14. PROTOPLASTS: A UNIQUE MATERIAL FOR PLANT VIRUS RESEARCH*

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Whole plants, attached or detached leaves, and excised tissue pieces are usually employed as a host material for experiments with plant viruses. While these materials are useful for studying many aspects of plant virology, they are unsuitable for investigating other problems, particularly those related to the virus infection cycle in plant cells.

Tremendous difficulty is thus encountered when one wishes to study with these materials the infection events in primary-infected cells because such cells are extremely small in number (less than one thousandth of total leaf cells).

Since virus moves from one cell to another, larger numbers of cells become infected a few days later. However, the materials at such stages are of limited use for following the sequence of events in infected cells because individual cells within them are no longer at the same stage of infection cycle.

In addition, the complex tissue materials contain cells of diverse types which possibly behave differently to infection and exert their influence on cells of other types. Also, it is difficult with these materials to obtain uniform samples or to ensure uniform chemical environments to their constituent cells. These features obviously cause complications in the experiments of biochemical type which are requisite for studying the process of virus multiplication.

We have been able to develop a new experimental materials for plant virus research, protoplasts of leaf cells, which largely overcomes the disadvantages of the conventional plant materials. An outline of the protoplast system will be presented in this article, and some of its implications to the studies of plant virus infection and multiplication will be discussed. The subject was reviewed in more depth in some recent papers (Takebe, 1975a, b).

Protoplasts from leaf mesophyll

Plant cells are surrounded by rigid cellulosic walls which resist penetration by plant viruses. The cells undergo plasmolysis in a medium of high osmotic pressure; the cellular entity enclosed within the cytoplasmic membrane, the protoplast, shrinks and retracts from the cell walls.

Under such conditions, protoplasts can be released into medium, if the walls are dissolved by cell wall-degrading enzymes. The protoplasts thus isolated are spherical in shape and persist in spite of the absence of walls, if cultured under appropriate conditions.

We have worked out an enzymatic method by which large amounts of protoplasts of mesophyll cells are isolated from tobacco leaves (Takebe et al., 1968; Otsuki et al.,

* Abbreviations for virus names: AMV (alfalfa mosaic virus), BMV (brome mosaic virus), CCMV (cowpea chlorotic mottle virus), CGMMV (cucumber green mottle mosaic virus), CMV (cucumber mosaic virus), CPMV (cowpea mosaic virus), PEMV (pea enation mosaic, virus), PVX (potato virus X), TMV (tobacco mosaic virus), TRV (tobacco rattle virus), TYMV (turnip yellow mosaic virus)

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1974). The starting material is mature tobacco leaves whose mesophyll tissues are exposed by manually peeling the epidermis off with the aid of a forceps. The peeled leaves are treated first with pectinase mesophyll cells are then suspended in a cellulase solution and are incubated to digest the walls.

The treatments with both enzymes are performed in a medium containing D-mannitol at a plasmolyzing concentration. About 10^7 protoplasts are thus obtained within two hours from 1 gram (fresh weight) of tobacco leaves. The method is applicable in principle also to other species than tobacco, and protoplasts have been isolated from the leaves of some 50 dicotyledonous and 20 monocotyledonous species (Takebe, 1975b).

The protoplasts isolated from tobacco leaves are largely normal in their ultrastructure, except that the wall structures are absent (Takebe et al., 1973). They actively synthesize RNA and protein (Sakai and Takebe, 1970) or regenerate surface walls (Nagata and Takebe, 1970). Under appropriate nutritional conditions, the isolated leaf protoplasts can even undergo cell division to form callus-like colonies of daughter cells (Nagata and Takebe, 1971).

Infection of isolated protoplasts

Since plant viruses cannot penetrate the walls of plant cells, inoculation of plant materials requires wounding of the cell wall as effected by rubbing the leaves with abrasives, for exampple. We thought that the chance of virus to get into cells may be greater if the walls are removed, and this was indeed one of the rationales of our attempt to develop the protoplast system for plant virus research.

The idea turned out to be correct when we found that tobacco leaf protoplasts briefly incubated with a solution of TMV became infected at high frequencies, provided that the virus was pretreated with poly-L-ornithine (Takebe and Otsuki, 1969). The polymer of a basic amino acid L-ornithine is thought to form a complex with the negatively charged virus particles and to help them to adsorb to the surface of protoplasts.

For inoculation, a solution of purified TMV is preincubated with poly-L-ornithine and is then added to a suspension of protoplasts. Virus adsorbs to and penetrates into

Table 1. Plant viruses inoculated to isolated leaf protoplasts

Protoplasts from	Virus	Percentage of protoplasts infected	Reference
Tobacco	TMV	90	Otsuki et al., 1972
	CGMMV	70	Sugimura and Ushiyama, 1975
	TRV	98	Kubo et al., 1975
	PVX	70	Otsuki et al., 1974
	CMV	90	Otsuki and Takebe, 1973
	BMV	77	Motoyoshi et al., 1974
	CCMV	65	Motoyoshi et al., 1973
	AMV	35	Motoyoshi et al., 1975
	PEMV	84	Motoyoshi and Hull, 1974
Tomato	TMV	83	Motoyoshi and Oshima, 1976
Cowpea	CPMV	96	Hibi et al., 1975
	CMV	95	Koike et al., 1975
	TMV	57	Koike et al., 1976
Chinese cabbage	TYMV	90	Renaudin et al., 1975
Barley	BMV	30	Okuno et al., 1975

protoplasts during a subsequent incubation of the mixture for 10 minutes. Protoplasts are then separated from unadsorbed virus by low speed centrifugation and are cultured in a simple liquid medium to allow virus multiplication.

