Some Properties of a Temperature-sensitive Mutant of Cowpea Chlorotic Mottle Virus*

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SUMMARY

The behaviour and properties of a temperature-sensitive mutant of cowpea chlorotic mottle virus are described. The mutant multiplied well at 21° but, unlike the wild-type, only slightly at 32° although considerable amounts of uncoated RNA accumulated in inoculated leaves at this temperature. The specific infectivity of the mutant was much lower than that of the wild-type virus because the largest species of encapsidated mutant RNA was almost completely degraded even in virus from plants grown at 21°. The temperature sensitivity and low specific infectivity of the mutant were related to properties of its coat protein, which was much less heat stable than that of the wild-type virus. Glutamic acid and alanine replaced lysine and valine respectively in the mutant coat protein and these replacements, in addition to affecting thermal stability, influenced the polymerization of isolated protein at pH 6.7.

INTRODUCTION

Although tobacco mosaic virus (TMV) temperature-sensitive (ts) mutants have been obtained (Jockusch, 1966a), and analysed (Jockusch, 1966b; Jockusch, 1968; Jockusch, Koberstein & Jaenicke, 1969; Wittmann & Wittmann-Liebold, 1966) no comparable mutants have been isolated and characterized for spherical plant viruses. In this paper, we wish to describe a ts mutant of the spherical cowpea chlorotic mottle virus (CCMV).

METHODS

Virus culture and purification. The mutant was obtained after CCMV-RNA had been treated with nitrous acid according to Siegel (1960) to a survival level of about 10⁻² as described (Bancroft et al. 1970). The mutant was passed through three serial single lesion transfers in Chenopodium hybridum L. and was grown in cowpea (Vigna unguiculata (L.) Walps Var Blackeye).

Cowpea infected either with wild-type CCMV or the ts mutant was blended in 2 to 3 vol. (w/v) 0.2 M-acetate buffer pH 4.7 containing 0.01 M-ascorbic acid. The homogenate was expressed through cheesecloth and was kept at 4° overnight before centrifugation at 10,000 rev./min. for 15 min. in a Servall or MSE centrifuge. Virus in the clear supernatant fluid was concentrated and further purified by 2 cycles of differential ultracentrifugation (Bancroft, Hiebert, Rees & Markham, 1968). Alternatively, the clarified solution was brought to 0.2 M with respect to NaCl with and 10 % (w/v) polyethylene glycol 6000 (Hebert, 1963) was added with stirring. After the polyethylene glycol had dissolved, the mixture was kept for 1 hr at 4°

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to precipitate the virus which was collected by centrifuging at low speed and was then subjected to a cycle of differential centrifugation.

Virus, and RNA obtained by phenol extraction, were assayed for infectivity on soybean (Glycine max (L.) Merr. Var Lindarin), using at least six half-leaves per treatment.

**RNA and protein characterization.** Polyacrylamide gel electrophoresis was done with RNA, heated for 10 min. at 50°C in 1 M-urea, 0.05 M-mercaptoethanol, 0.2% (w/v) sodium dodecyl-sulphate, according to Loening (1967) in 2.6% gels. These were monitored after electrophoresis in a Joyce–Loebl ‘Polyfrac’ ultraviolet densitometer. Protein was prepared according to Bancroft & Hiebert (1967) and the amount of denaturation determined by light scattering at 380 nm. and/or colorimetrically (Lowry et al. 1951) on nonprecipitated protein.

Analyses of coat protein composition were made as before (Bancroft et al. 1971). Two new tryptic peptides called ‘D’ and ‘E’ (see Results) were found in the mutant and these were purified by two successive preparative high voltage electrophoretic separations on Whatman 3 mm. paper (27 x 13 cm.). Each sample containing 9 mg. of a tryptic digest of the protein of the ts mutant dissolved in 0.2 ml. of 0.2 N-ammonium hydroxide was applied as a line across the papers 1.5 cm. from each side. Electrophoresis was for 2 hr at 40 v/cm. using pyridine–acetic acid–water buffer, pH 6.5 (100:4:896 by vol.). After location by staining of side strips, the components were eluted with 0.2 N ammonia and lyophylized. The E peptide was further purified by ascending paper chromatography using butanol–acetic acid–water–pyridine (15:13:12:10 by vol.) as solvent.

