Cucumber Green Mottle Mosaic Virus Infection and its Bearing on Cytological Alterations in Tobacco Mesophyll Protoplasts

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SUMMARY
Protoplasts isolated from *Nicotiana tabacum* (cv. Xanthi) leaves were infected with cucumber green mottle mosaic virus (CGMMV). Progeny CGMMV particles were first detected in the protoplasts at 24 h after inoculation. They were in small crystalline arrays confined in the ground cytoplasm. The earliest detectable cytological modification in the protoplasts was the presence of peripheral vesicles in the mitochondria at 24 h after inoculation. No other effects on the cytoplasm or organelles were noted.

INTRODUCTION
Cucurbitaceous plants become systemically infected with cucumber green mottle mosaic virus (Inouye *et al.* 1967; Komuro *et al.* 1971), and virus infection induces numerous small vesicles in the perimitochondrial spaces between outer and inner membranes (Hatta *et al.* 1971; Hatta & Ushiyama, 1973). Although *Nicotiana tabacum* (cv. Samsun, White Burley, Xanthi) plants inoculated with the cucumber strain of CGMMV showed no symptoms, the virus could be detected, in inoculated leaves only by back transmission to *Cucumis sativus* and to *Datura stramonium* (Inouye *et al.* 1967; Komuro *et al.* 1971; Y. Sugimura, unpublished data). The present work was undertaken to examine the infection of tobacco mesophyll protoplasts isolated from *N. tabacum* cv. Xanthi by CGMMV and showed that virus multiplication induces small peripheral vesicles in mitochondria of infected protoplasts. Recent research on tobacco mesophyll protoplasts infected with various other viruses has been reviewed by Takebe (1975).

METHODS

**Virus.** CGMMV-C (cucumber strain) was purified from systemically infected leaves of *Cucumis sativus* L. grown in the glasshouse. The procedure for virus purification was that of Nozu *et al.* (1971), except that 0.1 M-phosphate buffer containing 0.1 M-thioglycollic acid was used instead of 0.01 M-KCN.

**Preparation and inoculation of protoplasts.** Fully expanded leaves (15 to 20 cm long) of *Nicotiana tabacum* cv. Xanthi grown in the glasshouse at 25±3 °C were used for the isolation of protoplasts. The protoplasts were isolated from palisade parenchyma cells by

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the procedures reported previously (Takebe, Otsuki & Aoki, 1968; Takebe & Otsuki, 1969). Inoculum contained purified CGMMV (2 µg/ml) in 0.02 M-potassium citrate buffer (pH 5.2) containing mannitol (0.7 M) and poly-L-ornithine (mol. wt. 130000, Pilot Chemicals, USA; 2 µg/ml). After standing at 25 °C for 10 min, the inoculum was added to an equal volume of protoplast suspension in 0.7 M-mannitol, and the mixture was incubated at 25 °C for 10 min. After incubation, the protoplasts were separated from the inoculum virus and were washed several times with sterile 0.7 M-mannitol containing 0.1 mM-CaCl₂. The washed protoplasts were transferred aseptically into the incubation medium (Aoki & Takebe, 1969) and cultured at 28 °C for 48 h under continuous illumination from fluorescent lamps (about 3000 lux).

Preparation of antisera and fluorescent antibody staining. The purified virus was suspended in 0.01 M-phosphate buffer (pH 7.0) containing 0.85 % NaCl for injection. Intramuscular injections of 10 mg CGMMV emulsified with Freund's complete adjuvant were made into the hind-foot pads of a rabbit three times at intervals of 7 days. Finally an intravenous injection of 10 mg CGMMV was made into the marginal ear vein. The rabbit was bled 20 days after the last injection. Antibody to CGMMV was prepared and conjugated with fluorescein isothiocyanate (FITC) by the technique of Otsuki & Takebe (1969). The immunoglobulin G fraction was isolated by ammonium sulphate precipitation from antiserum with a ring test titre of 1/2000 to 1/4000. The conjugated CGMMV antibody had a FITC/protein molar ratio of 2.7 as calculated from the extinction at 280 and 495 nm. Protoplasts were fixed in 95 % ethanol for 10 min at room temperature and were then stained with a 1/20 dilution of the fluorescent antibody for 90 min at 36 °C. The stained protoplasts were examined under a fluorescence microscope.

Infectivity assay of protoplast extracts. At 12, 24 and 48 h after inoculation, protoplasts were collected by low speed centrifuging from 20 ml of protoplast suspension (3 to 4 x 10⁵ protoplasts/ml). The collected protoplasts were washed several times with sterile 0.7 M-mannitol containing 0.1 mM-CaCl₂ and frozen at −5 °C. They were then homogenized in 10 ml of 0.1 M-phosphate buffer (pH 7.0). The crude homogenate was centrifuged at 6000 g for 15 min, and the supernatant solution was assayed for infectivity on half leaves of Datura stramonium. The opposite half leaves were mechanically inoculated with a purified CGMMV preparation.

Electron microscopy. At 12, 24 and 48 h after inoculation, the protoplasts were harvested and fixed for 4 h in 6 % glutaraldehyde in 0.025 M-sodium phosphate buffer (pH 7.0) containing 10 % sucrose. They were washed twice with 0.1 M-sodium phosphate buffer containing 10 % sucrose and then post fixed in 1 % OsO₄ in 0.1 M-sodium phosphate buffer for 90 min at room temperature. Dehydration was carried out through a graded ethanol series beginning at 70 % ethanol. After dehydration, fixed protoplasts were transferred to Epon via propylene oxide and finally embedded in Epon. Thin sections were cut on an LKB Ultratome 88014 with a glass knife and then stained with uranyl acetate and lead citrate. Sections were examined with a JEM-100 electron microscope at 80 kV.

