Interference in Infections of Tobacco Protoplasts with Two Bromoviruses

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SUMMARY

Freshly prepared tobacco mesophyll protoplasts behave as if they contain two classes, one of which is resistant to infection but the other is susceptible and can readily be doubly infected with brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV). BMV dominates in all double infections and although both types of virus particles are produced, only those of BMV are infectious. The defective CCMV particles contain normal amounts of RNA but only RNA 3 could be detected in them. No CCMV RNA 2 could be detected in doubly infected protoplasts, showing that there was only partial replication of the genome of CCMV. The proteins encoded by CCMV RNA 3 were produced in mixed infections. It is possible that BMV may exploit the gene products of CCMV while suppressing synthesis of infectious CCMV particles. There was no evidence that BMV RNA was encapsidated in CCMV coat protein.

INTRODUCTION

Isolated plant protoplasts are well suited for the study of mixed virus infections (Dawson et al., 1975; Otsuki & Takebe, 1976, 1978; Barker & Harrison, 1978; Watts & Dawson, 1980). They can be efficiently infected with two or more viruses and virus development is synchronous. In a previous paper (Watts & Dawson, 1980) we described experiments with the two related bromoviruses brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) in tobacco protoplasts. Mixed infections were readily produced and interference between the two viruses was observed. When both viruses developed in a protoplast, BMV always dominated and there was little if any synthesis of infectious CCMV, although fluorescent antibody staining, specific for intact virus (Bancroft, 1970), showed that CCMV capsids were present. Attempts to rescue a temperature-sensitive (ts) strain of CCMV with BMV were unsuccessful and it was found that BMV suppressed the synthesis of infectious CCMV RNA. We report here further studies of interactions of CCMV and BMV in mixed infections designed to clarify these results.

METHODS

Protoplast preparation and infection

Protoplasts were prepared from leaves of Nicotiana tabacum (cv. White Burley or Xanthi) and cultured by the methods used previously (Motoyoshi et al., 1974a, b). Type strain CCMV and V5 strain BMV were used throughout. Protoplasts were doubly infected with viruses by one of the following methods.

Consecutive inoculation. Freshly pelleted protoplasts (2 \times 10^6) were resuspended in 20 ml citrate-buffered mannitol (CMB, 0.01 M-potassium citrate in 0.7 M-mannitol, pH 5.2) containing poly-L-ornithine (tool. wt. 120000; 1 \mu g/ml) and either CCMV (1 \mu g/ml) or BMV (5 \mu g/ml). The inoculum was preincubated for 10 min before use. After 10 min the protoplasts were collected by centrifugation (600 rev/min for 2 min), washed with 0.7 M-mannitol, and resuspended in a similar volume of inoculum containing the other virus. After a further 10 min the protoplasts were washed twice with 0.7 M-mannitol and cultured.

Simultaneous inoculation. Freshly pelleted protoplasts (2 \times 10^6) were resuspended in 20 ml CMB containing poly-L-ornithine (1 \mu g/ml), CCMV (1 \mu g/ml) and BMV (5 \mu g/ml) for 10 min. The inoculum was preincubated for 10 min before use. The protoplasts were then washed twice with 0.7 M-mannitol and cultured.

Polyethylene glycol inoculation. Freshly pelleted protoplasts (2 \times 10^6) were resuspended in 1 ml 40% (w/v) polyethylene glycol 1500 (PEG) containing 25 \mu g CCMV, 20 \mu g BMV and 3 mM-CaCl_2 and immediately after
mixing diluted to 40 ml with 0.7 M-mannitol. After 30 min the protoplasts were washed three times with 0.7 M-mannitol and cultured (Dawson et al., 1978).

Culture and labelling of protoplasts. Protoplasts were cultured at a density of 1 \times 10^5 to 2 \times 10^5/ml in conical flasks. When radioactivity was present, 5 ml cultures in 25 ml flasks were used. ^14C-labelled compounds were added at 2 \muCi/ml, and ^3H-labelled compounds at 25 \muCi/ml. L-[U-^14C]leucine (300 mCi/mmol), L-[4,5-^3H]leucine (50 Ci/mmol), [U-^14C]uridine (500 mCi/mmol), and carrier-free [32p]orthophosphate were obtained from Amersham International.

Assay of infectivity. The percentage of infected protoplasts was determined, after at least 24 h of culture, by staining with fluorescent antibody; 300 protoplasts were scored (Motoyoshi et al., 1974 b). Biological infectivity was assayed by the methods of Watts & Dawson (1980).

Separation of viruses by electrophoresis. To separate particles of BMV from those of CCMV, infected protoplasts were homogenized in 0.1 M-acetate buffer (pH 5.0) and the clarified homogenate was fractionated by electrophoresis in cylindrical 2.5% polyacrylamide gels using the procedure of Hull & Lane (1973).

