INFECTION OF TOBACCO MESOPHYLL PROTOPLASTS BY TOBACCO MOSAIC VIRUS*

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Abstract.—It was proved that the protoplasts prepared from mesophyll of *Nicotiana tabacum* are infected by tobacco mosaic virus. The infection occurred when purified tobacco mosaic virus particles were added to a protoplast suspension in the presence of poly-L-ornithine. The virus multiplied in these protoplasts to a level of 10^6 virus particles per infected protoplast during 24 hours of incubation. The efficiency of infection was remarkably high, exceeding that by mechanical inoculation of tobacco leaves.

Protoplasts can be prepared from plant cells by mechanical or enzymatic removal of cell walls.^{1, 2} These isolated naked cells should be far more suitable than conventional tissue materials for experiments of quantitative nature, since they can be used as a homogeneous suspension in liquid medium.³ The isolation of protoplasts from mesophyll was accomplished for the first time in this laboratory by a newly developed enzymatic technique.^{3, 4} Mesophyll protoplasts were prepared on a large scale by cellulase treatment of isolated mesophyll cells which had been obtained by digesting leaves with pectinase. The effectiveness of this technique prompted us to explore the potentialities of mesophyll protoplasts as a new experimental material for studying various activities of plant cells.

Recently, RNA from tobacco mosaic virus (TMV) inoculated into tobacco mesophyll protoplasts was shown to give rise to a synchronous multiplication of this virus in these protoplasts.⁵ This finding indicated that the mesophyll protoplasts should be invaluable for studying plant virus multiplication at a cellular level. However, it is obvious that studies of the entire process of infection, including penetration and uncoating of infecting virus, are possible only when infection is caused by complete virus particles. In the following, we present evidence to show that complete particles of TMV infect tobacco mesophyll protoplasts and multiply within them.

Materials and Methods.—Protoplasts of palisade parenchyma cells were prepared from mesophyll of healthy tobacco plants (*Nicotiana tabacum* var. Bright Yellow) according to the procedures previously reported³ with slight modifications.⁵ Figure 1 shows microscopical appearance of these protoplasts. The protoplasts were washed with and resuspended in 0.8 M mannitol at a concentration of 1 to 4×10^6 /ml, as measured by a Coulter electronic counter.⁵ For infection, purified TMV (OM, a common strain⁶) was dissolved to a concentration of $2 \mu\text{g}/\text{ml}$ in 0.02 M potassium citrate buffer, pH 5.0, containing mannitol to 0.8 M and poly-L-ornithine (mol. wt. $\sim 130,000$, Pilot Chemicals, Inc., Watertown, Mass.) to $2 \mu\text{g}/\text{ml}$. After standing at 25°C for 10 min, the TMV solution was added to an equal volume of the protoplast suspension, and the mixture was kept at 25°C with occasional gentle swirling. After 60 min of infection, the protoplasts were separated from the inoculum virus by low speed centrifugation⁵ and were washed several times with sterile 0.8 M mannitol containing CaCl_2 to 0.1 mM. The washed protoplasts were transferred aseptically into an incubation medium⁵ to yield a concen-

tration of 1 to 4×10^5 /ml, and incubated in 10 ml portions as described in a previous paper.³ After an appropriate period of incubation, the protoplasts were harvested by centrifugation, washed, and homogenized as described previously.⁵ The extract thus prepared was made up to 10 ml with 0.1 M phosphate buffer, pH 7.0, and assayed for infectivity on a half leaf of *Nicotiana tabacum* var. *Xanthi* nc. Each assay was calibrated with a standard TMV solution inoculated on the other half leaf.³

FIG. 1.—Protoplasts isolated from tobacco mesophyll and suspended in 0.8 M mannitol. Cytoplasm around central vacuole is packed with chloroplasts, which obscure nucleus and other structures. The scale represents 50 μ .

FIG. 2.—Fluorescence micrograph of protoplasts infected by TMV. Protoplasts were incubated for 27 hr after infection and stained with TMV-antibody labeled with fluorescein isothiocyanate. Four protoplasts in this figure contain stained material, while two in the upper part (arrows) do not. The scale represents 50 μ .

Results.—Development of infectivity: During infection under the conditions described above, only 1 per cent of the added virus became associated with protoplasts and was found in their extract as measured by infectivity assay. Correspondingly, the added virus was recovered, without detectable decrease in infectivity, in the infection medium from which the protoplasts were removed. It was thus found that approximately 1 per cent of the inoculum TMV particles were adsorbed to protoplasts under these conditions. Average number of virus particles adsorbed per protoplast was roughly estimated to be 10^2 .

