An Electron Microscope Study of Starch Lesions in Cucumber Cotyledons Infected with Tobacco Mosaic Virus

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ABSTRACT

Starch lesions in cucumber cotyledons became evident under the light microscope 2.0 - 2.5 days after inoculation. At this time, many of the chloroplasts were markedly swollen, without necessarily containing starch grains. At 21 C, lesions reached their final size 5 days after inoculation.

Paradermal sections taken through the palisade layer 5 days after inoculation revealed tobacco mosaic virus (TMV) particles in two types of cells.

In central cells of the lesion, chloroplasts were extremely swollen, and contained large starch grains. Virus particles, scattered or in crystalline arrays, were present in the cytoplasm. At the periphery of the lesion about half the cells, although containing virus, were otherwise normal. The others carried virus and contained chloroplasts with large starch grains. Structures, resembling plasmodesmata, connect these cells with neighboring normal noninfected cells. Observations were similar when lesions were sampled 7-8 days after inoculation.

The finding that in a mature lesion virus-carrying, though otherwise normal-appearing, cells directly border noninfected cells, suggests that in cucumber cotyledons localization of TMV is not due to ultrastructural changes in advance of the infection, or to blocking of plasmodesmata.

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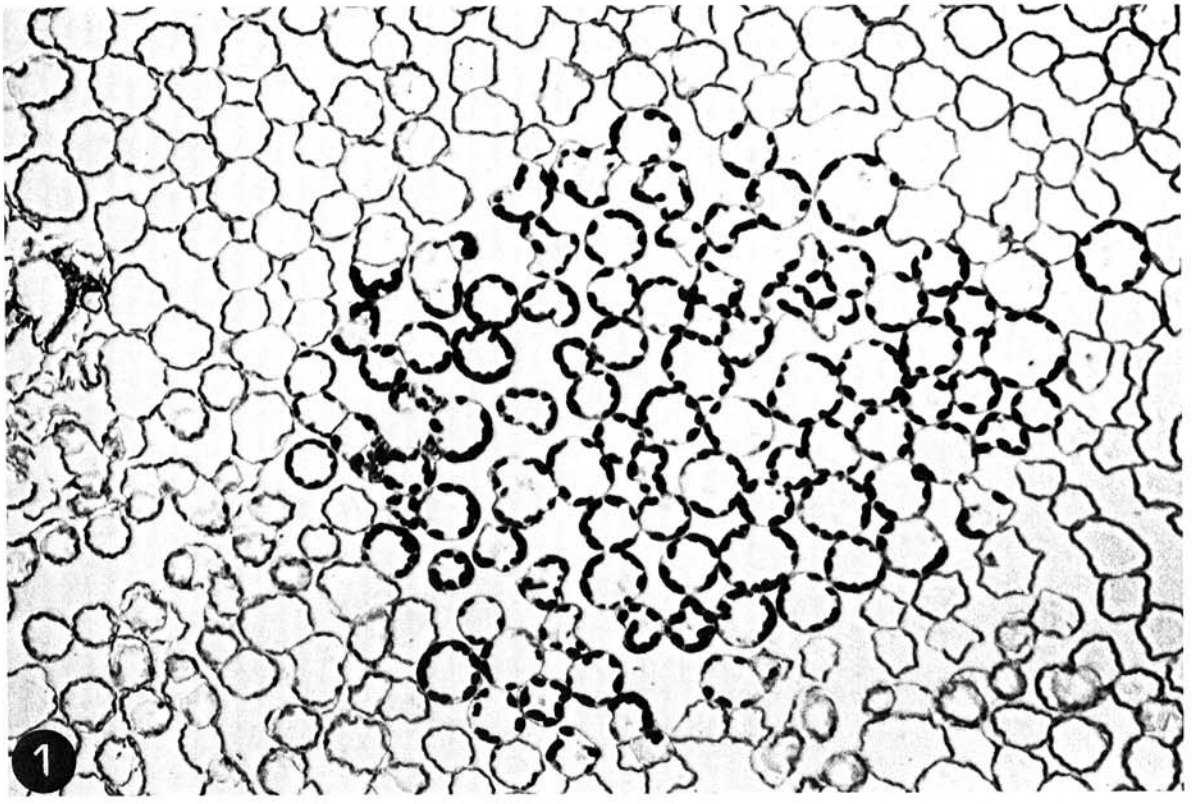
Cytological and ultrastructural alterations in response to virus infection have been associated with blocking mechanisms which effectively checks virus spread beyond necrotic local lesions (6). Thus, based on electron microscope examination of the region surrounding a necrotic tobacco mosaic virus (TMV) lesion, Ross and Israel (10) postulated that structural changes in advance of infection lead to cell collapse, and thus may be instrumental in virus localization. Subsequently, it was suggested that this collapse is largely due to quinone accumulation (11). In the uninfected zone surrounding a TMV lesion in Pinto bean leaves, membrane-bound vesicular bodies were observed between the plasmalemma and cell wall. Plasmodesmata in the cell wall adjacent to these paramural bodies appeared to be broken (12). Blocking of plasmodesmata was also observed as a result of callose deposition, which appeared as a secondary cell wall thickening in the periphery of lesions incited by potato virus M (4).

