Limits to the Independence of Bottom Component RNA of Cowpea Mosaic Virus

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SUMMARY

Electron microscopic analyses have revealed that the bottom (B) component of cowpea mosaic virus alone induces cytopathic structures in cowpea mesophyll protoplasts, similar to those induced by the complete virus [i.e. B plus middle (M) components]. This indicates that the development of such structures is not linked to accumulation of virus particles but to virus RNA replication and expression. When purified B component was inoculated to primary cowpea leaves, symptoms were not produced and B component RNA was incapable of spreading to surrounding cells. The results are discussed in terms of limits to the independence of B component RNA and of possible functions for M component RNA-encoded proteins.

INTRODUCTION

Cowpea mosaic virus (CPMV) is a plant virus with an RNA genome distributed between two nucleoprotein particles, the bottom (B) and middle (M) component (Van Kammen, 1972). Both components, or their RNAs (denoted B RNA and M RNA, with mol. wt. 2.02 x 10^6 and 1.37 x 10^6 respectively; Reijnders et al., 1974) are necessary for virus multiplication (Bruening, 1977; Van Kammen, 1968). We have shown previously that B component RNA is able to replicate independently (i.e. in the absence of M component), a property not associated with M component RNA (Goldbach et al., 1980). Direct studies on the expression of B component RNA in vivo have therefore been possible (Rezelman et al., 1980; Goldbach et al., 1982).

CPMV infection is accompanied by the appearance of characteristic cytopathic structures (Assink et al., 1973; De Zoeten et al., 1974). These structures consist of amorphous electron-dense material and a large number of vesicles. We have now investigated whether the B component alone is able to induce these cytopathic structures in protoplasts. Furthermore, as the replication and expression of B component RNA has mainly been studied in isolated mesophyll protoplasts, we have now followed its fate in the intact host. We have studied whether B RNA is capable of spreading to surrounding cells in the absence of M RNA. This property has been reported for the large RNA (RNA-1) of another two-component virus, tobacco rattle virus (TRV) (Sänger & Brandenburg, 1961; Cadman, 1962; Lister, 1968, 1969).

The results presented in this paper demonstrate that the independence of the B component is limited.

METHODS

Virus purification and separation of B and M components. CPMV was propagated in
cowpea plants (*Vigna unguiculata* L. 'California Blackeye') and purified as described previously (Van Kammen, 1967). B and M components were separated in a linear 15 to 30% sucrose gradient by zonal centrifugation (Beckman Ti 15 rotor, 16 h, 23,000 rev/min at 10 °C). This procedure was repeated twice to give an M component free of B, and a B component contaminated with less than 0.2% of M, as determined by the local lesion infectivity test (De Jager, 1976).

**Protoplast isolation and inoculation.** Cowpea mesophyll protoplasts were isolated and inoculated with CPMV components [5 µg of M components, 5 µg of B components, or 10 µg of a 1:1 (w/w) mixture of both components per 5 × 10⁵ protoplasts per ml inoculum] or left untreated as described in detail previously (Hibi *et al.*, 1975; Rottier *et al.*, 1979). Inoculated protoplasts were incubated in culture medium at 25 °C as described by Rottier *et al.* (1979). In some experiments protoplasts were reinoculated with CPMV components 24 h after their preparation. For that purpose, protoplasts incubated for 23 h in culture medium were sedimented and resuspended in a solution of 0.5% (w/v) cellulase in 0.6 M-mannitol pH 5.6. After 1 h incubation at 25 °C the protoplasts were washed twice with 0.6 M-mannitol and resuspended in 0.6 M-mannitol, 0.01 M-potassium citrate pH 5.2, containing 0.5 or 1.0 µg poly-L-ornithine (PLO) (Pilot Chemicals, New England Nuclear) per ml. Concurrently, a solution of unFractionated virus (5 µg/ml) or purified M component (2.5 µg/ml) was made in the same buffer (also containing 0.5 or 1.0 µg/ml PLO). Both the virus solution and the protoplast suspension were kept for 5 min at room temperature. The protoplasts were then sedimented and resuspended in the virus solution. After 15 min the protoplasts were washed three times in 0.6 M-mannitol containing 10 mM-CaCl₂, and were finally resuspended and incubated in culture medium.

