Cowpea Mosaic Virus Infection of Protoplasts from Samsun Tobacco Leaves

By R. HUBER, G. REZELMAN, T. HIBI* AND A. VAN KAMMEN

Department of Molecular Biology, Agricultural University, Wageningen, The Netherlands

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SUMMARY

Palisade parenchyma protoplasts isolated from the tobacco varieties Samsun and Samsun NN were inoculated in vitro with cowpea mosaic virus (CPMV). By fluorescent antibody staining it was demonstrated that about 75% of the protoplasts became infected. Poly-l-ornithine was required for infection. The cytopathic structures shown previously to be characteristic for CPMV-infected cowpea cells were also produced in CPMV-infected tobacco protoplasts. The optimum inoculum concentration for CPMV was 4 to 20 μg/ml. Compared with CPMV multiplication in cowpea protoplasts and tobacco mosaic virus multiplication in tobacco protoplasts, the growth of CPMV in tobacco protoplasts had a longer lag period but the virus concentrations in all three kinds of infection finally reached similar values. The difficulty with which tobacco leaves are infected with CPMV contrasts with the ease of infection of tobacco mesophyll protoplasts.

INTRODUCTION

Recently Hibi, Rezelman & Van Kammen (1975) reported the isolation of mesophyll protoplasts from the primary leaves of cowpea and the infection of these protoplasts with cowpea mosaic virus (CPMV). It appeared interesting to compare the infection of protoplasts from two different plant families with the same virus. Therefore, we have studied the infection of tobacco mesophyll protoplasts (Takebe & Otsuki, 1969) with CPMV. The infection of tobacco protoplasts with CPMV is furthermore significant because the Samsun tobacco varieties used for the isolation of leaf cell protoplasts were not known to be hosts for the strain of CPMV used to infect the protoplasts (Swaans & Van Kammen, 1973).

METHODS

Culture of tobacco plants. Nicotiana tabacum L. cv. Samsun or cv. Samsun NN were grown in a greenhouse for 6 weeks after sowing under artificial light: 14 h light of 6000 to 7000 lux from Philips HPLN mercury vapour lamps; 10 h darkness, 22 to 28°C and 60 to 80% humidity. The soil used was Trio 17S (Triomf, Vroomshoop, Netherlands) consisting of a mixture of compost, leaf mould, farmyard manure, peat dust, sand and lime. Seedlings were transplanted 2 weeks after sowing, and 2 weeks later the young plants were transferred to 10 cm diam. pots. After 6 weeks the plants were transferred to 20 cm diam. pots, put in containers with moist peat dust, placed in a phytotron and grown given 14 h light, at 25°C

* Present address : Plant Virus Research Institute, Chiba, Japan.
and 80 to 90% relative humidity. The Samsun plants were grown under HPLN lamps at 5000 to 7000 lux. In contrast, Samsun NN plants had to be grown under Philips SON lamps at 4500 to 5500 lux to obtain plants suitable for the isolation of protoplasts. The specified light intensities were measured at the top of the plants. The light was passed through a 5 mm Perspex plate which removed wavelengths below 380 nm. Plants grown in these conditions yielded protoplast preparations which could be reproducibly infected throughout the year. When the tobacco plants were 60 to 70 days old, the leaf which had just fully expanded was suitable for the isolation of protoplasts. Up to 1 week later another leaf could be harvested from the same plant.

**Preparation of protoplasts.** Palisade parenchyma protoplasts were isolated from the tobacco leaves essentially by the two step procedure (Takebe, Otsuki & Aoki, 1968; Takebe & Otsuki, 1969). The chemicals used were: Macerozyme R-10 (Yakult Biochemicals Co. Ltd., Japan), potassium dextran sulphate, mol. wt. approx. 2800 (Meito Sangyo Co., Japan), Cellulase Onozuka R-10 (Yakult Biochemicals Co. Ltd., Japan). For cellulase treatment the cells were suspended in 2% cellulase and 0.7 M-mannitol at pH 5.6, and incubated for 90 min at 30°C in a shaking water bath at a frequency of 70 excursions/min. The protoplasts after the cellulase treatment were washed four times by sedimenting at 100 g for 1 min and resuspending in 0.7 M-mannitol. The concentration of protoplasts was determined using a Fuchs–Rosenthal haemocytometer. The yield of palisade protoplasts was in the range of 2 to 6 × 10⁶ protoplasts/leaf and the percentage of living protoplasts was 80 to 90%.

