Strain-specific Pathways of Cytological Change in Individual Chinese Cabbage Protoplasts Infected with Turnip Yellow Mosaic Virus

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(Accepted 10 July 1979)

SUMMARY

Two mutually exclusive pathways of cytological change in the chloroplasts of Chinese cabbage cells infected with turnip yellow mosaic virus have been defined by light microscopic examination of individual infected protoplasts. In the first, all the chloroplasts in a cell became rounded and clumped together. This was followed by the development of a large vacuole, giving the chloroplast a sickled appearance. In the second pathway the chloroplasts became angular in outline before clumping, and subsequently fragmented to yield small pieces of chloroplast. Both these responses are controlled by the virus genome because some virus strains gave rise to the sickling response, while others cause fragmentation. Photosynthetic activity is a prerequisite for both pathways since they occur only when the protoplasts are illuminated, and both are inhibited by an inhibitor of photosynthetic electron transport.

INTRODUCTION

The pathogenesis of virus disease is a complex process, or series of processes, that is not yet understood for any plant virus. A long-term research objective is to delineate the biochemical steps by which the virus genome and the process of virus replication gives rise to the diseased state. For certain diseases, studies using light microscopy provide a useful bridge between macroscopic observations and electron microscopy and biochemical studies of diseased cells.

Turnip yellow mosaic virus (TYMV) infection of Chinese cabbage is a particularly favourable example for the use of light microscopy because the virus induces several characteristic gross changes in the chloroplasts of infected cells in addition to biochemical changes and structural changes seen only by electron microscopy. All strains of the virus induce numerous, very small vesicles near the periphery of the chloroplasts which are sites of virus RNA replication (Laflèche & Bové, 1969, 1971; Ushiyama & Matthews, 1970).

For all strains of the virus that have been described, chloroplasts in infected cells became rounded and subsequently clumped together. The rounding process occurs at an early stage following infection, while clumping occurs later, soon after virus production has begun (Hatta & Matthews, 1974). Furthermore it is known that different strains of the virus may differ in the other cytological effects they induce in the chloroplasts and that these changes may be associated with diseases of varying severity. Additional abnormalities noted in fresh sections from infected plants included angular plate-like chloroplasts, very large vacuoles and fragmented chloroplasts (Chalcroft & Matthews, 1967; Matthews, 1973, 1979). These latter abnormalities became apparent late in infection after most virus replication had already occurred.

Isolated protoplasts offer the possibility of studying the sequential development of cytological changes. Thus Matthews & Sarkar (1976) showed that when protoplasts isolated
from TYMV-infected leaves were exposed to light, a proportion of the rounded and clumped chloroplasts developed large clear vesicles, giving rise to a sickled appearance. The transition from rounding to sickling was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU). These experiments were limited by the fact that observations were made at various times on samples taken from a bulk culture of protoplasts.

In the work described here we have developed a method which permits the repeated examination by light microscopy of the changes taking place in individual protoplasts. Using this method, alternative and mutually exclusive pathways of disease development in chloroplasts have been delineated which we have termed sickling and fragmentation. Both processes are dependent on active photosynthetic electron transport and are dependent on some strain-specific function of the virus genome.

**METHODS**

**Plants and viruses.** The stock culture of TYMV used in this laboratory was maintained in Chinese cabbage plants (*Brassica pekinensis* Rupr. c.v. Wong Bok) grown in the glasshouse. Strains of the virus varying in the extent to which they affected chloroplast structure were isolated by the method of Chalcroft & Matthews (1967). Experimental plants were maintained in the glasshouse at 21 ± 3 °C under natural lighting, supplemented during winter with light from Philips HLRG 400 W lamps to give an 8 h dark period. TYMV was isolated from infected plants by the method of Matthews (1960) and stored at 4 °C in 50% glycerol.

**Chemicals and enzymes.** Macerozyme R 10 and cellulose ‘Onozuka’ R 10 were from Yakult Biochemical Co. Ltd, Nishinomiya, Japan. DCMU was from Serva, Heidelberg. Mannitol was from E. Merck, Darmstadt.

**Electron microscopy.** Protoplasts in 0.4 M-mannitol were fixed and post-fixed using the method of Takebe et al. (1973), in half-strength Millonig’s phosphate buffer, pH 7.5, containing glucose (Pease, 1964). Protoplasts were then embedded in agar using the method of Kellenberger et al. (1958) and passed through an acetone dehydrating series at room temperature. Embedding, sectioning and staining procedures have been described previously (Ushiyama & Matthews, 1970). Sections were examined with a Philips EM 301 electron microscope.