However, these studies, as well as others (15) were done in necrotic lesion hosts. The question was therefore raised, whether these structural changes are not a response to necrosis and not necessarily related to the localizing process (6). We were therefore interested in studying the ultrastructure in a starch-lesion host, where the infection remains localized without causing necrosis. Changes in such a host can be related to the localizing process, without having to consider their possible association with the necrotic reaction.

MATERIALS AND METHODS.—*Cucumis sativus* L. 'Bet Alpha' plants were grown in 10-cm diameter pots in a greenhouse. Eight days after seeding, the first leaf was trimmed and the plants were transferred to a 21 C greenhouse. One day later, the cotyledons were uniformly inoculated on their upper side with a purified preparation of TMV to obtain a density of about 120 starch lesions per cotyledon. Density of starch lesions in each batch was checked on ten cotyledons, after removing the chlorophyll by heating in 70% ethanol at 80 C. Starch lesions were developed by placing the cotyledons in a I-KI-lactic acid mixture, according to Lindner et al. (5). Uninoculated plants, for control, were kept similarly. Lesion size was determinated by measuring two diameters, at right angles to each other, under a stereoscopic microscope equipped with an ocular micrometer. One hundred lesions selected at random from 7-8 cotyledons were measured and the mean diameter calculated.

For electron microscopy, 3-mm diameter disks were punched out from the cotyledons under 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7. The disks were transferred to fresh fixative solution for 30 min at room temperature under vacuum, and then for 90 min at 4 C. The samples were rinsed and postfixed overnight in 2% (w/v) osmic tetroxide in the same buffer at 4 C. After dehydration through graded ethanol, and several passages through propylene oxide, samples were embedded in Epon mixture (8) and polymerized at 60 C for 72 hours. Starch lesions were located with the aid of a phase or bright-field microscope on 2- to 3-μm-thick sections cut paradermally or across the 3-mm disk, after it had been stained for 2 min in a dilute aqueous I-KI mixture (Fig. 1). Ultrathin sections (ca. 60 nm) were then cut with glass knives on an LKB Ultrotome III. Sections were stained with aqueous 6% uranyl acetate for 10-15 minutes, counterstained with lead citrate for 5 minutes, and examined with a JEM 7A electron microscope operating at 80 kv. About 50 lesions were sampled and examined.