Extraction of RNA. RNA was prepared by a method similar to that described by Aoki & Takebe (1975) and fractionated by electrophoresis (Loening, 1969).

When the RNA in virus separated by gel electrophoresis was required, the relevant gel sections were homogenized in the buffer used to extract whole RNA (bands from six gels in 1 ml buffer). The homogenates were allowed to stand for 1 h at room temperature before being loaded onto polyacrylamide gels for electrophoresis.

Electrophoresis of proteins. Proteins were fractionated by the methods of Sakai et al. (1979).

RESULTS

Improving the efficiency of double infection

The susceptibility of protoplasts to infection depends on their physiological condition (Motoyoshi et al., 1974 b). As experience was gained with double infections it became apparent that even when the final level of infection was quite low, e.g. 25%, over 90% of these would be doubly infected. That is to say, provided there was a minimum of delay between inoculations, the protoplasts that became infected were doubly infected (Table 1). When there is a delay, even of minutes, between inoculations, there is always an appreciable risk that singly infected protoplasts will be present. The method of inoculation was therefore modified to reduce this possibility. BMV and CCMV have opposite electrical charges under the conditions used for inoculation (citrate-buffered mannitol, pH 5.2) and readily associate. Both viruses were therefore preincubated together in the presence of poly-L-ornithine to produce mixed aggregates, and the mixed inoculum was used for what was literally a simultaneous double inoculation. In this way, variations caused by destruction of protoplasts, inactivation or activation through washing etc. were reduced or eliminated. In practice these gave cultures in which all infected protoplasts were reliably doubly infected and simplified analysis of the results, even when the batch of protoplasts did not have a very high susceptibility to infection.

Attempts to recover infectious CCMV from mixed infections

When care was taken to ensure that virtually all infected protoplasts were doubly infected with BMV and CCMV, as measured by fluorescent antibody staining, infectivity assays on Chenopodium hybridum, barley and cowpea showed that there were large amounts of infectious BMV but no detectable infectious CCMV. This result was in agreement with the previous observations that a ts strain of CCMV could not be rescued with BMV at 35 °C because synthesis of infectious CCMV RNA had been suppressed in mixedly infected protoplasts (Watts & Dawson, 1980).

Production of CCMV particles in mixed infections

The absence of infectious CCMV from protoplasts that fluorescent antibody staining had shown to be infected with both CCMV and BMV suggested that only CCMV coat protein might be formed. Alternatively, the level of CCMV production might be below the limits of sensitivity of the biological assays; this is however unlikely because assay directly onto the systemic host cowpea was extremely sensitive. The differing charges on CCMV and BMV allow a simple physical separation of the viruses by polyacrylamide gel electrophoresis. Under the conditions
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Table 1. Mixed infection of tobacco protoplasts

<table>
<thead>
<tr>
<th>Method of inoculation*</th>
<th>Number of experiments</th>
<th>Fluorescing protoplasts (%)</th>
<th>Range of % total infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consecutive</td>
<td>7</td>
<td>Total 64 BMV 62 CCMV 63</td>
<td>47-85</td>
</tr>
<tr>
<td>Simultaneous</td>
<td>7</td>
<td>Total 74 BMV 74 CCMV 71</td>
<td>50-94</td>
</tr>
<tr>
<td>PEG</td>
<td>2</td>
<td>Total 66 BMV 63 CCMV 67</td>
<td>65-67</td>
</tr>
</tbody>
</table>

* For experimental details, see Methods.
† Error (standard deviation) for results with consecutive or simultaneous inoculation were ± 3%; for PEG inoculation ± 5%.

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used routinely for electrophoresis of the bromoviruses (Hull & Lane, 1973) BMV travelled towards the cathode and CCMV towards the anode. When homogenates of doubly infected protoplasts were fractionated on polyacrylamide gel, substantial amounts of material that co-migrated with BMV or CCMV were detected. These bands corresponding to the viruses were cut out and assayed on C. hybridum, barley and cowpea leaves. The band migrating with BMV behaved as BMV uncontaminated by CCMV but that co-migrating with CCMV possessed no infectivity, either of BMV or CCMV. Electrophoresis of the proteins in these bands in SDS-polyacrylamide gels (Weber & Osborn, 1969) showed that they corresponded to the respective coat proteins. There was therefore production of non-infectious CCMV particles and no transcapsidation either of CCMV RNA into BMV capsids or vice versa.

