Infection of Protoplasts from Tobacco Suspension Cultures by Tobacco Mosaic Virus

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SUMMARY

Protoplasts isolated from suspension-cultured tobacco cells were inoculated with tobacco mosaic virus (TMV) using a procedure similar to that for tobacco mesophyll protoplasts. Fifty to 70% of protoplasts were infected, as determined by staining with fluorescent TMV antibody. The formation of progeny virus particles was substantiated by electron microscopy of sectioned protoplasts, and the virus yield was assessed by assaying the infectivity of protoplast extracts. The course of TMV multiplication was studied by following the incorporation of [3H]uridine into virus RNA. The results indicated that infection in this system is synchronous and that the rate of virus multiplication is comparable to that in mesophyll protoplasts. A system of undifferentiated, growing plant cells was thus established for one-step growth of TMV.

INTRODUCTION

Protoplasts isolated from the mesophyll tissues of plants are extensively used as a system for one-step growth of plant viruses, since they are synchronously infected with, and support vigorous multiplication of, a number of plant viruses (Takebe, 1975, 1977, 1980). In contrast, protoplasts from plant cells cultured in vitro have only rarely been employed as a host cell system for plant viruses (Jarvis & Murakishi, 1980; Lesney & Murakishi, 1981), although suspension-cultured cells provide a good source of protoplasts (Vasil & Vasil, 1980). This may be due in part to the long-standing idea that plant viruses multiply only slowly and to a limited extent in plant cells growing in vitro (Kassanis, 1967). Nevertheless, protoplasts from suspension cultures may be useful for plant virus studies, because they are uniform and contamination-free and because there are reports of active virus multiplication in cultured cells (Motoyoshi & Oshima, 1968; Murakishi et al., 1970).

This laboratory has shown that protoplasts isolated from suspension cultures of periwinkle (Fukunaga et al., 1981) and tobacco (Nagata et al., 1981) become infected at high frequencies when they are inoculated with RNA of tobacco mosaic virus (TMV) with the aid of liposomes. The present work was undertaken to establish a standard one-step growth system for TMV, using complete virions, and protoplasts from suspension-cultured tobacco cells.

METHODS

Isolation of protoplasts. Suspension culture of the BY-2 cell line of tobacco (Nicotiana tabacum L., cv. Bright Yellow-2) (Kato et al., 1972) and protoplast isolation were performed as described by Nagata et al. (1981).

Inoculation of protoplasts. In the standard inoculation procedure, purified TMV (OM, a common strain) was suspended at a concentration of 0·6 μg/ml in 5 ml 0·02 M-potassium citrate buffer pH 5·2 containing 0·4 M-mannitol and 1·6 μg/ml Polymun P (polyethyleneimine, mol. wt. 30000 to 40000; Bethesda Research Laboratories). After incubation at 25 °C for 10 min, 5 ml of protoplast suspension (2 × 10⁶ cells) in 0·4 M-mannitol solution was added to the inoculum, and the mixture was kept at 25 °C for a further 10 min. Concentrations in the final inoculation mixture were thus (per ml) 0·3 μg TMV, 0·8 μg Polymun P, 0·01 M-citrate buffer and 2 × 10⁶ protoplasts. It was our practice to centrifuge protoplasts and resuspend them in mannitol solution immediately before they were added to the inoculum solution.
Culture of protoplasts. The inoculated protoplasts were washed three times with 0.4 M-mannitol solution and were cultured at 28 °C as 10 ml suspensions of 2 × 10^5 cells/ml. The culture medium was that of Nagata et al. (1981) except that cephaloridine (to 300 μg/ml) and mycostatin (to 10 μg/ml) were added after autoclaving.

Determination of infection level. After 20 h of culture, the inoculated protoplasts were stained with fluorescent antibody to TMV as described by Fukunaga et al. (1981). The level of infection was expressed as the percentage of protoplasts containing the specific yellowish-green fluorescence.

Infectivity assay. Protoplasts (2 × 10^6 cells), harvested 24 and 48 h after inoculation, were washed with 0.4 M-mannitol solution and suspended in 2 ml of 0.01 M-potassium/sodium phosphate buffer pH 7.0. Homogenate obtained by the method of Nishimura & Beevers (1978) was centrifuged at 8000 g for 10 min, and the supernatant solution was diluted 20- (24 h sample) or 50-fold (48 h sample) with 0.1 M-phosphate buffer pH 7.0. Infectivity of the diluted samples was assayed on half-leaves of N. tabacum L. cv. Xanthi-nc according to Otsuki et al. (1972b).