RESULTS.—Starch lesions became evident under the light microscope 2.0 - 2.5 days after inoculation, having a slight reddish coloration. Lesion diameters increased from 0.36 mm on the third day to 0.45 mm on the fifth day after inoculation (average from three experiments). No further increases in lesion diameters were detected up to 14 days after inoculation. When sampled 5 days after inoculation and viewed perpendicular to the surface, about 70-100 darkly stained palisade cells were observed in an average lesion (Fig. 1). In a cross section, darkly stained cells were observed in the epidermal and palisade



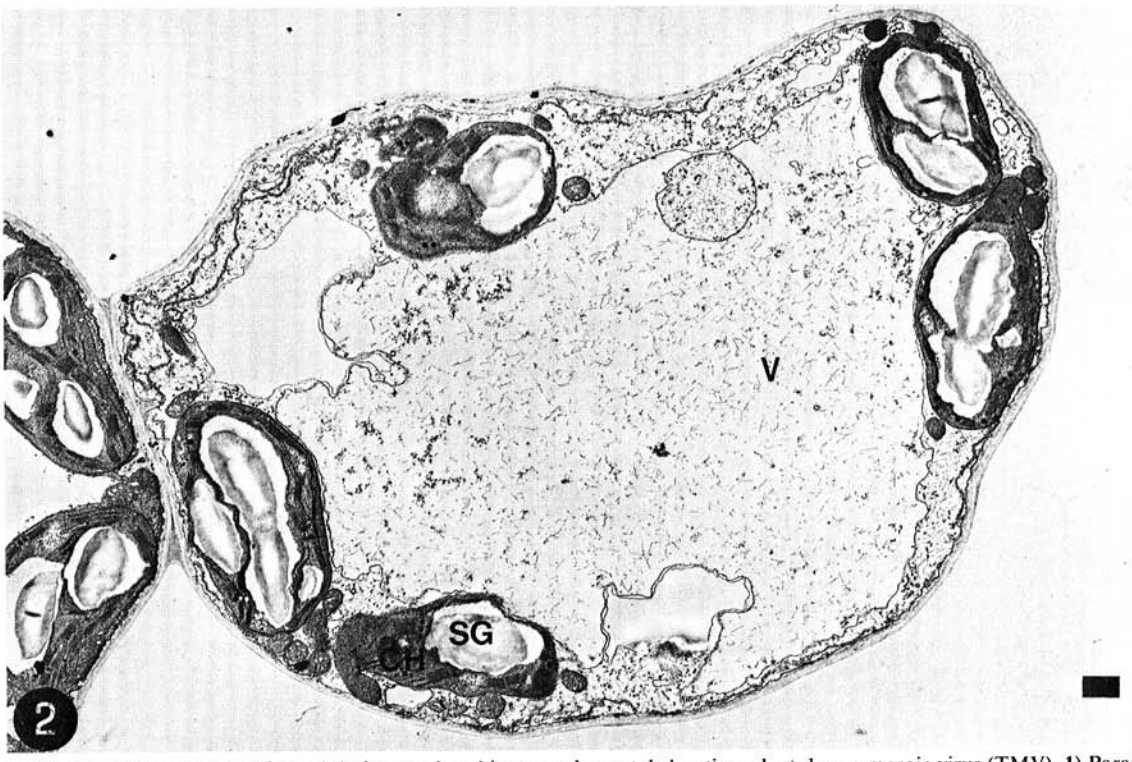
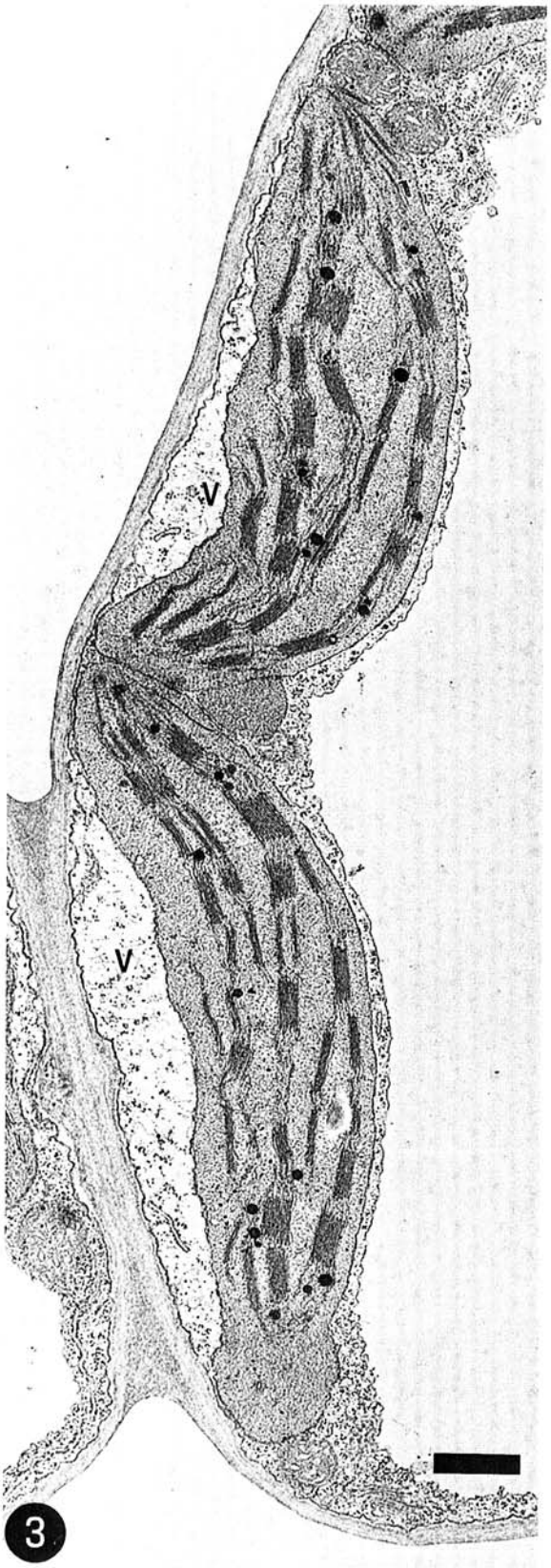