The presence of RNA in non-infectious CCMV particles

CCMV coat proteins spontaneously reassociate to form 'empty' capsids (Bancroft et al., 1968). The u.v. absorption spectrum of the particles separated on polyacrylamide gels showed, however, that they contained nucleic acid. The precise ratio of coat protein to nucleic acid was not easily determined directly because of the difficulties of preparing sufficient purified virus to allow analysis. Doubly infected protoplasts were therefore cultured in medium containing [3H]leucine and [14C]adenine to label proteins and RNA. The protoplasts were homogenized and the viruses separated on cylindrical polyacrylamide gels. The gels were cut into slices and the ratio of 3H : 14C determined in each slice. If the specific radioactivities of the pools of protein and RNA precursors are assumed to be the same for both viruses, then the ratio should give some indication as to whether the CCMV particles were rich or poor in RNA relative to BMV. The results (Fig. 1) showed that the ratios were similar for both infectious BMV and non-infectious CCMV. The samples indicated by the arrows contained in the case of BMV (Fig. 1 a) 905 ct/min in 3H (protein) and 291 ct/min in 14C (RNA) giving a ratio of 3H : 14C of 3-1; in the case of CCMV (Fig. 1 b) the corresponding figures were 429 and 188 ct/min, giving a ratio of 2-3. There was therefore no less RNA in the ‘CCMV’ than in BMV particles.

The nature of the RNA in non-infectious CCMV particles

Protoplasts that had been infected with both BMV and CCMV were cultured in medium containing [32P]orthophosphate, homogenized and the viruses separated on cylindrical polyacrylamide gels as before. The CCMV band was then subjected to electrophoresis on 2-5% gels in the presence of SDS and autoradiographs of the gels were prepared. The result is shown in Fig. 2. The autoradiograph of the total virus before fractionation showed bands characteristic of BMV RNA, i.e. RNAs 1 and 2 running close together and poorly resolved and RNA 3 running somewhat faster and well resolved from the other two; RNA 4 was obscured by labelled material of unknown origin, perhaps degradation products, and this section of the gel has been omitted. In contrast, the autoradiograph of the ‘CCMV’ showed one band corresponding to RNA 3; the amount of material recovered was too small to determine whether RNA 4 was also present.

Synthesis of RNA and protein in doubly infected protoplasts

Doubly infected protoplasts were cultured in medium containing [14C]uridine, the total (i.e. host and viral) RNA was prepared and separated on 2-5% polyacrylamide gels. An
Fig. 1. Relative amounts of protein and RNA in virus particles isolated from doubly infected protoplasts. Protoplasts doubly infected with CCMV and BMV were cultured for 24 h in medium containing [3H]leucine (10 μCi/ml) and [14C]adenine (2 μCi/ml). Virus particles were separated on cylindrical gels, the gels were sliced, and the amounts of 3H and 14C in BMV (a) and CCMV (b) were determined. Unlabelled carrier virus was added to the samples to assist location of the virus band. ▲, Counts in 14C (RNA); ●, counts in 3H (protein); ○, ratio 3H:14C. The arrows show the virus band.

It is possible that failure to detect CCMV RNA 2 was due to lack of sensitivity of the experimental procedures. The corresponding results for protein synthesis are therefore of some interest (Fig. 4). This is the same experiment illustrated in Fig. 3 but the protoplasts were cultured in medium containing [14C]leucine and the proteins were separated on SDS–polyacrylamide gels (Sakai et al., 1979) using a Tris–glycine running buffer. Under these conditions BMV and CCMV coat proteins and virus-specific proteins of mol. wt. 35 000 can be completely resolved. It can be seen that in the doubly infected protoplasts both coat proteins were present, although there was considerably more BMV coat protein than CCMV coat.
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Fig. 2. RNA isolated from CCMV particles in doubly infected protoplasts. Protoplasts infected with CCMV alone or BMV and CCMV together were cultured for 24 h in medium containing \( ^{32}P \) orthophosphate (10 \( \muCi/ml \)). Virus particles were separated by electrophoresis into 2.5% polyacrylamide gels. RNA was isolated from CCMV-like particles and run in 2.5% polyacrylamide gel. Traces of autoradiograms of (a) total virus and (b) CCMV from a mixed infection are shown. Numbers indicate RNA species 1 to 3.

Fig. 3. RNA synthesis in doubly infected protoplasts. Protoplasts were irradiated with u.v. to reduce host protein synthesis, inoculated with BMV, CCMV or both viruses, and cultured for 20 h in medium containing actinomycin D (10 \( \mug/ml \)) to suppress host RNA synthesis, and chloramphenicol (100 \( \mug/ml \)) to reduce host protein synthesis (Sakai et al., 1977), and \(^{14}C\)uridine (2 \( \muCi/ml \)) to label nucleic acids. Traces of autoradiograms of nucleic acids separated on 2.5% polyacrylamide gels are shown. (a) CCMV-infected; (b) BMV-infected; (c) doubly infected. Numbers indicate RNA species 1 to 4. Uninfected controls showed no incorporation of label.