**Protoplast isolation.** Protoplasts were isolated from healthy or infected leaves by the method of Matthews & Sarkar (1976). The final pellet of protoplasts was washed three times with 0.4 M-mannitol and finally suspended in medium to give a protoplast density of 1 × 10³ protoplasts/µl. The medium used was that of Aoki & Takebe (1969), modified in the following ways: 0.4 M-mannitol, 5 x final concentration of Mg and half strength with respect to other components.

**A microdrop method for incubating protoplasts.** Study by light microscopy of time-dependent processes in infected protoplasts should be facilitated by a system with the following properties: (i) containment of a small number of protoplasts sufficiently immobilized in a confined space for the repeated recognition of individual protoplasts; (ii) adequate culture conditions for survival of protoplasts for a minimum period of 1 to 2 days; (iii) ready observation using the light microscope at any magnification.

Trials with various procedures and methods for microculture led to the simple system shown in Fig. 1 and 2 which was used in all subsequent work.

The following factors were found to be important for successful microculture. (i) Drop size: if the diam. of the drop when spread on the slide was too large (e.g. several mm) cells near the centre died within 24 h, presumably due to lack of oxygen. (ii) Shear: great care was needed when transferring the protoplasts by syringe. A very slow transfer rate was essential to avoid mechanical damage. (iii) Light intensity: survival was best in the light intensity
TyMV strains in protoplasts

RESULTS

Rounding, clumping and sickling

Protoplasts were prepared from Chinese cabbage leaves at various times after these had been inoculated and incubated in microdrops. At the time of isolation, the protoplasts were in three states with respect to TYMV infection: (i) infected; (ii) apparently normal but developing cytological signs of infection during the incubation period; (iii) apparently healthy.
Fig. 3. Two effects of TYMV infection on Chinese cabbage chloroplasts. A comparison of rounded clumped chloroplasts (a) and angular clumped chloroplasts (c). In the light rounded clumping gives rise to sickling (b) and angular clumping to fragmentation (d).

Observations on protoplasts which developed rounding and clumping during the period of observation showed that all the chloroplasts in a cell became involved in the process at an early stage. Within a given cell, the time between the earliest signs of rounding and full rounding and clumping was about 2 to 4 h.

By contrast, the development of sickling within the chloroplasts of any particular cell took 4 to 10 h and there was no detectable synchrony in the process for the chloroplasts within a single cell. Sickling was observed to begin only in protoplasts in which rounding
and clumping had fully developed. Chloroplasts retained a bright yellow-green colour throughout the sickling process. In some preparations of protoplasts from healthy leaves about 0.5 to 10% of cells showed or developed a contraction and clumping of the chloroplasts, but this was readily distinguished from the smooth rounding of chloroplasts in TYMV-infected cells.

Observations on many individual protoplasts confirmed that the development of sickling from clumped chloroplasts was dependent on light and inhibited by DCMU (e.g. Table I). The effects of light intensity and the time course of development in a population of individual protoplasts were very similar to those obtained by Matthews & Sarkar (1976) using flask cultures of protoplasts. Fig. 3(a) and (b) illustrate the sickling pathway.

Electron microscopic examination of protoplasts with sickled chloroplasts suggested that the vacuole formation which leads to the sickled appearance may be accompanied by a substantial swelling of the chloroplast, but we have no quantitative data on this point. The vacuole is bounded by a lipid bilayer membrane. It is unlikely that this membrane arises from the external chloroplast membrane since the latter in infected cells always contains numerous small vesicles which have never been observed in the membrane of the large vesicle. Observations on many large vesicles (not illustrated here) suggest that the membrane arises from distortions of stroma lamellae within the sickled chloroplast. The interior of the large vesicles is electron lucent (Fig. 4) except where the membrane has been ruptured allowing an influx of cytoplasmic material.

**Angular clumped chloroplasts and fragmentation**

In protoplasts isolated from leaves inoculated with the stock culture of TYMV most infected protoplasts showed rounded and clumped chloroplasts. However, in some, the clumped chloroplasts were angular rather than round (Fig. 3c). The time taken for the angular outline and clumping to develop was about the same as for rounded chloroplasts (2 to 4 h).