Labelling and extraction of RNA. RNA synthesized in the inoculated protoplasts was labelled by adding [5-3H]uridine (29 Ci/mmol, Amersham International) at the start of culture to a concentration of 10 to 30 μCi/ml. Actinomycin D was also added to the medium at a concentration of 10 to 50 μg/ml to suppress synthesis of host RNA (Sakai & Takebe, 1970).

After various periods of culture, protoplasts were harvested, washed with 0.4 M-mannitol solution, and solubilized with 1.25 ml of the extraction buffer of Bourque et al. (1973). The extract was shaken with an equal volume of water-saturated redistilled phenol, centrifuged and the aqueous phase was extracted twice more with a half-volume of phenol. Nucleic acid was ethanol-precipitated from the aqueous phase, the precipitate collected by centrifugation, dissolved in 0.5 ml of 0.15 M-sodium acetate pH 6.0 containing 0.5% SDS, and re-precipitated. The cycle was repeated two or three times.

Gel electrophoresis. Gels (0.7 x 12 cm) containing 2.4% acrylamide and 0.12% bisacrylamide were prepared in acrylate tubes according to the method of Loening (1968). Electrophoresis buffer contained 36 mM-Tris-HCl, 30 mM-NaH2PO4, 1 mM-disodium-EDTA, 1 mM-n-propylamine, and 0.2% SDS, the pH being adjusted to 7.8. Gel polymerization was performed in the same buffer but in the absence of SDS.

Nucleic acid (30 to 50 μg in 10 to 50 μl electrophoresis buffer containing 10% sucrose) was electrophoresed for 3 h at a constant current of 5 mA/gel at 5 °C in gels which had been pre-run for 1 h. Gels were then washed in distilled water at 5 °C for 2 h and scanned at 260 nm in a Gilford spectrophotometer 250. Gels were then weakly stained with a dilute solution of toluidine blue, placed on Scotch mending tape stuck on a glass plate, and dried by heating at 60 °C for 30 min. The dried gels were then pasted on to strips of sectioned paper, and were cut into 1 mm sections using a paper cutter. Each section was placed in 0.2 ml of 31% H2O2 in a glass scintillation vial, digested by overnight incubation at 60 °C, and radioactivity was determined in a liquid scintillation counter after the addition of 5 ml ACS II (Amersham International).

RESULTS

Protoplasts of suspension-cultured BY-2 cells

The tobacco BY-2 cell line grew very rapidly in suspension culture as fine clusters of 10 to 20 cells (Fig. 1a). The cell mass (dry weight) increased from 0.3 to 15 mg/ml within a week. Protoplasts from BY-2 cells in the exponential phase of growth were rich in cytoplasm (Fig. 1b), as is characteristic of actively growing cells. Yield of protoplasts was 3 × 10^7 to 4 × 10^7 from 100 ml culture.

Inoculation conditions

BY-2 protoplasts were inoculated with TMV according to a procedure based on that previously developed for tobacco mesophyll protoplasts (Otsuki et al., 1972a). As described below, various factors in the inoculation conditions were systematically studied for their effects on infection. The standard procedure described in Methods was developed from the results of these experiments, and consistently gave infection in 50 to 70% of protoplasts.

Infection of BY-2 protoplasts by TMV depended on the addition to inocula of macromolecular polycations such as poly-L-ornithine, as was the case with tobacco mesophyll protoplasts (Takebe & Otsuki, 1969). Polymine P, a commercial preparation of polyethylenimine, gave higher rates of infection of protoplasts than did poly-L-ornithine (mol. wt. 250000; Pilot Chemicals, Watertown, Mass., U.S.A.). For example, Polymine P (1 μg/ml) gave infection in 54% of protoplasts as compared with 41% for poly-L-ornithine (1 μg/ml). Although the maximum proportion of protoplasts was infected at 1-0 to 1-5 μg/ml Polymine P, 0-8 μg/ml was
TMV in protoplasts from cultured cells

Fig. 1. (a) Fluorescence micrograph of suspension-cultured tobacco BY-2 cells used as the source of protoplasts. Only the cell walls are visible because the cells were stained with Calcofluor White ST (Nagata & Takebe, 1970). Bar marker represents 100 μm. (b) Micrograph of protoplasts of BY-2 cells. Bar marker represents 50 μm.