When these protoplasts were incubated for 27 hours, the infectivity extractable from the protoplasts increased strikingly (Table 1). The magnitude of increase varied somewhat from one experiment to another, but an increase of over 1000-fold was frequently obtained. As shown in Table 1, the development of infectivity was prevented by adding either 2-thiouracil or cycloheximide to the incubation medium. In contrast, actinomycin D showed apparently no effect

TABLE 1. *Influence of inhibitors on rise of infectivity in protoplasts infected by TMV.**

Inhibitor added	Infectivity of Extract of Protoplasts†	
	0 hr	27 hr
	1.5 ± 1.7	
None‡		426 ± 174
Actinomycin D (10 µg/ml)†		558 ± 312
2-Thiouracil (10 µg/ml)†		52 ± 26
None		636 ± 198
Chloramphenicol (100 µg/ml)		636 ± 240
Cycloheximide (10 µg/ml)		3.5 ± 3.2

* Infected protoplasts were suspended in 70 ml of incubation medium at a concentration of 3.9×10^5 /ml. Ten ml were removed as 0 hr sample and the rest was incubated in 10 ml portions for 27 hr with and without addition of inhibitor.

† Incubation was performed in the dark.

‡ Number of lesions produced by extract of protoplasts on a half leaf of *Nicotiana tabacum* var. *Xanthi* nc. Mean of nine determinations with standard deviation.

at the concentration which inhibited RNA synthesis in non-infected protoplasts by 85 per cent.⁷ A high concentration of chloramphenicol also did not interfere with the development of infectivity (Table 1). These results show that the development of infectivity in the protoplasts requires DNA-independent RNA synthesis as well as cytoplasmic protein synthesis. The same pattern of response to these inhibitors is known for TMV multiplication in tobacco leaves,⁸⁻¹⁰ in isolated tobacco leaf cells,³ and in tobacco protoplasts infected by TMV-RNA.⁵ The results in Table 1 thus indicate that TMV multiplied in the protoplasts as the result of infection by the added virus particles.

Fluorescent antibody staining of protoplasts: The protoplasts infected with TMV particles and subsequently incubated for 27 hours were stained with fluorescent antibody against TMV by the method recently described.¹¹ As illustrated in Figure 2, a number of protoplasts contained tiny masses of material which were stained by the fluorescent antibody. None of the protoplasts contained such material when they were sampled immediately after infection. Three types of control staining experiments¹¹ confirmed that this staining is specific for TMV antigen. It was thus demonstrated that the protoplasts infected under the present conditions accumulated TMV antigen within them during subsequent incubation.

Percentage of the protoplasts in which infection was established could be estimated by scoring, under the fluorescent microscope, those protoplasts which contained the stained material. The percentage was 13.8, 25.1, 25.3, 30.4 and 31.2 in five separate infection experiments.

Demonstration of progeny particles: Production of complete progeny TMV particles in these protoplasts was ascertained by electron microscopic examination of their extract. Numerous particles of the same morphology as TMV were found in the extract of protoplasts which were incubated for 27 hours, after infection (Fig. 3). Extracts of protoplasts prepared immediately after infection rarely contained similar particles, which should represent the infecting virus.

Time course of TMV multiplication: The multiplication of virus in the protoplasts infected by TMV particles followed a time course illustrated in Figure 4. During the initial period of incubation, there was a drop of infectivity in protoplasts. This was followed by a very rapid rise of infectivity between 8 and 22

FIG. 3.—Electron micrograph of particles found in the extract of protoplasts infected by TMV and incubated for 27 hr. Negatively stained with phosphotungstic acid.⁵ The scale represents 10 μ .

FIG. 4.—Time course of TMV multiplication in protoplasts. Protoplasts infected by TMV were suspended in 150 ml of incubation medium at a concentration of 2.0×10^5 /ml and incubated in 10-ml portions. At indicated times one tube was harvested, extract of protoplasts was prepared and assayed for infectivity. To determine infectivity in medium, the incubation medium from which protoplasts were removed by centrifugation was dialyzed first against water and then against 0.1 M phosphate buffer, pH 7.0, and assayed for infectivity. Each point in the figure represents mean of eight to ten determinations.

hours of incubation. The infectivity in protoplasts reached a maximum at 24 hours and then decreased. The infectivity in medium, which probably reflects disintegration of some protoplasts, increased roughly in parallel to that in protoplasts during early periods of incubation, but reached a maximum somewhat later.