Fig. 1-2. Ultrastructure of starch lesion produced in cucumber cotyledon tissue by tobacco mosaic virus (TMV). **1)** Paradermal section after staining with dilute I-KI water mixture. Light microscope view (× 160). **2)** Section of a palisade cell in the center of the lesion, 5 days after inoculation. Chloroplasts (CH) are distorted and heavily invested with starch grains (SG); V = virus; calibration bar = 1 μm.



layers, as well as in the next two or three spongy parenchyma layers. However, the area of darkly stained cells decreased from the palisade layer towards the spongy parenchyma, giving the vertical section of the lesion a trapezoidal appearance.

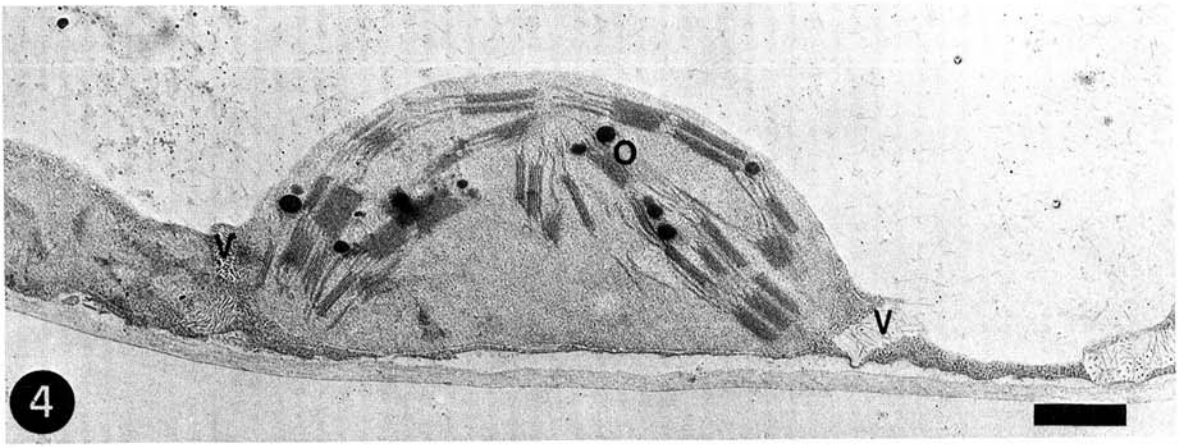
Within a lesion sectioned paradermally through the palisade layer, TMV particles were observed in two types of cells. All the cells in the center of the lesion had marked changes in their chloroplasts (Fig. 2), whereas about half the cells in the periphery of the lesion, though containing virus particles, were otherwise normal (Fig. 3). The other cells in the periphery contained chloroplasts with large starch grains and virus particles. No ultrastructural changes were observed in the zone of apparently noninfected cells surrounding the lesion. No differences in the appearance of the cells from the center of the lesion were observed when plants were kept in the dark for 24 h before sampling.