Table 1. Effects of inoculation buffers on infection of BY-2 protoplasts by TMV*

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Protoplasts infected (%)</th>
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<tbody>
<tr>
<td>Potassium citrate</td>
<td>55.7</td>
</tr>
<tr>
<td>Potassium succinate</td>
<td>36.6</td>
</tr>
<tr>
<td>MES†</td>
<td>11.0</td>
</tr>
<tr>
<td>MOPS‡</td>
<td>0</td>
</tr>
</tbody>
</table>

* Protoplasts were inoculated with 1 μg/ml TMV in the presence of 1 μg/ml poly-L-ornithine and various buffers at 0.01 M and pH 5.2. Procedure of inoculation was otherwise as described in Methods.
† 2-(N-morpholino)ethanesulphonic acid.
‡ 3-(N-morpholino)-2-hydroxypropanesulphonic acid.

used, because at higher concentrations protoplasts tended to aggregate, causing decrease in viability.

The number of infected protoplasts was proportional to the logarithm of virus concentration up to 0.1 μg/ml. Infection was maximal at a virus concentration of 0.3 μg/ml, higher concentrations yielding lower levels of infection.

Infection of BY-2 protoplasts was favoured by low pH, the highest level of infection being at pH 5.2, the lower limit for protoplast stability, and the proportion of infected protoplasts diminished with increasing pH of the medium. Table 1 shows that citrate was the best of the buffers which were tested at pH 5.2. Aminosulphonic acid-type buffers, which were previously reported to effect cowpea mosaic virus infection of soybean protoplasts in the absence of poly-L-ornithine (Ferguson et al., 1980), gave low rates of infection or none at all (Table 1). Infection of BY-2 protoplasts by TMV was influenced also by the concentration of buffer, with 0.01 M being optimal for citrate buffer, pH 5.2.

Incubation of protoplasts with inocula for varying lengths of time showed that 10 min was sufficient for maximal infection.

TMV multiplication in BY-2 protoplasts

Electron microscopy of ultrathin sections of inoculated BY-2 protoplasts, processed by the method of Kajita et al. (1980), showed that typical aggregates of TMV rods were present in large numbers of protoplasts harvested 20 h after inoculation. Yield of virus was assessed by comparing the infectivity of protoplast extracts with that of standard TMV solution of known concentration. Assuming that virus in both types of preparations has the same specific infectivity, it was estimated that an average infected protoplast contained 4.5 × 10⁵ and 2.0 × 10⁶ TMV particles at 24 and 48 h after inoculation, respectively.

In the preliminary phase of this study, inoculated protoplasts were cultured in a medium similar to that used for maintaining tobacco mesophyll protoplasts (Takebe, 1977). This medium
Fig. 2. Time course of increase in the proportion of infected protoplasts. Protoplasts harvested at indicated times were stained with fluorescent antibody to TMV to determine the proportion of infected cells.

Fig. 3. Profiles of polyacrylamide gels ($A_{260}$ lines; radioactivity, ○) of nucleic acid extracted from BY-2 protoplasts inoculated with TMV. Inoculated protoplasts were cultured for 20 h in the presence of 10 μCi/ml [3H]uridine and 10 μg/ml actinomycin D. Nucleic acid (45 μg) extracted from the protoplasts was subjected to electrophoresis. Small radioactivity peaks at fractions 2 to 8 and those at fractions 11 to 17 are assumed to represent replicative intermediate and replicative form of TMV RNA, respectively.

Fig. 4. Time course of [3H]uridine incorporation into TMV RNA by BY-2 protoplasts. Inoculated protoplasts were cultured for 48 h in the presence of 10 μCi/ml [3H]uridine and 46 μg/ml actinomycin D. Nucleic acid extracted from the protoplasts at indicated times was subjected to electrophoresis. The radioactivity in the region of TMV RNA was plotted against the time of culture after normalization to a fixed number of protoplasts ($1.4 \times 10^6$).

was essentially a dilute solution of major inorganic salts and contained neither a metabolizable carbon source nor vitamins. Virus yield in infected protoplasts, as judged by the amount of virus antigen visualized by immunofluorescence, was markedly improved when we switched from this poor medium to the Linsmaier and Skoog medium, as modified by Nagata et al. (1981). This indicated that TMV multiplication in BY-2 protoplasts depends significantly on the nutrients supplied by the latter medium.
TMV in protoplasts from cultured cells

