situation the symptoms would be determined predominantly by RNA 1, as found with mutants M-A, M-B and M-C.

The spontaneous mutants of TpM-34 described here, which are likely to differ much less in their nucleotide sequence from TpM-34 than do other RCNMV isolates from nature, should be valuable for further studies on the molecular basis of RCNMV pathogenicity and the induction of symptoms.

We are grateful to the Egyptian Ministry of Higher Education for a scholarship to support Mr T. A. M. Osman and to the AFRC for a grant. RCNMV was held under MAFF licence no. PHF29B/115(107).

REFERENCES


(Received 9 August 1988)