Protoplasts from the leaves of several species have been successfully inoculated with a number of viruses (Table 1) using procedures essentially similar to that for tobacco protoplasts and TMV.

The pretreatment of virus with poly-L-ornithine is essential for all the viruses except BMV both of which have much higher isoelectric points than other viruses. These viruses apparently adsorb to protoplasts without the help of poly-L-ornithine because of their less negative charge.

Infection of isolated protoplasts is unique in that it does not require mechanical wounding of cells. Electron microscopy of the inoculated protoplasts showed that TMV rods adsorb endwise to the surface of protoplasts. The cytoplasmic membrane developed invagination at the site of virus adsorption and trapped virus into intracellular vesicles. A process similar to the endocytosis by animal cells thus appears to be involved in the entry of virus into protoplasts (Takebe et al., 1975).

Another unique feature of the infection of protoplasts is the very high frequencies at which it occurs. When inoculated under optimal conditions, some viruses infect over 90% of protoplasts (Table 1). This is demonstrated by sampling the inoculated protoplasts after one or two days of culture and by treating them with viral antibody labeled with fluorescein isothiocyanate.

Infected protoplasts are readily identified under a fluorescence microscope because they contain virus masses which react with the labeled antibody and show specific fluorescence. The infection of high proportion of cells is obviously one of the great advantages of the protoplast system over conventional plant materials in which less than one thousandth of cells are primary-infected.

Virus multiplication in protoplasts can be followed by sampling protoplasts at intervals after inoculation, breaking them open and assaying the infectivity in extracts. With TMV in tobacco protoplasts, virus starts to increase exponentially at 6 hours after inoculation. Then, the rate of virus multiplication becomes linear, and virus continues to multiply for 2–3 days. Over 10^6 TMV particles are produced in a protoplast, a yield comparable to that of cells in leaves. Electron microscopy also reveals accumulation of virus in large amounts.

When we follow the course of virus multiplication in a population of protoplasts, we should be looking at the infection cycle in a single representative protoplast being amplified by a factor equal to the number of infected protoplasts. This is so because protoplasts represent a population of relatively uniform mesophyll cells and because infection occurs simultaneously in the majority of protoplasts. In other words, virus infection is synchronous in the protoplast system.

Some of the uses of protoplasts in plant virus research

As an experimental material for plant virus research, the leaf protoplast system has many unique features which are not shared by the conventional plant materials.

First, it is a single cell system and consists of only one type of cells. Second, nearly all the cells are infected simultaneously and a synchronous virus multiplication ensues. Third, it is possible to place all the constituent cells in an identical chemical environment and to take samples of uniform quantity. Finally, isolation of subcellular structures and substances is easy because of the absence of rigid walls. As will be exemplified below, these features can be exploited to obtain new insights into various problems of plant virology which have been difficult to attack with the existing materials.

We followed the course of synthesis of viral RNA and proteins in TMV-infected

tobacco protoplasts to correlate it to virus multiplication (Aoki and Takebe, 1975; Sakai and Takebe, 1974). Synthesis of viral RNA started as early as 4 hours after inoculation and was initially exponential in its rate. Most of viral RNA made in the initial period of infection remained unassembled into virus rods for some time until active synthesis of coat protein ensued.

In the later periods of infection cycle, viral RNA synthesis proceeded linearly, being closely paralleled by the production of virus particles. Double-stranded forms of TMV-RNA were also detected in infected protoplasts and the course of their synthesis was consistent with their postulated role as intermediates of viral RNA synthesis.

Viral coat protein was produced in large amounts and its synthesis followed a course very similar to that of virus particles production, suggesting that coat protein synthesis limits the rate of assembly.

In addition, infected protoplasts synthesized two high molecular weight proteins which are not found in healthy protoplasts, and one of them is assumed to be an enzyme responsible for the synthesis of viral RNA.

The high molecular weight proteins were synthesized earlier but in much smaller amounts than coat protein, indicating that the genes in TMV-RNA are translated separately.

A hypothesis has been developed using leaf materials that TMV particles exclude each other during infection so that only one virus can participate in the infection of a cell at one time. The hypothetical exclusion phenomenon may be considered to be a basis for the cross protection known for related viruses. It is clear that a single cell system is needed to test the validity of the exclusion hypothesis without ambiguity.

We inoculated tobacco protoplasts with a mixture of a common and a tomato strain of TMV to see if they infect the same protoplast. After 24 hours, the protoplasts were examined for their reaction to the fluorescent antibodies specific to each of the strains. It was thus found that about 80% of protoplasts accumulated both of the common and the tomato strain (Otsuki and Takebe, 1974).