**Virus.** An isolate of the yellow strain of CPMV was propagated in cowpea (*Vigna unguiculata* (L.) Walp. var. ‘Blackeye Early Ramshorn’) and purified as described by Van Kammen (1967). Only freshly purified virus was used for inoculation of protoplasts.

**Infection of tobacco protoplasts with CPMV.** A virus suspension with a concentration of 10 μg/ml in 0.7 M-mannitol, 0.01 M-potassium citrate at pH 5.2, containing 0.5 μg/ml poly-L-ornithine (Pilot Chemicals Inc., Boston, Massachusetts; mol. wt. approx. 130000) was pre-incubated for 5 min at 25°C. In the meantime, protoplasts were sedimented at 100 g for 2 min. After removing the supernatant fluid the sedimented protoplasts were directly suspended in the pre-incubated virus inoculum. The concentration of protoplasts in the infection mixture was 1 to 3 × 10⁶ protoplasts/ml. The infection mixture was kept for 15 min at 25°C with occasional gentle swirling. Thereafter the protoplasts were washed three times with sterile 0.7 M-mannitol, 10 mM-CaCl₂ solution. The washed inoculated protoplasts were resuspended and incubated in sterile culture medium. The culture medium was the same as that used by Aoki & Takebe (1969) except that the mannitol concentration was 0.7 M instead of 0.8 M and 6-benzyladenine was omitted. The concentration of protoplasts was 1 to 3 × 10⁶/ml. Incubation was in 10 ml portions in 100 ml Erlenmeyer flasks at 25°C under continuous illumination (approx. 2200 lux) from fluorescent tubes.

**Fluorescent antibody staining of infected protoplasts.** Samples of infected protoplasts were stained with fluorescent antibody to CPMV (Hibi *et al.* 1975) according to the staining procedure described for TMV-infected protoplasts (Otsuki & Takebe, 1969), except that 96% ethanol was used for fixation, and examination was with a Wild fluorescence microscope.

**Infectivity assay of protoplast extracts.** After appropriate periods of incubation, the protoplasts from a 10 ml sample were collected by sedimentation, washed once with 0.7 M-mannitol containing 10 mm-CaCl₂, and finally suspended in 0.1 M-phosphate buffer, pH 7.0. The suspension of protoplasts was homogenized in an all-glass microsize Potter–Elvehjem tissue grinder and the homogenate was clarified by centrifuging at 15000 g for 15 min. The
Infection of tobacco protoplasts with CPMV

Table 1. Frequency of infection of Samsun and Samsun NN tobacco mesophyll protoplasts with CPMV and TMV

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Inoculum concentration/ml</th>
<th>% infected protoplasts*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5µg CPMV + 0.75µg poly-L-ornithine</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>1µg TMV + 0.75µg poly-L-ornithine</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>5µg CPMV + 1µg poly-L-ornithine</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>1µg TMV + 1µg poly-L-ornithine</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>10µg CPMV + 0.5µg poly-L-ornithine</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>1µg TMV + 1µg poly-L-ornithine</td>
<td>46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Inoculum concentration/ml</th>
<th>% infected protoplasts*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10µg CPMV + 0.5µg poly-L-ornithine</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1µg TMV + 1µg poly-L-ornithine</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>8µg CPMV + 0.5µg poly-L-ornithine</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>1µg TMV + 1µg poly-L-ornithine</td>
<td>95</td>
</tr>
</tbody>
</table>

* Percentage infected protoplasts was determined by staining with fluorescent antibodies against CPMV and TMV after 36 to 42 h incubation.

Infectivity of the supernatant was tested by local lesion assay on six half leaves of Phaseolus vulgaris L. cv. Pinto as described by Hibi et al. (1975).