protein. In contrast, this relation was reversed for the 35000 mol. wt. proteins; there was considerably more of the CCMV-coded than of the BMV-coded protein. No conclusion could be drawn about the proteins of mol. wt. about 10^5 other than that two at least were present, one of which must be coded by BMV (Sakai et al., 1977, 1979); the CCMV-coded protein co-migrated with the faster of the two BMV-coded proteins (mol. wt. 10^5).
Fig. 4. Protein synthesis in doubly infected protoplasts. The experiment was as described in Fig. 3 except that [14C]leucine (2 μCi/ml) was used to label proteins. Traces of autoradiograms of proteins separated on 9% polyacrylamide gels are shown. (a) Uninfected control; (b) BMV-infected; (c) CCMV-infected; (d) doubly infected. P1, Coat proteins; P2, mol. wt. 35000; P3, P4, mol. wt. 10^5.

Table 2. Relative amounts of virus-specific proteins in doubly infected protoplasts*

<table>
<thead>
<tr>
<th>Experiment no. (label)</th>
<th>Radioactivity in protein (ct/min)</th>
<th>Coat proteins</th>
<th>35000 mol. wt. proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCMV</td>
<td>BMV</td>
<td>CCMV : BMV</td>
</tr>
<tr>
<td>1 (14C)</td>
<td>324</td>
<td>1544</td>
<td>0.21</td>
</tr>
<tr>
<td>2 (14C)</td>
<td>288</td>
<td>904</td>
<td>0.32</td>
</tr>
<tr>
<td>3 (3H)</td>
<td>650</td>
<td>11600</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Protoplasts were inoculated first with CCMV followed by BMV and cultured for 24 h in medium containing 3H- or 14C-labelled leucine. Proteins were fractionated on 9% polyacrylamide gels which were sliced and counted.

Table 2 illustrates similar experiments in which doubly infected protoplasts were cultured in medium containing [14C]leucine or [3H]leucine and the proteins were prepared, separated on gels and the gels were sliced and counted. Again it can be seen that the amount of BMV coat protein was always greater than that of CCMV but that the reverse was true for the 35000 mol. wt. proteins. There was therefore appreciable but unbalanced expression of the CCMV genome in the mixed infection.

DISCUSSION

A number of points emerge from the results. The data on inoculation shown in Table 1 suggest that a population of protoplasts consists of two classes, one of which is susceptible to infection, so that a sequential or simultaneous inoculation infects only one part of the population and gives few singly infected protoplasts.

Turning to the phenomenon of mixed infections it can be seen that BMV dominance is a complex process which permits only limited expression and replication of the CCMV genome, suppressing production of infectious particles of CCMV and perhaps even exploiting those gene products of CCMV that are produced. In the results presented here BMV consistently produced infectious particles but those of CCMV were defective and may have lacked RNAs 1 and 2.
There are technical problems against recovering 100% of all species of RNA from CCMV and the infection and other assays may be too insensitive to detect small amounts of RNAs 1 and 2 in the presence of RNA 3; therefore, some caution is necessary in accepting this interpretation. Other work (Trim et al., 1977) has shown that RNAs 1 and 2 are limiting factors in infection, requiring only trace amounts of RNA 3 to cause infection. What is clear, however, is that there is no mixing of RNA and capsid proteins: CCMV particles isolated from mixed infections never gave BMV infections, and vice versa. It is known that transcapsidation can be produced in vitro (Bancroft, 1970) so that virus assembly in protoplasts must be restricted to specific regions of the cytoplasm, presumably within the vesicles that are observed to develop during infection (Burgess et al., 1974).

The pattern of protein synthesis in doubly infected protoplasts shows that although no infectious CCMV is obtained, the proteins that are coded by RNA 3 are both produced. Remarkably, the quantitative data show that the amounts of the 35000 mol. wt. protein coded by CCMV in some cases exceeded that coded by BMV. There is therefore the suggestion of an unbalanced translation of the genomes of both viruses in mixed infections; BMV coat protein was always produced in larger amounts than that of CCMV. It is possible that BMV does not simply suppress production of CCMV but exploits the machinery to assist its own synthesis. Bancroft (1972) has shown that a hybrid of CCMV and BMV can be constructed in one direction only, CCMV RNA 3 + BMV RNAs 1 and 2. It is thus possible that in the mixed infection the 35000 mol. wt. protein of CCMV may be exploited by BMV. It is, however, just as likely that the effect is a consequence of unbalanced translation and replication of the CCMV genome.

The function of the proteins, other than coat, synthesized in bromovirus infections remains unclear and differing opinions have been expressed (Hariharasubramanian et al., 1973; Sakai et al., 1979; Bujarski et al., 1982). Until this problem has been resolved it will be difficult to explain why there is so much expression of the CCMV infection without production of infectious virus.

REFERENCES


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