Furthermore, analysis of the antigen constitution of virus particles produced in such protoplasts revealed the presence in the same virus particles of the coat protein of both strains (Otsuki, 1975). These results are clearly incompatible with the exclusion hypothesis and show unequivocally that two closely related strains of TMV can infect the same cell.

Our experiments showed at the same time, however, that the common and the tomato strain interfere with each other during the multiplication in the same protoplast (Otsuki and Takebe, 1976). The cross protection in plants may possibly be explained by the interference between related viruses for multiplication in the same cell.

Tobacco varieties carrying the N gene (Xanthi nc or Samsun NN) respond to TMV infection by the necrotic death of infected cells, thereby localizing infection. Although this reaction has been known for many years and formed the basis of local lesion assay of TMV, its mechanism is still obscure.

We showed that TMV multiplies normally in the protoplasts of Xanthi nc and Samsun NN. The N gene has, therefore, nothing to do with the capacity of leaf cells to produce virus. To our surprise, the infected Xanthi nc or Samsun NN protoplasts did not show any necrotic reaction (Otsuki et al., 1972).

One of the plausible explanations for the lack of necrotic reaction in protoplasts may be that the expression of the N gene requires interaction of cells in tissues. We hope that further studies with protoplasts will give a clue to the mechanism of action of the N gene.

Some viruses induce the formation of characteristic inclusion bodies in infected cells. Studies with leaf materials have provided little information as to when during

the infection cycle such inclusion bodies are formed and their function remains also obscure.

We showed with tobacco protoplasts that the genesis of the laminate inclusion body characteristic of PVX infection is preceeded by active virus multiplication (Honda et al., 1975). This finding excludes the possibility that the structure is somehow involved in virus production. Protoplasts should be useful similarly for defining the nature and the function of inclusion bodies induced by other viruses.

Several genes are known in tomato which confer resistance to TMV infection. Protoplasts from tomato lines carrying one such gene were immune to TMV, indicating that the function of this gene is to interfere with the establishment of infection (Motoyoshi and Oshima, 1975). It is possible that the latter gene controls the spread of virus within leaf tissues. These results illustrate the way in which protoplasts may be effectively used to characterize the genetically controlled resistance to virus infection.

A completely different area of protoplast research may be pertinent in the future to the control of virus diseases of plants. We have shown that individual tobacco leaf protoplasts are capable of forming callus-like colonies on an agar-containing medium (Nagata and Takebe, 1971). Organ differentiation can subsequently induced in the colonies and complete plants can eventually be regenerated. With the availability of haploid plants and their protoplasts in mind, this finding points to the possibility that the techniques of microbial genetics are applicable to higher plants.

In addition, the unusual properties of protoplasts to take up nucleic acids (Aoki and Takebe, 1969) or to undergo interspecific cell fusion (Kao and Michayluk, 1974) might provide opportunities of genetically modyfying plants by other means than sexual crosses. Many new possibilities may thus be envisaged using protoplasts to breed useful plants, including those resistant to virus infection.

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Discussion

E. W. Kitajima, Brazil: (7) Have you considered the possibility that plant cell would produce interferon or interferon-like substance?

(2) Is it possible to inoculate protoplast with viral RNA? What is the efficiency? Is it possible to detect any differences in virus growing curve when you inoculate complete virus or RNA?

Answer: (1) No, I have not. It seems to be unlikely that plant cells produce interferon or similar substance.

(2) Yes, but unfortunately with very low efficiency. We have not followed the growth curve of TMV in protoplasts inoculated with viral RNA, because of low level of infection achieved.

E. *W.* Kitajima, Brazil: You did it successively on tobacco leaf. Is there any difficulty in preparing protoplast of the other plant materials, such as rice plant?

Answer: Protoplasts can be obtained from leaves of many herbaceous species including some grasses. Unfortunately, however, rice is one of the most difficult species to isolate protoplasts. Rice leaves are extremely refractory to digestion by pectinase and cellulase.

D. A. Benigno, Philippines: In what form, as complete virus particle or only the RNA, does the TMV enter into cells? Is a single particle enough to cause infection in protoplast?

Answer: Strictly speaking, we have no definite information on this question, but there seems to be no indication to suggest that TMV enters into cells after it is uncoated. Theoretically, single virus particle should be enough to cause infection in a protoplast, but we have not proved it experimentally yet.

T. Soelaeman, Indonesia: Can the "isolated protoplast method" be used to culture mycoplasma?

Answer: It is unlikely that mesophyll protoplasts will be useful for culturing mycoplasma, because the pathogen does not multiply in mesophyll cells. To my knowledge, protoplasts have not been obtained from phloem cells where mycoplasma grows.

W. P. Ting, Malaysia: Once you have obtained protoplasts from leaf mesophyll of tobacco, can you regenerate more protoplasts from the isolated ones?

Answer: We can not. When isolated protoplasts are placed under such conditions that encourage cell division, they rapidly form cell walls on their surface, so that they are no longer protoplasts when they divide.