**RESULTS**

**Symptoms, yield and infectivity**

The ts mutant produced a mild mottle on cowpea at 21°C but symptoms were not noticeable at 32°C whereas the wild-type incited marked symptoms at both temperatures (Fig. 1). An average lesion diameter 7 days after inoculation on C. hybridum of 0.5 mm. was found for the mutant at 21°C and 32°C, and also for the wild-type at 21°C. At 32°C, the average lesion diameter for the wild-type was 1.6 mm.

The yields and specific infectivities of wild-type and ts CCMV and their RNA’s from all the leaves of plants grown under permissive and restrictive conditions are shown in Table 1. The mutant yield was only about 1% of that found for the wild-type at an average temperature of 32°C whereas it was 70% of that found for the wild-type at 21°C. The specific infectivities of ts mutant preparations were 1%, 13% and 20% of those of the wild-type grown at 32°C, 24°C and 21°C respectively. The differences in the specific infectivities were reflected by those of the RNA’s indicating that the reduced infectivities did not result from uncoating difficulties.

**RNA and protein stability**

The RNA’s from the wild-type and ts mutant were extracted from virus purified from plants grown at the same time at 21°C for 10 days. Polyacrylamide gel electrophoresis revealed that the largest RNA species of the wild-type (mol. wt 1.1 x 10^9) was almost completely missing from the mutant virus (Fig. 2). The large piece of RNA is one of the species essential for infection (Bancroft, 1971), so this discrepancy would account for the low specific infectivity of the mutant virus. The other RNA’s were clearly present, although in different relative amounts. The ts RNA species 2 (1.0 x 10^9 mol. wt) and 3 (0.8 x 10^9 mol. wt) have been identified as such in complementation experiments against the appropriate wild-type species (unpublished).
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The wild-type and the mutant had the same densities in CsCl, so the mutant $1.1 \times 10^6$ mol. wt RNA had probably been synthesized but was subsequently degraded in the virus particle. The relative proportions of the three density populations observed in CsCl of wild-type and mutant nucleoprotein preparations did, however, differ (Fig. 3). The ratio of the three density components reflects the relative amounts of the encapsidated RNA species; the densest nucleoprotein contains the $1.1 \times 10^6$ mol. wt RNA, the least dense contains the $1.0 \times 10^6$ mol. wt RNA and the middle nucleoprotein contains jointly encapsidated 0.8 and $0.3 \times 10^6$ mol. wt RNA (Bancroft & Flack, 1972). It therefore appears that the $ts$ mutant synthesized less of the two large RNA species than did the wild-type virus. The reason that
Table 1. The effect of temperature on the yield and infectivity of wild-type (WT) and ts CCMV

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Yield*</th>
<th>Specific infectivity†</th>
<th>Specific infectivity of virus particle/RNA§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>ts</td>
<td>WT</td>
</tr>
<tr>
<td>21</td>
<td>346</td>
<td>247</td>
<td>414</td>
</tr>
<tr>
<td>24</td>
<td>348</td>
<td>112</td>
<td>282</td>
</tr>
<tr>
<td>32</td>
<td>441</td>
<td>5</td>
<td>195</td>
</tr>
</tbody>
</table>

* mg. virus/kg. tissue. Plants were infected for 15 days.
† Number of local lesions produced in 8 half-leaves of soybean at $E_{80} = 0.1$.
§ Local lesions as for † except that the assays for the 21 and 24° preparations were made at different times. The RNA infectivity assays were done on the same 15 day samples as used for the purified virus assays.

Fig. 2. RNA from wild-type (a) and ts (b) CCMV, from virus from plants infected for 10 days at 21°, after electrophoresis for 2½ hr at 4 mA/tube on 2-6 % polyacrylamide gels. The numbers 1, 2, 3 and 4 refer to RNA's with mol. wt of 1.1, 1.0, 0.8 and 0.3 × 10⁶, respectively. The RNA loads were 40 µg.

the relative amount of the 0.8 × 10⁶ mol. wt RNA species from the wild-type is low in Fig. 2a is that this species particularly is not always extracted efficiently from wild-type virus (Bancroft & Flack, 1972).

The degradation of the RNA in the virus particle may reflect the stability of the coat protein. Temperature sensitivity and low infectivity accompany the ts mutant coat protein gene on RNA species 3 in complementation tests (unpublished). Accordingly, unpolymerized isolated protein was heated at different temperatures in 1 m-NaCl pH 7.4 for 3 min.
Temperature-sensitive mutant of CCMV

Fig. 3. Schlieren patterns of ts (upper) and wild-type CCMV (lower) at 0.15 mg./ml. in CsCl. \( \bar{\rho} = 1.35 \text{ g./cm.}^3 \) after centrifuging for 20 hr at 40,000 rev./min.