When cells in the center of the lesion were observed 2.0 - 2.5 days after inoculation, many of the chloroplasts were markedly swollen, without containing starch grains (Fig. 4). TMV particles could easily be observed in the central cells of the lesion. When viewed 5 days after inoculation, when lesions had reached their final size, some cells, especially in the palisade layer, were distorted. In extreme cases shrinkage of cells resulted in the formation of large intercellular spaces, so that cells at some points became separated from each other (Fig. 5). Almost all chloroplasts at 5 days were extremely distorted (Fig. 6), their width being twice that of a normal chloroplast. Osmiophilic globules were seen in some of the chloroplasts. The chloroplasts contained large starch grains changing the order of the thylakoid system. Grana were displaced towards the periphery of the chloroplast. TMV particles, scattered (Fig. 8) and in crystalline (Fig. 6) arrays, were present in the vacuole and the cytoplasm. Occasionally, the starch grains appeared to be broken down into fragments (Fig. 7). More microbodies, between 5 and 10, in a cross section of a palisade cell, were observed, compared with from zero to two microbodies found in a comparable noninfected cell (countings from 45 infected and control cells).

Encircling the lesion center, there was a layer of one-to-two cells, in which the virus content appeared to be lower (Fig. 9). Otherwise, these cells resembled those in the center of the lesion. Their chloroplasts were distorted and contained large starch grains. Some of the cells were shrunken. Occasionally structures resembling paramural bodies (12) were observed in this layer, as well as in the central cells of the lesion.

Towards the periphery of the lesion, viewed perpendicular toward the surface, cells that contained virus, but without any apparent ultrastructural alterations, were evident (Fig. 10). Cell wall thickenings were occasionally observed, which however, did not differ from similar thickenings in healthy control cells. No

Fig. 3. Portion of a virus (V)-containing palisade cell in the periphery of the lesion 5 days after inoculation. No distortion of chloroplasts; calibration bar = 1 μm.