**Electron microscopy.** Samples of protoplasts were collected by centrifugation (2 min, 600 g) 24 h or 40 h after inoculation and incubated in 2% glutaraldehyde, 0.1 M-sodium phosphate pH 7, 0.6 M-mannitol for 1 h at 4 °C. After five successive washes with 0.1 M-sodium phosphate pH 7, they were fixed in 1% osmium tetroxide in 0.1 M-sodium phosphate pH 7 for 1 h at 4 °C, washed in double-distilled water and in 0.14 M-veronal acetate pH 5 (containing 0.577 g sodium barbiturate and 0.38 g sodium acetate per 100 ml) successively, and stained for 1 h in 2% uranyl acetate in 0.14 M-veronal acetate pH 5. Finally, the fixed protoplasts were washed in veronal acetate (0.14 M, pH 5), dehydrated in ethanol and acetone, and embedded in a prepolymerized mixture of methacrylate and divinyl benzene by the method of Kushida (1961). Polymerization took place in gelatin capsules at 50 °C for 48 h. Ultrathin sections made with an LKB Ultratome III ultramicrotome were stained with uranyl acetate and lead citrate by the method of Reynolds (1963), and examined in a Siemens Elmiskop 101 electron microscope, operated at an accelerating voltage of 80 kV.

**Assay for spreading of CPMV components in cowpea leaves.** Primary leaves of 8- or 9-day-old cowpea plants were inoculated with B component (5 µg in 100 µl 0.1 M-sodium phosphate pH 7 per leaf) or with a mixture of M + B components (5 µg of each in 100 µl of the same buffer). After 24 or 48 h protoplasts were isolated from the inoculated leaves. Protoplasts prepared from B-inoculated leaves were divided into three aliquots at a concentration of 5 × 10⁵ protoplasts per ml. One portion was inoculated with M component (5 µg per 5 × 10⁵ protoplasts), and, to verify whether the protoplasts were infectable with CPMV, a second portion was inoculated with a mixture of B and M components (5 µg of each per 5 × 10⁵ protoplasts). A third portion was left untreated. Protoplasts isolated from M + B-infected leaves were left untreated to measure the spreading of the complete virus. To determine the percentage of cells containing virus particles, samples of protoplasts were stained with fluorescent antibodies against CPMV (Hibi *et al.*, 1975) 0, 25 and 42 h after inoculation.
Independence of CPMV B RNA

Fig. 1. Electron micrographs of cowpea mesophyll protoplasts: (a) uninoculated; (b) inoculated with B component; (c) inoculated with a 1:1 (w/w) mixture of B + M components. Portions of protoplasts were mounted for electron microscopy 24 h after inoculation. Arrows indicate the characteristic cytopathological structures (i.e. electron-dense material); N, nucleus. Bar marker represents 1 μm.

RESULTS

Electron microscopy

Cowpea mesophyll protoplasts, inoculated with B, M, or B + M components, were analysed by electron microscopy 24 h and 40 h after inoculation. Cytopathic structures were
found in protoplasts inoculated with complete virus and in protoplasts inoculated with only B component, but not in protoplasts inoculated with M component (Fig. 1, 2). The morphology of the cytopathic structures in the inoculated protoplasts changed during the time after inoculation. Twenty-four h after inoculation (Fig. 1) these structures contained amorphous
Independence of CPMV B RNA

Table 1. Induction of cytopathic structures in cowpea protoplasts by components of CPMV

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>c.p.s.*</th>
<th>CPMV particles†</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>71</td>
<td>4</td>
</tr>
<tr>
<td>M</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B + M</td>
<td>68</td>
<td>67</td>
</tr>
</tbody>
</table>

* c.p.s., Cytopathic structures.
† As determined by staining with fluorescent anti-CPMV serum.

electron-dense material clustered within membrane structures. Forty h after inoculation (Fig. 2) the amount of electron-dense material diminished, but the number of vesicles surrounding the remaining electron-dense material increased. Such structures were absent in both healthy protoplasts (Fig. 1a, 2a) and in M component-inoculated protoplasts (data not shown). The results indicate that B component alone is capable of inducing the cytological alterations typical of CPMV infection. The data presented in Table 1 show that in the case of inoculation with B + M particles the percentage of protoplasts containing cytopathic structures (68%) and the percentage of infected protoplasts as determined by fluorescence (67%) were in good agreement. This correlation strongly suggests that all virus-containing cells contain cytopathic structures. In the case of B-inoculated protoplasts, the percentage of cells containing cytopathic structures was as high (71%) as for the B + M-inoculated cells, but the percentage of fluorescent cells was only 4%. Production of capsid proteins by these cells must have resulted from minor (0.2% or less) contamination of the B component preparation with M components. Therefore, it appears that B component alone is capable of inducing cytopathic structures in protoplasts with the same efficiency as the complete virus.