![Schlieren patterns of ts (upper) and wild-type CCMV (lower) at 0.15 mg./ml. in CsCl.](image)

Fig. 4. Denaturation curves of wild-type (wt) (○) and ts (●) CCMV protein in 1 M-NaCl + 0.02 M-tris, pH 7.4 or in 1 M-NaCl + 0.02 M-acetate, pH 5.0 after heating at the various temperatures for 3 min. The dashed lines refer to scattering measurements at 380 nm. at 0.32 and 0.30 mg./ml. for wild-type and ts protein respectively at either pH 7.4 or pH 5.0. The solid lines refer to the protein content of the supernatant fluids, measured colorimetrically (see text). After heating the protein was kept overnight at 4°C at pH 7.4, and was centrifuged at 3000 rev./min. for 5 min. The final protein concentrations tested were 0.069 and 0.064 mg./ml. for wild-type and ts protein, respectively.

![Denaturation curves of wild-type (wt) (○) and ts (●) CCMV protein in 1 M-NaCl + 0.02 M-tris, pH 7.4 or in 1 M-NaCl + 0.02 M-acetate, pH 5.0 after heating at the various temperatures for 3 min.](image)
Fig. 5. Inactivation kinetics of ts (●) and wild-type (○) CCMV after heating in 0.1 M-acetate buffer pH 5.0 at 59°C.

Fig. 6. Free RNA (---) and virus (--) levels obtained after ts and wild-type (wt) CCMV were grown for various lengths of time at either 21 or 32°C in the primary leaves of cowpea. The triangles and circles designate different experiments.
### Temperature-sensitive mutant of CCMV

**Table 2. The effect of high temperature on the yield of wild-type and ts CCMV from inoculated leaves kept at low temperature initially**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Temperature</th>
<th>Plants kept for 8 days at 21°</th>
<th>Plants kept for a further 6 days at either 21° or 32°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>wt 30 leaves* (g.) mg. virus/ kg. tissue</td>
<td>wt 30 leaves* (g.) mg. virus/ kg. tissue</td>
</tr>
<tr>
<td>ts</td>
<td>21°</td>
<td>48</td>
<td>626</td>
</tr>
<tr>
<td></td>
<td>32°</td>
<td>--</td>
<td>257</td>
</tr>
<tr>
<td>Wild-type</td>
<td>21°</td>
<td>51</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>32°</td>
<td>--</td>
<td>750</td>
</tr>
</tbody>
</table>

* Only primary leaves were used.

**Table 3. Amino acid composition of the proteins of wild-type and ts mutant strains of cowpea chlorotic mottle virus**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per sub-unit of mol. wt 19,400</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative molar ratio Wild-type ts Mutant Wild-type ts Mutant</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.9 10.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.0 1.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>9.1 8.9</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>11.2 11.1</td>
</tr>
<tr>
<td>Threonine*</td>
<td>17.3 17.2</td>
</tr>
<tr>
<td>Serine*</td>
<td>15.8 16.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>16.0 17.2</td>
</tr>
<tr>
<td>Proline</td>
<td>7.2 7.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.0 9.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>25.2 26.2</td>
</tr>
<tr>
<td>Half cystine†</td>
<td>1.9 1.9</td>
</tr>
<tr>
<td>Valine</td>
<td>19.2 18.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.0 1.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.9 6.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>15.8 15.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.9 5.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.9 4.0</td>
</tr>
<tr>
<td>Tryptophan§</td>
<td>2.9 2.9</td>
</tr>
</tbody>
</table>

* Threonine has been corrected 5% and serine 10.5% for losses during acid hydrolysis.
† Determined from performic oxidized protein.
§ Determined by the method of Spies & Chambers (1949).

periods from 0 to 55°C. The amount of light scattering was recorded, the samples centrifuged and the amount of protein left in the supernatant fluids estimated colorimetrically. The resulting denaturation curves (Fig. 4) showed that the one-half denaturation temperature for the wild-type protein was 46°C whereas that for the mutant was 39°C. Thus, the ts mutant protein was much more sensitive to heat than was the wild-type protein. Both proteins were more heat stable at pH 5, where the subunits formed capsids, than at pH 7.4 where they were in the unpolymerized state. This marked pH dependent stability has also been observed in analogous experiments with tobacco mosaic virus protein (Jockusch, 1966c) and probably results from the configuration of the protein subunits being mutually changed upon capsid formation under conditions where the virus or its capsid is normally stable.
Fig. 7. Two-dimensional separations of peptides obtained by tryptic digestion of wild-type CCMV protein. Peptides located by the chlorination method (top) and peptides stained specifically for arginine by the Sakaguchi reaction (bottom). The arrows point to the peptides labelled A, B and C missing in the mutant.