Electron microscopy. Samples of infected and healthy protoplasts were prepared and stained for electron microscopy as described by Hibi et al. (1975).

RESULTS

Infection of tobacco protoplasts

When Samsun or Samsun NN tobacco protoplasts were inoculated with 5 to 10µg/ml CPMV, usually more than 50% of the protoplasts became infected as determined by staining with fluorescent CPMV antibody after 36 to 42 h incubation in the culture medium. For comparison, separate samples of the protoplast preparations were inoculated with 1µg/ml TMV. It appeared that the frequency of infection obtained with CPMV and TMV was similar in all cases (Table 1), indicating that the tobacco protoplasts were equally susceptible to infection by both viruses. The high frequency of infection of tobacco protoplasts with CPMV was rather surprising as Swaans & Van Kammen (1973) showed that intact leaves of Samsun and Samsun NN tobacco do not develop symptoms after inoculation with CPMV. Whereas poly-L-ornithine is not needed for the infection of cowpea protoplasts with CPMV, it was an absolute requirement for the infection of tobacco protoplasts with CPMV. Best results were obtained with 0.5 to 1µg/ml poly-L-ornithine in the inoculum. Higher concentrations decreased the percentage of living protoplasts and the percentage of infected protoplasts. Therefore 0.5µg/ml poly-L-ornithine was used in all further experiments.
There was no sharp pH optimum for infecting tobacco protoplasts with CPMV. At inoculum pHs lower than 5.0 or higher than 5.4 variable results were obtained with respect to the percentage of living protoplasts and the amount of infectious virus extractable after incubation. However, no consistent variation of the frequency of infection was observed, showing that the pH is important for the stability rather than for infectibility of protoplasts.

The effect of virus concentration in the inoculum on the percentage of Samsun NN tobacco protoplasts infected with CPMV is shown in Fig. 1. Similar results were obtained with protoplasts from Samsun tobacco leaves. The percentage of infected protoplasts increased with the concentration of CPMV in the inoculum up to 4 μg/ml and then remained at the same level with higher inoculum concentrations. A similar concentration of CPMV in the inoculum has been found necessary to obtain the maximum percentage of infected cowpea protoplasts (Hibi et al. 1975). As the concentration of cowpea and tobacco protoplasts in the inoculum mixture was the same, these results indicate that there is no difference between the two species in the CPMV concentration needed to achieve infection.

The virus antigen observed in the infected tobacco protoplasts by antibody staining occurred in amorphous fluorescent masses which were very similar in appearance to the CPMV antigen masses seen in infected cowpea protoplasts.

Previously it was shown that cytopathic structures consisting of vesicular membranes embedded in electron dense material are characteristic of CPMV-infected cells (De Zoeten, Assink & Van Kammen, 1974; Hibi et al. 1975). Fig. 2 shows that such cytopathic structures are also found in CPMV-infected tobacco protoplasts. This suggests that the structural changes induced in CPMV-infected cells are mainly directed by the virus.

A growth curve of CPMV in Samsun NN tobacco protoplasts is shown in Fig. 3. The amount of virus produced was determined by local lesion assay of extracts of infected protoplasts on Pinto bean leaves. Simultaneously, the percentage of protoplasts that stained with fluorescent antibody after different times of incubation was determined. It was conspicuous that the percentage of tobacco protoplasts stainable with fluorescent antibody and
Infection of tobacco protoplasts with CPMV

Fig. 2. Cytopathic structure found in a Samsun NN tobacco mesophyll protoplast infected with CPMV 36 h after inoculation. The characteristic features of the structure are the many vesicles (Ve) surrounded by a large amount of amorphous electron dense material (A).