The time course of TMV multiplication in BY-2 protoplasts was studied in two ways. First, the development of virus antigen in protoplasts was followed by staining with fluorescent antibody at intervals after inoculation (Fig. 2). Materials reacting with virus antibody became detectable in some protoplasts 8 h after inoculation as weakly fluorescing specks. The proportion of protoplasts containing virus antigen, as well as the amount of antigen in individual protoplasts, increased markedly during the subsequent 12 h. After 25 h of culture, the proportion of stained protoplasts remained unchanged, while fluorescence in these protoplasts continued to intensify until at least 40 h after inoculation. The sharp increase in the proportion of infected protoplasts followed by its complete cessation (Fig. 2) indicated synchrony of infection in this system.

TMV multiplication in BY-2 protoplasts was also followed by determining \(^{3}\text{H}\)uridine incorporation into virus RNA. Fig. 3 depicts a gel electrophoresis profile of nucleic acid extracted from protoplasts after 20 h of labelling. The absence of chloroplast ribosomal RNAs in these cells was apparent in the absorbance curve, whereas the radioactivity curve showed that the synthesis of 25S and 18S cytoplasmic ribosomal RNAs was effectively inhibited by actinomycin D. The major RNA species synthesized in these protoplasts had the same mobility as TMV RNA, was also detectable by absorbance, and was absent in non-inoculated protoplasts. Thus, it was assumed to be TMV RNA. Some radioactivity was found also in the regions of gel where the replicative form and replicative intermediate of TMV RNA are known to migrate (Aoki & Takebe, 1975). The time course of \(^{3}\text{H}\)uridine incorporation into TMV RNA is shown in Fig. 4. Significant incorporation had occurred 6 h after inoculation, and radioactivity increased exponentially with time during the subsequent 10 h. In later periods, increase in the radioactivity in TMV RNA was more or less linear. The time course of precursor incorporation into TMV RNA was largely similar to that previously observed in tobacco mesophyll protoplasts inoculated with TMV (Aoki & Takebe, 1975).

DISCUSSION

Inoculation with TMV of the protoplasts from suspension-cultured tobacco cells resulted in infection of 50 to 70\% of the protoplasts. The optimal conditions for inoculation were similar to those developed for tobacco mesophyll protoplasts (Otsuki et al., 1972a), but polyethyleneimine replaced poly-L-ornithine, because it is inexpensive and can be obtained to better-standardized specifications. The efficiency of infection (5 x 10^4 TMV particles added for each infected protoplast) was similar to that recorded with tobacco mesophyll protoplasts (Takebe, 1977). However, the protoplasts from suspension cultures were saturated at lower virus concentration (0.3 \(\mu\)g/ml) than mesophyll protoplasts (1 \(\mu\)g/ml) under otherwise similar conditions.

Jarvis & Murakishi (1980) and Lesney & Murakishi (1981) showed that a critical factor in infecting protoplasts from soybean suspension cultures with legume viruses was the addition to the inoculum of Ca\(^{2+}\), which increased the levels of infection up to six-fold. They suggested that Ca\(^{2+}\) acts primarily on the virus rather than on protoplasts, since preincubation with virus was a condition necessary for the stimulation of infection by Ca\(^{2+}\). In the present work, we found that Ca\(^{2+}\) has no influence on the infection of BY-2 protoplasts by TMV (data not shown). Obviously, the stimulation of infection by Ca\(^{2+}\) is not a common property of protoplasts of cultured cells.

The course of \(^{3}\text{H}\)uridine incorporation into virus RNA (Fig. 4) and the results of infectivity assays of protoplast extracts showed that the rate of TMV multiplication in BY-2 protoplasts is comparable to that in tobacco mesophyll protoplasts. This indicates that the undifferentiated state of plant meristem cells is not necessarily a hindrance to infection and multiplication of viruses. Thus, protoplasts from tobacco suspension cultures provide an alternative to the mesophyll protoplast system in that they permit one-step growth of TMV. Advantages associated with the protoplasts of cultured cells include (i) that uniform and contamination-free protoplasts are obtained consistently and in large amounts, and (ii) that isolation of virus-related molecules is not complicated by the presence of the chloroplasts and large vacuoles which characterize mesophyll protoplasts. Furthermore, the availability of the two types of host cell system permits comparative studies on the behaviour of viruses in undifferentiated growing cells and in highly differentiated leaf cells.
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REFERENCES


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