That the *ts* coat is probably less effective in protecting RNA than is that of the wild-type can also be demonstrated by following the inactivation kinetics of the two isolates. The mutant heated at 59° in 0.1 M-acetate buffer pH 5 is inactivated at about 1.7 times the rate of wild-type virus (Fig. 5).

**RNA production in vivo**

The reason that the *ts* mutant did not multiply well at 32° could either be due to a defective replicative function or to a maturation fault linked with the coat protein. If the latter were true, free RNA should be present in *ts* mutant-infected plants grown under restrictive conditions. To test this hypothesis, the primary leaves of cowpea were separately inoculated with wild-type and *ts* virus adjusted to the same infectivities as measured by local lesion tests and
the plants were placed at 21° and 32°. Thirty leaf discs (17 mm. diameter) were cut from different leaves from 3 to 12 days after inoculation and 10 discs were extracted immediately in phenol. The remaining 20 discs were ground separately in water in two groups of 10 and were kept for 2 hr at 25°. Both the wild-type and ts mutant virus are stable for this length of time but free RNA is inactivated. One of the groups which had been aged for 2 hr was extracted in phenol. The zero time phenol extracts and the two extracts which had been aged for 2 hr (along with an internal standard used so that samples taken at different times could be directly compared) were then assayed. The differences in lesion numbers from the phenol extracts taken at 0 and 2 hr gives an estimate of free RNA present in the tissue. The water (non-phenolized) extracts assayed after 2 hr give a direct measure of virus content. Fig. 6 shows that the ts mutant RNA from plants kept at 21° was mostly encapsidated by 8 days after inoculation. However, relatively little RNA was encapsidated in plants grown at 32° and more free RNA accumulated under restrictive than permissive conditions. These results
clearly differ from those found with wild-type virus (Fig. 6) and suggest that the malfunction of the mutant is coat associated.

Stability of mutant in vivo

Although the quantities of coat protein made by the mutant under restrictive conditions are not yet known, some protein must be made because virus nucleoprotein can be isolated, although in very low yields. It is most probable from the in vitro denaturation experiments that the protein would be most susceptible to denaturation before capsid assembly. However, it is also possible that mature virus becomes insoluble in vivo at high temperatures. Accordingly, wild-type and mutant virus were grown in primary leaves of cowpea for 8 days at 21°C. Some of the leaves were harvested and the virus in them purified, and the remaining plants were then divided into groups kept for a further 6 days at either 21 or 32°C before virus purification. Table 2 shows that less virus was isolated from the mutant-infected plants kept at 32°C for 6 days than from those kept at 21°C or from plants infected for 8 days at 21°C. This did not happen with wild-type virus. These results indicate that the mutant virus can be denatured in vivo.

Protein composition

The amino acid composition of the protein of the ts mutant is given in Table 3 as the mean value from 12 analyses of 24 hr HCl hydrolysates. Eight analyses of type CCMV protein were interspersed with the mutant analyses and are also presented in Table 3. The figures indicate that the ts mutant had one glutamic acid and one alanine residue more and one lysine and one valine residue less than wild-type protein.

Fig. 7 and 8 show two-dimensional peptide maps obtained from peptides produced by 3½ hr tryptic digestions of the S-β-aminoethylated proteins of type and ts mutant CCMV, respectively, and stained by the chlorination technique of Reindel & Hoppe (1954). Fig. 7 and 8 bottom are similar separations stained for peptides containing arginine using the specific Sakaguchi reaction. The three peptides indicated by the letters A, B and C in the type protein peptide map are not present in the ts mutant protein peptide map but the two new peptides indicated by the letters D and E are apparent.

From unpublished work in progress on the amino acid sequence of wild-type CCMV protein, the amino acid composition and sequence of peptides A, B and C are known. Peptide A is lysyl-asparaginyl-lysine, peptide B is asparaginyl-lysyl-arginine and peptide C is valyl-glycyl-arginine. The amino acid analysis of peptide D obtained from the ts mutant gave 1.0, 1.0 and 1.0 residues respectively of alanine, glycine and arginine and most likely represents the alanine for valine replacement.