Is B RNA capable of spreading to surrounding cells?

In protoplasts inoculated with B component alone, B RNA is replicated and expressed to the same extent as it is in protoplasts inoculated with B + M components (Goldbach et al., 1980; Rezelman et al., 1980). To investigate whether B RNA is also able to act independently from M RNA in the host plant, we have followed the fate of this RNA upon infection of primary cowpea leaves with B component. Since transfer of B RNA from cell to cell is necessary for detectable expression of B component RNA in leaves, we have determined the number of leaf cells containing B RNA upon inoculation with B component. For that purpose, protoplasts from B-inoculated leaves were prepared at various times after inoculation, and these protoplasts were subsequently inoculated with excess M particles. The rationale was that B RNA-containing cells which become inoculated with M particles should begin production of B and M particles, which can easily be detected by staining these cells with fluorescent antibodies against CPMV. Table 2 shows that the complete virus (M + B) rapidly spreads throughout the leaf, reaching up to 40% of the cells within 48 h. In contrast, the number of fluorescent protoplasts from B-inoculated leaves hardly increased (from 1 to 4%, Table 2 and Fig. 3) within the first 48 h after inoculation of leaves. Since addition of excess M particles to these cells did not further increase the number of fluorescent cells (Table 2 and Fig. 3) we conclude that the number of cells containing only B component RNA was negligible. The reliability of the assay used in this experiment was verified by inoculating one portion of protoplasts from B-inoculated leaves with a 1:1 (w/w) mixture of B + M components. Approximately 40 to 50% (Table 2) of these protoplasts produced virus particles, demonstrating that they were accessible to infection by CPMV particles.
Fig. 3. Transport of CPMV components throughout the cowpea leaf. Leaves were inoculated with a 1:1 (w/w) mixture of B + M components, or with B component alone. Protoplasts were prepared from these leaves at the times indicated. ■, B + M-inoculated leaves; , B-inoculated leaves. One portion of protoplasts prepared from B-inoculated leaves was inoculated with excess M component (■). After 42 h incubation of the protoplasts, percentages of infected cells were determined by staining with fluorescent anti-CPMV serum.

Table 2. Spreading of CPMV components throughout the cowpea leaf

<table>
<thead>
<tr>
<th>Leaf inoculum</th>
<th>Preparation of protoplasts (h post-inoculation)*</th>
<th>Protoplast inoculum</th>
<th>CPMV-infected cells (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>24</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B + M</td>
<td>48.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.4</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B + M</td>
<td>41.7</td>
</tr>
<tr>
<td>B</td>
<td>48</td>
<td>–</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B + M</td>
<td>41.7</td>
</tr>
<tr>
<td>B + M</td>
<td>2</td>
<td>–</td>
<td>5.5</td>
</tr>
<tr>
<td>B + M</td>
<td>24</td>
<td>–</td>
<td>8.2</td>
</tr>
<tr>
<td>B + M</td>
<td>48</td>
<td>–</td>
<td>35.5</td>
</tr>
</tbody>
</table>

* Protoplasts were isolated from leaves at the times indicated.
† As determined by staining with fluorescent anti-CPMV serum.
‡ Time of incubation of protoplasts.

Table 3. Reinoculation of cowpea protoplasts with CPMV components

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Reinoculum†</th>
<th>PLO (μg/ml)‡</th>
<th>CPMV-infected cells (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>B + M</td>
<td>–</td>
<td>35</td>
</tr>
<tr>
<td>–</td>
<td>B + M</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td>–</td>
<td>B + M</td>
<td>1.0</td>
<td>35</td>
</tr>
<tr>
<td>B</td>
<td>–</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>B + M</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>B + M</td>
<td>1.0</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>M</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>M</td>
<td>1.0</td>
<td>20</td>
</tr>
</tbody>
</table>