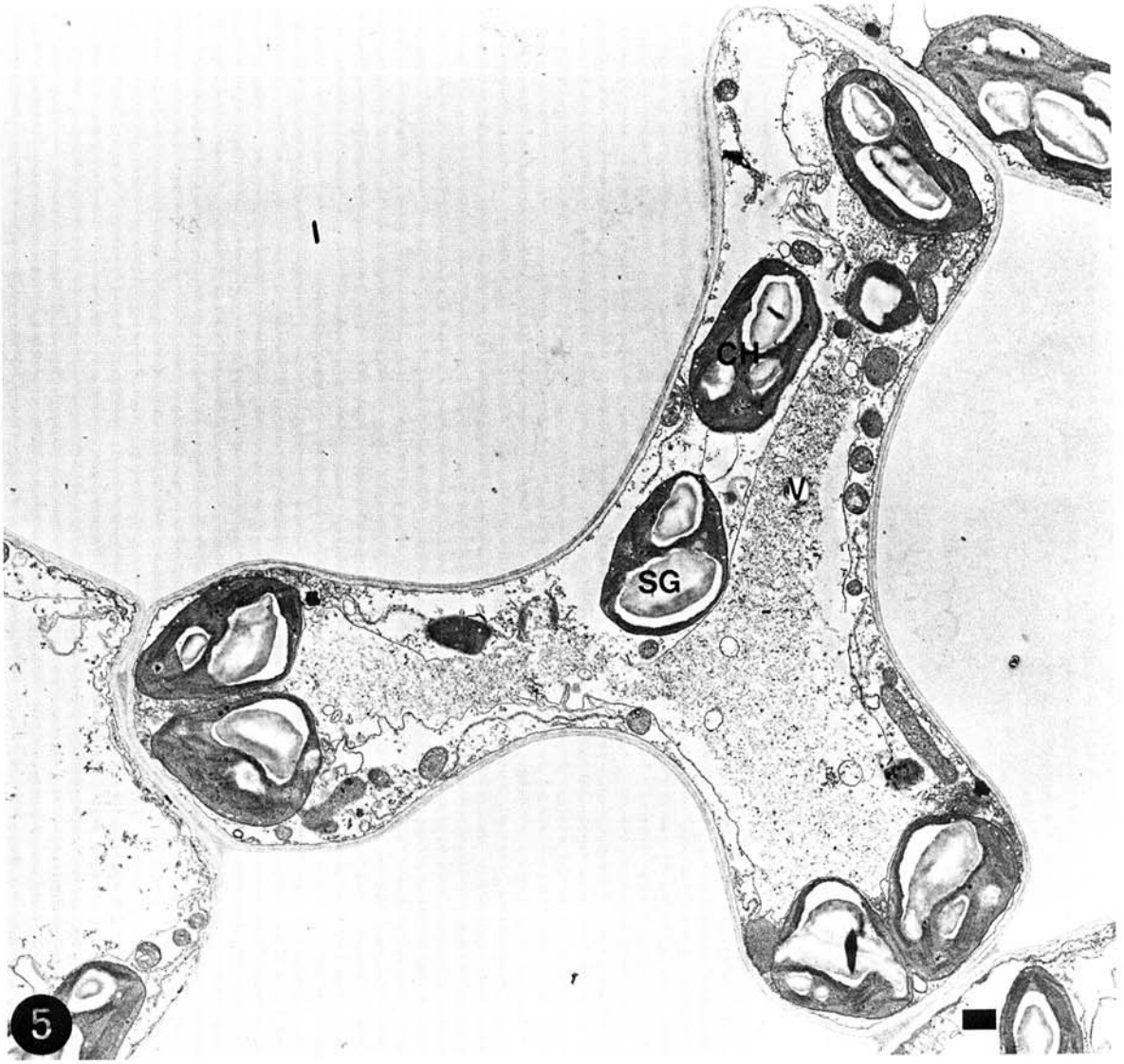


Fig. 4-5. Ultrastructure of starch lesions. **4)** Swollen chloroplast without starch grains in palisade cell, 2-1/2 days after inoculation with TMV. **5)** Shrinkage of palisade cell from the center of the lesion 5 days after inoculation; CH = chloroplast, O = osmiophilic material, SG = starch grain, V = virus; calibration bar = 1 μm.