Infectivity both began to increase later with CPMV in tobacco protoplasts than with CPMV in cowpea protoplasts or TMV in tobacco protoplasts (Fig. 4). In the last two cases the titre of virus extractable from infected protoplasts started to increase between 10 to 12 h after inoculation when incubation took place under the conditions described in Methods, whereas the CPMV content of infected tobacco protoplasts started to increase 20 h after inoculation. The amount of CPMV produced per infected protoplast was estimated by comparing the infectivity of extracts of samples of infected protoplasts at various times of incubation with that of purified virus (0.5 μg CPMV/ml). The final percentage of infected protoplasts as determined by fluorescent antibody staining (74% in Fig. 3) was used to calculate the amount of CPMV produced at various times after inoculation. Such estimates are necessarily crude as it is assumed that the specific infectivity of the virus in the protoplast extract and in the purified virus sample used as a control, is the same. They make possible, however, the comparison of the average yield of progeny virus per infected tobacco or cowpea protoplast. The final amount of CPMV produced per infected tobacco protoplast was estimated to be about $10^{-5}$ to $10^{-4}$ μg (approx. $7 \times 10^{-5}$ μg/protoplast in Fig. 3), which is similar to the amount of CPMV synthesized in infected cowpea protoplasts (Hibi et al. 1975) and that of TMV produced in tobacco protoplasts (Fig. 4).

Infection of tobacco plants

Swaans & Van Kammen (1973) suggested that Samsun and Samsun NN tobacco are not susceptible to CPMV because no symptoms developed in the inoculated plants. The infection of leaves of these tobacco varieties with CPMV was therefore re-investigated. Fully
Fig. 3. CPMV multiplication in Samsun NN tobacco protoplasts. •—•, % protoplasts staining with fluorescent antibody; •---•, virus concentration determined by measuring the infectivity of protoplast extracts. The amount of CPMV in the protoplasts was calculated from the infectivity of the extracts and the final percentage of infected protoplasts.

Fig. 4. Comparison of the multiplication of TMV (▲—▲) and CPMV (●—●) in Samsun NN tobacco protoplasts.

expanded leaves from 21 to 28-day-old plants of both tobacco varieties were inoculated with 1 mg CPMV/ml. A very high inoculum concentration was used to ensure maximum infection. Samples of the leaves 1, 4 and 8 days after inoculation were homogenized in 0.1 M-phosphate buffer pH 7.0 (1 ml/g leaf tissue) and the clarified sap assayed for infectivity by local lesion assay on six half leaves of Pinto beans. No virus infectivity was found 1 day
Infection of tobacco protoplasts with CPMV

after inoculation, indicating that no detectable amounts of inoculum virus survived. But on days 4 and 8 after inoculation lesions were produced by the homogenates of inoculated leaves. For example, the number of lesions in six half leaves of Pinto beans was 122 for day 4 and 12 for day 8, whereas CPMV at 0.2 μg/ml produced respectively 27 and 17 lesions on the control halves. The extractable infectivity decreased with longer periods after inoculation. No systemic infection occurred, and inoculated plants did not develop symptoms of disease. Thus CPMV apparently multiplied in the inoculated leaves, but only to a limited extent. Similar amounts of infectivity were produced in inoculated leaves of 60-day-old plants, as used for protoplast isolation. To examine the extent of CPMV-infection further leaves suitable for the isolation of protoplasts were inoculated with 10 mg CPMV/ml and 4 days later protoplasts were isolated. Five to 10% of the protoplasts showed bright fluorescence on staining with fluorescent antibody. These results show that at very high inoculum concentrations cells within leaves become infected with CPMV, but infection does not spread to all cells and virus multiplication is therefore limited. The effect of poly-l-ornithine on the CPMV infection of tobacco leaves was not examined.

DISCUSSION

The infection of tobacco mesophyll protoplasts by several plant viruses has been reported (TMV by Takebe & Otsuki, 1969; cucumber mosaic virus by Otsuki & Takebe, 1973; cowpea chlorotic mottle virus by Motoyoshi et al. 1973; potato virus X by Otsuki et al. 1974; pea enation mosaic virus by Motoyoshi & Hull, 1974; a variant of brome mosaic virus by Motoyoshi, Bancroft & Watts, 1974; tobacco rattle virus by Kubo et al. 1975, 1976; alfalfa mosaic virus by Motoyoshi, Hull & Flack, 1975; and cucumber green mottle mosaic virus by Sugimura & Ushiyama, 1975). Our study was aimed at the comparison of CPMV infection of tobacco protoplasts with that of cowpea protoplasts (Hibi et al. 1975).