Peptides A and B in the type CCMV protein tryptic digest are replaced by the one peptide E in the ts mutant. This suggests that the wild-type peptide A and peptide B are adjacent in the polypeptide chain and the amino acid replacement has prevented the hydrolysis of the peptide bond linking the two peptides. The amino acid analysis of peptide E was one residue each of arginine, glutamic acid, aspartic acid and lysine. This would be consistent with the amino acid sequence for the A and B peptides being asparaginyl-lysyl-arginyl-lysyl-asparaginyl-lysine, the lysine of the fourth residue being replaced by a glutamic acid residue in the ts mutant peptide. Trypsin catalyses the hydrolysis of the peptide bond between the —COOH group of lysine, or the —COOH group of arginine and the —NH₂ group of the adjacent amino acid, but cleavage takes place much more slowly when the basic residue is adjacent in sequence to an acidic residue (Smyth, 1967). After prolonged (18 hr rather than 3½ hr) digestion with trypsin, peptides A and B are absent from the peptide map of wild-type
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Fig. 9. Electron micrographs, kindly taken by G. J. Hills, of ‘ropes’ made from ts CCMV protein after overnight dialysis against 0.05 M-phosphate buffer pH 6.7 at 4°C.

CCMV protein but have given rise to free lysine and free arginine plus asparaginyl-lysine. It is probable that the change in the environment around the lysyl-arginyl bond produced by the presence of a glutamic acid residue nearby has made this bond more susceptible to hydrolysis since a peptide corresponding to asparaginyl-lysine was present in the ts mutant peptide map after 3½ hr digestion.
**Self-assembly**

Protein obtained from the ts mutant behaved similarly to wild-type protein in reconstitution experiments (Bancroft & Hiebert, 1967) and in the formation of nucleic acid-free capsids (Bancroft, Wagner & Bracker, 1968). It did not, unlike wild-type virus protein, form tubes (Bancroft, Hills & Markham, 1967) in 0.05 M-phosphate buffer, pH 7.2. At pH 6.7, the mutant protein formed only the occasional tube at the protein concentrations at which the wild-type formed abundant tubes. Rather, the mutant protein tended to form helical structures, called ‘ropes’ (Fig. 9), which have never been observed in wild-type protein preparations. We have not been able to ascertain unequivocally whether the ropes are one or two start helices or a combination of both.

**DISCUSSION**

The behaviour of the ts CCMV mutant in comparison to its wild-type falls within the definition for temperature sensitivity introduced for plant viruses by Jockusch (1964) for tobacco mosaic virus mutants which produced only a small amount of virus at 32° as compared with 23°. Jockusch differentiated ts mutants which had coat malfunctions, such as found with the present CCMV mutant, from those with replicative malfunction, by the inability of the latter to spread locally or systemically under restrictive conditions. At 32° local lesions caused by the CCMV mutant spread less than wild-type lesions, but no difference was found at 21°. The systemic symptoms caused by the mutant at 21° are much less severe than those of the wild-type so the lack of symptoms found at 32° for the mutant does not constitute a good case for the contravention of Jockusch’s symptomatology rules. However, unlike TMV mutant Ni 118 which has a coat malfunction, the CCMV mutant accumulates large amounts of labile RNA in comparison with virus under restrictive conditions, at least in inoculated leaves. Furthermore, at least some already formed mutant CCMV seems, unlike analogous TMV mutants, to be degraded in vivo at high temperatures. CCMV is, however, much less heat stable than TMV and the mutant CCMV is probably considerably more labile than the TMV coat mutants. Mutants of both viruses are ‘leaky’ under restrictive conditions.

The replacement of lysine by glutamic acid is consistent with the action of nitrous acid but that of valine by alanine is not. The latter replacement would require the spontaneous alteration of only one nucleotide in the valine codon. It is not currently possible to know which replacement is critical in determining the properties of the mutant. An analogous interpretative difficulty has recently been reported for a ts mutant of TMV (Hariharasubramanian, Zaitlin & Siegel, 1970). However, neither CCMV replacement has so far been found in thermally unstable TMV protein but this is probably not surprising. Further, it is not known if one replacement is concerned with heat stability and the other with the formation of ropes or if there is a co-operative effect. However, the present data do identify two out of 182 amino acid residues that markedly influence the behaviour of CCMV protein, and such information must be obtained to assess what roles the various amino acids in particular locations have in determining why CCMV behaves as it does.
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REFERENCES


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