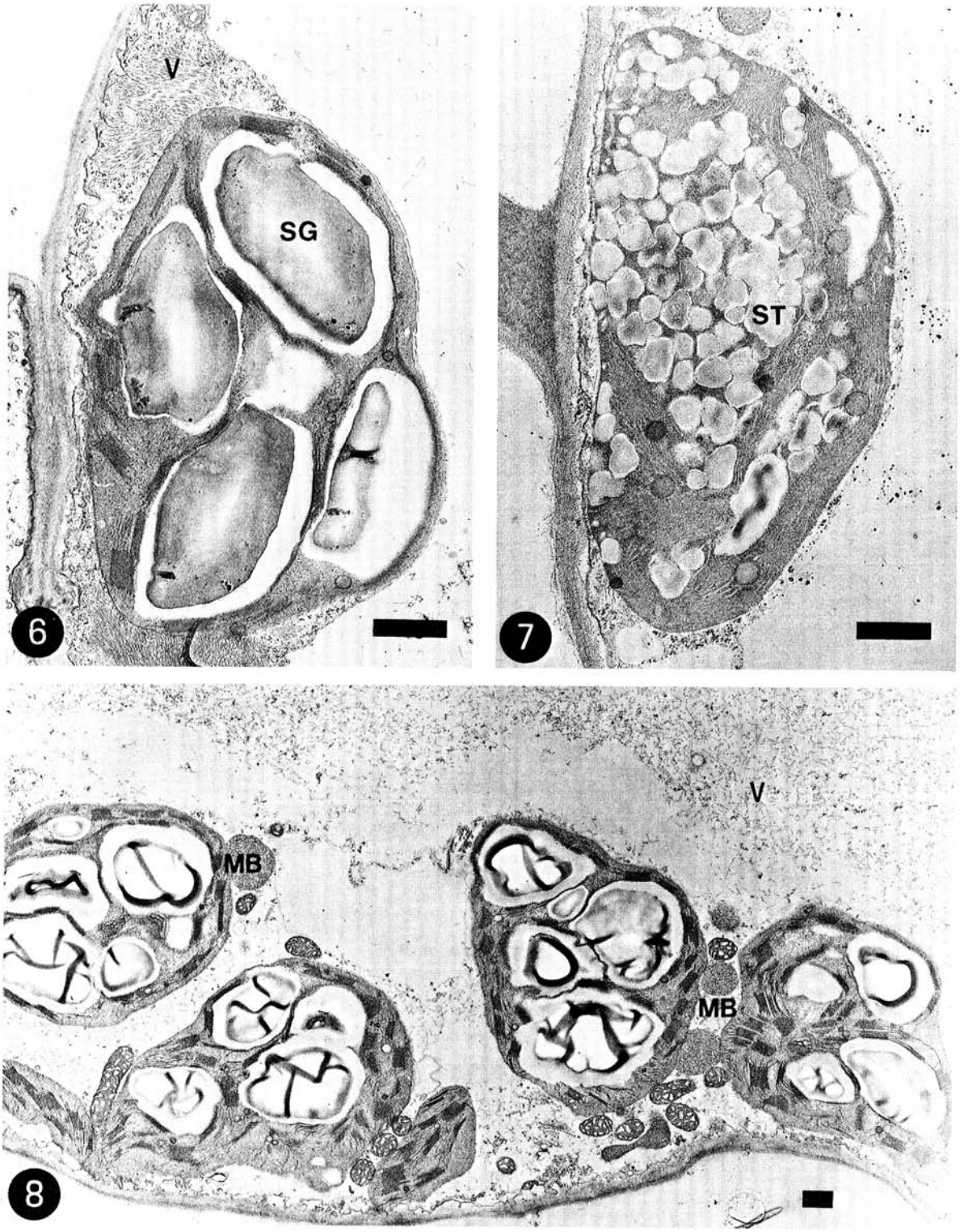


Fig. 6-8. 6-7) Chloroplasts from palisade cells from lesion center. **6)** Distorted chloroplast, whose thylakoid system is displaced by large starch grain (SG). Crystalline array of TMV (V) in the cytoplasm. **7)** Chloroplast with starch fragments (ST). **8)** Portion of a palisade cell from the center of the lesion with scattered TMV (V) particles in the vacuole; MB = microbody; calibration bar = 1 μm.