Samsun and Samsun NN tobacco were not known to be hosts for CPMV. Plants inoculated with CPMV do not produce symptoms of disease, and the virus does not spread systematically through the plants (Swaans & Van Kammen, 1973). In the present study we have shown that detectable, though limited, infection of the two tobacco varieties with CPMV can be brought about when very high inoculum concentrations are used. High percentages of mesophyll protoplasts isolated from Samsun and Samsun NN leaves could, however, be readily infected, but whereas CPMV infection of tobacco protoplasts requires poly-l-ornithine in the inoculum, that of cowpea protoplasts does not (Hibi et al. 1975). This shows that not only the charge properties of the infecting virus (Motoyoshi et al. 1973) are of importance for infection to occur, but also the properties of the plasma membrane of the protoplasts to be infected.

CPMV-infected tobacco protoplasts produced intracellular cytopathic structures similar to those found in CPMV-infected cowpea leaves or protoplasts (De Zoeten et al. 1974; Hibi et al. 1975). Evidently, the origin of these structures and their characteristics are determined by CPMV and very little, if at all, by the host cell.

In comparison with TMV multiplication in tobacco protoplasts or CPMV multiplication in cowpea protoplasts, CPMV multiplication in tobacco protoplasts showed a much longer lag period. The cause underlying this extended lag period is unknown. One might speculate that the longer lag period is due to a smaller number of virus particles entering the protoplasts so that it takes longer for virus multiplication to become detectable. In so far as the number of particles entering the protoplasts depends on the virus concentration in the inoculum, we have not noticed a difference between the effect of the CPMV concentration.
in the inoculum on the frequency of CPMV infection of tobacco protoplasts (Fig. 1) and cowpea protoplasts (Hibi et al. 1975). However, we have not examined the possible relation between virus concentration in the inoculum and lag period of the virus growth curve.

Another possibility might be that CPMV takes longer to start multiplying in tobacco protoplasts because an early step in the infection process, e.g. uncoating of the infecting virus particles (which sets free the virus RNA), or formation of the virus RNA replication complex (Zabel, Weenen-Swaans & Van Kammen, 1974), takes more time than in cowpea protoplasts.

The slow start of CPMV multiplication in tobacco protoplasts mimics the situation on infection of tobacco leaves with CPMV. In the case of tobacco leaves a very high CPMV inoculum concentration is necessary for virus infection. The inoculum concentration of 1 or 10 mg/ml which was used would be sufficient to demonstrate virus multiplication within 24 h in TMV-infected tobacco leaves or CPMV-infected cowpea leaves. With CPMV-inoculated tobacco leaves, only 5 to 10% of the palisade parenchyma protoplasts were found to be infected when isolated 4 days after inoculation. This indicates that virus has spread somewhat from the initially infected leaf cells, but this spread appears to be a rather slow process. At periods longer than 4 days after inoculation extractable infectivity did not increase further and in some cases decreased. Apparently, the spread and multiplication of CPMV is so slow that inactivation of virus outstrips the spread of infection. This might be due to either the large numbers of virus particles possibly necessary to infect neighbouring cells, or to the long time needed to start the virus multiplication process in each cell. It appears worth while to investigate the underlying cause of the extended lag period for CPMV in tobacco protoplasts, since it might help to elucidate the nature of virus specificity and host sensitivity in the early stages of infection.

The fact that CPMV infects high percentages of tobacco protoplasts and of cowpea protoplasts, and that similar amounts of virus particles are produced in protoplasts of both types suggests the two systems may be useful in work on identification of virus-specific proteins synthesized during infection. To distinguish the virus-coded proteins from host proteins, particularly those host proteins of which the synthesis is stimulated by virus infection, the availability of two different cell systems, which may become infected equally well, seems to be very helpful.

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REFERENCES
Infection of tobacco protoplasts with CPMV


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