Factors necessary for infection: When protoplasts were treated under the condition of infection without added TMV particles, no infectivity was found in the protoplasts throughout subsequent incubation (Table 2). This indicates that the inoculum TMV is responsible for the development of infectivity in protoplasts. The amount of infectivity developing in protoplasts during the incubation depended upon the amount of TMV added as inoculum. However, higher concentrations of inoculum TMV than specified above did not result in the development of larger amounts of infectivity.

Poly-L-ornithine was necessary for infection of protoplasts by TMV particles. As shown in Table 2, the adsorption of inoculum virus occurred even in the absence of this polycation. However, the adsorbed virus apparently failed to

establish infection, since no increase in infectivity was obtained by subsequent incubation of the protoplasts (Table 2). The effect of poly-L-ornithine depended upon its concentration, but it caused damage to the protoplasts at higher concentrations than used here.

TABLE 2. *Necessity of inoculum TMV and poly-L-ornithine for infection.**

Component omitted	Infectivity of Extract of Protoplasts†	
	0 hr	38 hr
None	1.2 ± 1.6	1315 ± 186
TMV	0	0
Poly-L-ornithine	1.9 ± 1.8	1.5 ± 1.6

* Protoplasts were treated under the condition of infection with full components, without addition of TMV, or without addition of poly-L-ornithine. The protoplasts were then incubated at a concentration of 1.6×10^5 /ml for 38 hr.

† Number of lesions produced by extract of protoplasts on a half leaf of *Nicotiana tabacum* var. *Xanthi* nc. Mean of eight determinations with standard deviation.

Discussion.—The experiments described above indicate that TMV particles added with poly-L-ornithine established infection and multiplied in the tobacco mesophyll protoplasts. The time course of development of infectivity in these protoplasts was similar to that observed in the protoplasts infected by TMV-RNA,⁵ suggesting that a synchronous multiplication of TMV is taking place also in the present system. The only difference was that, in the present system, the infectivity due to infecting virus was detectable in the protoplasts at the beginning of incubation and that it decreased prior to the development of infectivity due to progeny virus (Fig. 4). Whether this "eclipse" reflects uncoating of infecting virus particles is at present not clear. The nature of the decline of infectivity in protoplasts which occurred in the later phase of incubation also remains to be investigated.

Average yield of progeny virus per infected protoplast could be estimated from the maximal infectivity in protoplasts and the number of infected protoplasts which was determined by fluorescent antibody staining.⁵ The yield in the protoplasts infected by TMV particles ranged 0.5 – 1.1×10^6 particles per protoplast, being somewhat higher than that obtained by infection with viral RNA.⁵

The most remarkable aspect of infection in the present system is its high efficiency. In a typical experiment, 3×10^5 protoplasts were infected with $2 \mu\text{g}$ of purified TMV as inoculum. This amount of virus should contain 3×10^{10} particles, assuming 40 million for the "molecular weight" of TMV.¹² Infection in this system has, therefore, an efficiency of one infection per 10^5 inoculum particles, which is ten thousand times higher than that for the infection of protoplasts by TMV-RNA.⁵ The efficiency is higher even than that by mechanical inoculation of tobacco leaves, since the same amount of TMV produces only 10^3 necrotic lesions on a half leaf of *Nicotiana tabacum* var. *Xanthi* nc. By virtue of this high efficiency, as much as 30 per cent of whole protoplast population was infected in this system.

The infection of protoplasts by TMV particles requires the presence of poly-L-ornithine at the time of infection (Table 2). This polycation is known to strongly enhance pinocytotic uptake of proteins by cultured mammalian cells.¹³ On the

other hand, protoplasts of tomato fruit cells are reported to take up particles of TMV by a pinocytic process.¹⁴ It seems, therefore, that the penetration of inoculum particles into our system is also attributable to the pinocytic activity of plant protoplasts.

Cocking and Pojnar recently showed by an electron microscopic study that TMV is taken up by and multiplies in the protoplasts prepared from tomato fruits.¹⁵ Their system, however, requires the presence of a very high concentration of inoculum virus for a long period, indicating that the efficiency of infection is quite low. Moreover, the rate of virus multiplication in their system is apparently much slower than that in ours.

As discussed previously,⁵ the mesophyll protoplasts have definite advantages over conventional tissue materials as the experimental system for studying plant virus multiplication. The present study disclosed another advantage of this host cell system, namely that it can be inoculated with a high efficiency which was not attainable with systems thus far available. The mesophyll protoplasts should thus provide a powerful tool for elucidating the processes underlying the infection and the multiplication of plant viruses.

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