The angular chloroplasts underwent a slow colour change over a period of hours from a bright yellowish green to a grey-green colour. In the light such chloroplasts increased in diam. and gradually became webbed with fine lines dividing each chloroplast into sections. These sections rounded up (Fig. 3d) and became separated. The process was markedly
Table 1. Inhibition of fragmentation and sickling by DCMU*

<table>
<thead>
<tr>
<th>DCMU concentration</th>
<th>% infected protoplasts with fragmented chloroplasts</th>
<th>% infected protoplasts with sickled chloroplasts</th>
<th>% infected protoplasts showing only rounding and clumping</th>
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<td>oh 24 h</td>
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<td>0.0</td>
<td>0  36</td>
<td>6  28</td>
<td>94  36</td>
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<tr>
<td>$1 \times 10^{-4}$</td>
<td>0  1</td>
<td>2  4</td>
<td>98  95</td>
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* Protoplasts were prepared from leaves inoculated with a ‘yellow’ culture of TYMV which gave both fragmented and sickled cells. Slides were exposed to 2300 lux continuous illumination at 23 °C. Ten microdrops were assayed for each condition immediately after isolation and after 24 h.

Inhibition of fragmentation by DCMU

The experiment summarized in Table 1 confirms the observation of Matthews & Sarkar (1976) that the sickling response is inhibited by DCMU and shows that the fragmentation process is similarly inhibited.

Influence of virus strain on the proportion of sickled and fragmented chloroplasts

Six cultures of TYMV isolated from the stock culture of mixed strains and showing various local lesion types (and predominant systemic symptoms) were tested for the development of the sickling and fragmentation responses. The response in vitro by the isolated protoplasts was clearly dependent on the strain of virus used to inoculate the leaves. This is illustrated for two isolates in Fig. 5.

In synchronous with respect to the chloroplasts in a single cell. The fragmentation process did not occur in the dark. Fragmentation was an alternative to sickling, since each whole protoplast was committed to one or the other process. Angular clumping and fragmentation of chloroplasts like that found in TYMV-infected cells were not observed in chloroplasts of protoplasts derived from healthy plants.

Inhibition of fragmentation by DCMU

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Influence of virus strain on the proportion of sickled and fragmented chloroplasts

Six cultures of TYMV isolated from the stock culture of mixed strains and showing various local lesion types (and predominant systemic symptoms) were tested for the development of the sickling and fragmentation responses. The response in vitro by the isolated protoplasts was clearly dependent on the strain of virus used to inoculate the leaves. This is illustrated for two isolates in Fig. 5.
In this experiment and in others not described here there was a consistent correlation between the appearance of necrosis in the inoculated leaves and the development of the fragmentation response in isolated protoplasts. However, there was no evidence that strains producing necrosis in the leaf caused increased death of protoplasts in vitro. For example 80 to 90% of isolated protoplasts survived for 24 h at all leaf sampling times for both the strains illustrated in Fig. 5.

**DISCUSSION**

The development of a procedure for the repeated examination of individual protoplasts has enabled us to delineate alternate and apparently mutually exclusive pathways of disease induction by TYMV in the chloroplasts of infected cells. The usefulness of the technique will be limited of course to those rather uncommon host–virus combinations that give rise to cytological changes readily discernible by light microscopy.

Several biochemical changes associated with chloroplast activity have been reported for TYMV-infected tissue. Goffeau & Bové (1965) found that the Hill reaction and both cyclic and non-cyclic photophosphorylation were increased in chloroplasts of inoculated leaves compared with uninfected leaves. Bedbrook & Matthews (1972) found that, in systemically infected leaves, there was a diversion of the products of photosynthetic carbon fixation away from sugars and into organic acids and amino acids.

The fact that DCMU inhibits sickling and fragmentation shows that both processes require active photosynthetic electron transport, but the biochemical bases for the two kinds of disease process are not yet understood. Although both processes are seen only at a late stage of infection, the initiating biochemical activity may well occur very soon after infection. Since the sickling and fragmentation responses are markedly strain-dependent and since we have never observed the two responses in the same cell, it is likely that most individual cells in a diseased plant are infected entirely or primarily by one strain of the virus (Matthews, 1973).

Our results show that in further work aimed at exploring the biochemical basis for these cytological effects it will be necessary to use single strains of the virus or cultures containing predominantly a single strain. This may be a difficult objective, since it is known that single lesion isolates from the stock culture of TYMV rapidly revert to a mixture of strains.

We thank Mrs J. Keeling for assistance with electron microscopy.

**REFERENCES**


(Received 9 April 1979)