* Inoculated immediately after isolation of protoplasts.
† Reinoculated 24 h after isolation of protoplasts.
‡ Concentration of poly-L-ornithine used for reinoculation.
§ As determined by staining with fluorescent anti-CPMV serum 67 h after isolation of protoplasts.
Independence of CPMV B RNA

To exclude the possibility that, in protoplasts isolated from B-inoculated leaves, B RNA was not able to support the replication and expression of newly entered M RNA, the following control experiment was performed. Protoplasts inoculated with B component were first incubated for 23 h under standard conditions. They were then treated with cellulase (to remove the newly formed cell walls) and reinoculated with M component. Forty-four h later (i.e. 67 h after the first inoculation) they were stained with fluorescent anti-CPMV to determine the number of CPMV-producing cells. Whereas PLO is not necessary for efficient infection of freshly prepared protoplasts (Hibi et al., 1975; Rottier et al., 1979), its presence appeared to be a prerequisite for infection of protoplasts aged for 24 h. In the absence of PLO, only 1.5% of such protoplasts were infected upon inoculation with CPMV (B + M), whereas in the presence of PLO (0.5 or 1.0 µg/ml) this percentage was approx. 30 to 35% (Table 3). Using 1.0 µg/ml PLO, reinoculation of protoplasts with M component, 24 h after they had been inoculated with B component, resulted in 20% CPMV-producing cells, compared to only 6% if these protoplasts had not been inoculated with M component (Table 3). As the same percentage (20%) was reached after reinoculation with complete virus (Table 3) this result shows that at least in a major part of cells, which have been first inoculated with B component and 24 h later with M component, B RNA can support the replication and expression of M RNA. The low number of protoplasts from B-inoculated leaves which fluoresce after inoculation with M component (Table 2) therefore reflects the inability of B RNA to spread independently to surrounding cells.

DISCUSSION

It has been previously shown that the B component RNA of CPMV is capable of self replication (Goldbach et al., 1980) whereas M RNA is not. In studies on the expression of B RNA in mesophyll protoplasts, at least seven B RNA-coded proteins with sizes of 170K, 110K, 87K, 84K, 60K, 32K and 4K could be detected (Rezelman et al., 1980; Stanley et al., 1980). Although functions could not be assigned to any of these polypeptides, at least one of them should be involved in virus RNA replication (Goldbach et al., 1980) and another should represent the protease responsible for the in vitro cleavage of the M RNA-coded primary translation products (Pelham, 1979; Goldbach et al., 1981; Franssen et al., 1982). As it has recently been shown that M RNA encodes both capsid proteins (Franssen et al., 1982), B RNA should exist as an unencapsidated molecule in B component-inoculated protoplasts. In this paper we have shown that this unencapsidated B RNA and its products, rather than the accumulation of virus particles, are responsible for the induction of the characteristic cytopathic structures in CPMV-infected cells. Our results are consistent with the finding of Hibi et al. (1975) that RNA replication is associated with the appearance of the cytopathic structures. Furthermore, the electron microscopic analyses presented here show that 24 h after infection these structures mainly consist of electron-dense material as described previously (Assink et al., 1973; Hibi et al., 1975) but that with advancing time (i.e. 40 h after infection) the infected cell develops a large number of vesicles. Although B RNA appears to replicate independently in isolated protoplasts, our results demonstrate that non-packaged B RNA is unable to spread throughout leaves (Table 2 and Fig. 3). This indicates that M RNA encodes one or more proteins essential for the transport of the virus RNA molecules. In this context, it is worth mentioning that apart from the capsid proteins, two other polypeptides (58K and 48K) are translated from M RNA in vitro (Franssen et al., 1982). For TRV, a rod-shaped two-component virus, it has been shown (Lister, 1968, 1969) that the RNA from the long particle (which does not carry the information for the virus coat protein) can replicate by itself and this replication is accompanied by spreading of the virus RNA through the infected plant and by the production of symptoms. Although B RNA of CPMV is unable to cause local lesions in cowpea leaves, this does not mean a priori that B RNA is incapable
of spreading as expression of B RNA in the leaf may not have been associated with visible symptoms. We have now demonstrated that the lack of symptom development is related to the inability of B RNA to spread. The question as to whether encapsidation is solely essential for transport of CPMV RNAs or whether another M RNA-encoded function is involved in this process needs further investigation.

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REFERENCES


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