RESULTS

Fluorescent antibody staining of infected protoplasts

CGMMV-antigen was first observed by fluorescent antibody staining in the protoplasts at 24 h after inoculation, but the staining was very weak. At 48 h after inoculation, the small fluorescent masses had increased in number and were usually scattered in the
cytoplasm (Fig. 1). At this stage, about 60 to 70 % of the protoplasts contained specifically staining material. No such specific fluorescence was found in uninoculated protoplasts.

Detection of progeny virus particles in protoplasts

Extracts prepared from inoculated protoplasts taken at intervals after inoculation were assayed for infectivity. Local lesions were produced by protoplast extract taken at 48 h after inoculation, but not at 12 and 24 h. However, the local lesions were very few (3 to 6 local lesions per half leaf), suggesting that infected protoplasts might contain very small amounts of CGMMV.

In another electron microscopic examination to detect virus particles in the early stages of infection, extracts from protoplasts (20 ml of protoplast suspension) were subjected to two cycles of differential centrifuging at 5000 g for 10 min and 105,000 g for 90 min at 4 °C. The resuspended final pellet was negatively stained with 2 % uranyl acetate and then observed. CGMMV particles were observed in negatively stained preparations obtained at 24 and 48 h after inoculation, but not in protoplast extracts taken at 12 h after inoculation. Thus, CGMMV multiplied in the protoplasts during 24 h incubation, but the yield of virus was below the limit required for production of visible local lesions.

Electron microscopy

General features of uninoculated protoplasts

Isolated protoplasts were ultrastructurally quite similar to those observed by Otsuki et al. (1972) and Takebe et al. (1973). Some of the chloroplasts in the protoplasts appeared to contain inclusion bodies in their stroma, as described by the above authors. Lipid-containing bodies were more frequently observed in the cytoplasm of protoplasts than of cells in intact tobacco leaves. Internal structures of the protoplasts were well preserved during 48 h incubation, and there was no degenerative change in the nuclei or mitochondria, but some chloroplasts had a tendency to disintegrate.
Y. SUGIMURA AND R. USHIYAMA

Cytological changes in inoculated protoplasts

The earliest detectable cytological change in protoplasts inoculated with CGMMV was the appearance of small vesicles in the perimitochondrial spaces between outer and inner membranes (Fig. 2). These vesicles first formed between 12 and 24 h after inoculation. Most vesiculate mitochondria retained their normal shape, but they enlarged at 48 h after inoculation when large numbers of vesicles appeared and more mitochondria were affected. In the vesiculate mitochondria, the small vesicles were about 40 to 80 nm in diam. and contained fine stranded material which resembled nucleic acid in its ultrastructural appearance (Schreil, 1964). The vesicles were similar to those observed in cells of Cucumis sativus and other host plants infected with CGMMV (Hatta et al. 1971; Hatta & Ushiyama, 1973). In addition to these changes, electron-dense amorphous material was found in the area where the mitochondrion was cut tangentially and through the peripheral vesicles (arrow in inset of Fig. 2). The nature of the electron-dense substance is not known. No other c.p.e. of virus infection were observed in protoplasts cultured for up to 48 h after inoculation.

Accumulation of virus particles

Small arrays of CGMMV particles were occasionally observed in the ground cytoplasm of protoplasts sampled at 24 h after inoculation (Fig. 3), but not at 12 h. They were confined to the cytoplasm, and some were attached to the disrupted periphery of one mitochondrion (Fig. 4). Whether the virus particles attached to the mitochondrion after being formed in
Fig. 3 and 4. Sections of protoplasts showing a virus aggregate (V) in the ground cytoplasm (Fig. 3) and with some virus particles attached to an abnormal mitochondrion (Fig. 4) at 24 h after inoculation. Inclusion bodies (I) are seen in the stroma of the chloroplast (C); one is detached from the chloroplasts (Fig. 3).

the other regions of the cytoplasm, or whether the particles were produced there is not clear. Virus particles were more frequently found in protoplasts of the 48 h sample than in the 24 sample.

**DISCUSSION**

Our observations show that the virus antigen can be detected in about 60 to 70% of CGMMV-inoculated protoplasts after incubation for 48 h, but the yield of virus particles per protoplast seems to be low compared with that of tobacco mosaic virus (Otsuki &
Takebe, 1969; Hibi & Yora, 1972; Kagi & Ushiyama, 1972; Otsuki et al. 1972). Motoyoshi & Hull (1974) likewise observed that although tobacco protoplasts isolated from *Nicotiana tabacum* cv. White Burley were infected with pea enation mosaic virus (PEMV), they contained relatively few virus particles. It is assumed that CGMMV and PEMV multiply to a similarly limited extent in tobacco protoplasts.

In the course of CGMMV replication, the only structural modification of organelles in tobacco protoplasts is the appearance of small vesicles at the periphery of mitochondria. These vesicles resemble those induced in intact leaf cells of *Cucumis sativus* and other hosts infected with CGMMV (Hatta et al. 1971; Hatta & Ushiyama, 1973). Our work therefore suggests that the formation of mitochondrial vesicles in CGMMV-infected cells is independent of host species. Furthermore, the mitochondrial vesiculation seems to occur during the production of the first progeny virus particles. However, we have no evidence as to how these vesicles could be involved in the process of virus replication.

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REFERENCES


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