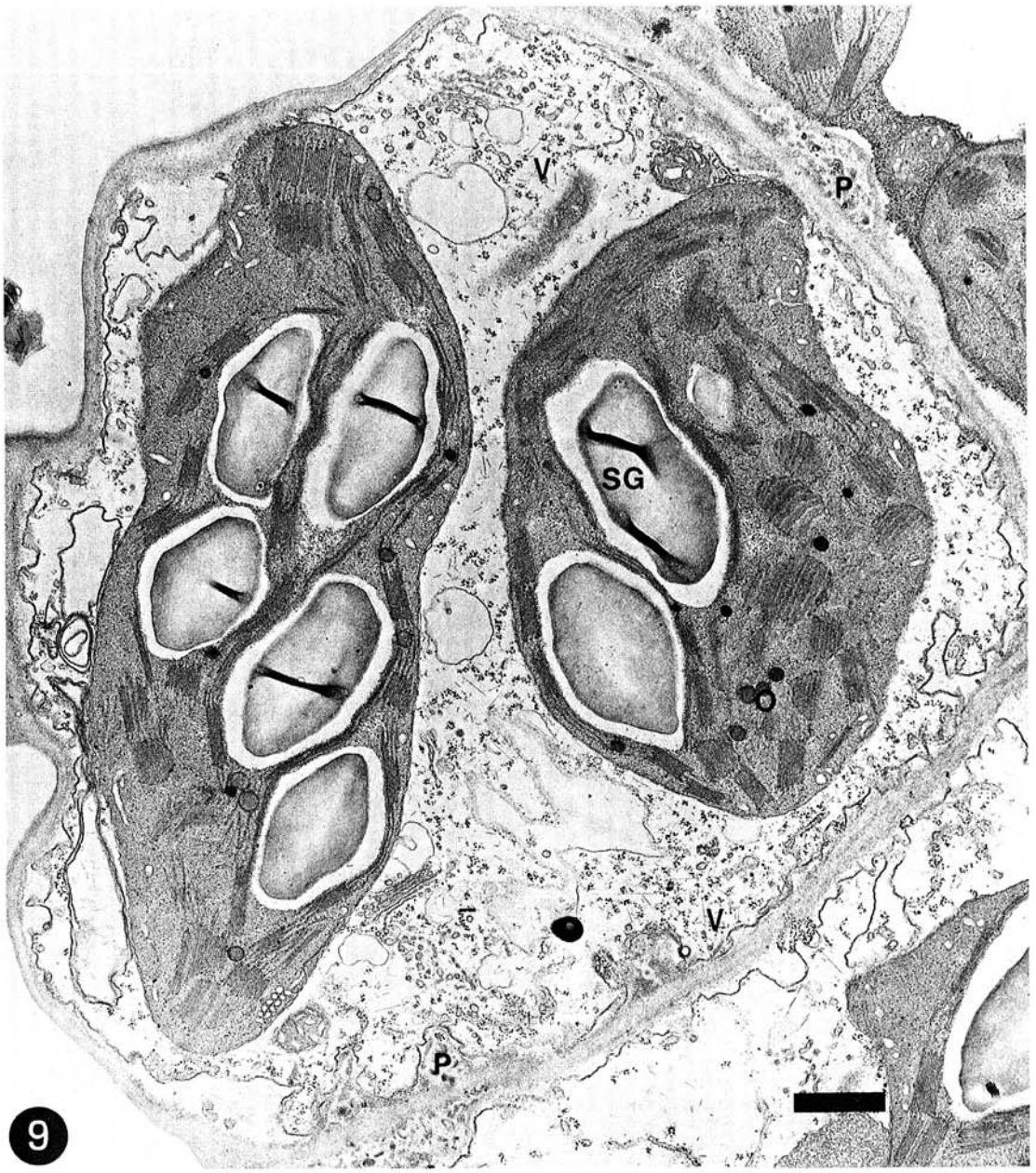


Fig. 9-10. 9) Section of a palisade cell with distorted chloroplasts and low virus (V) content from the zone surrounding the lesion core. 10) Portion of an infected palisade cell from the periphery of the lesion without distortion of chloroplasts: Note virus packet (V). O = osmiophilic material, P = paramural body, SG = starch grain; calibration bar = 1.0 μm.

paramural bodies were seen in these cells. Sometimes one or two layers of such cells were observed, although in some cases cells with marked changes in their ultrastructure bordered directly healthy, apparently uninfected cells. Virus in these cells was either scattered or in packets. Virus content in these cells was estimated in 24 sections. The number of particles in one plane averaged about 30-40 per cell compared to 300-400 particles in the central cells of the lesion (20 sections). In two series of serial sections through such cells, no ultrastructural changes were detected. Structures, resembling plasmodesmata, connected these cells with neighboring noninfected cells (Fig. 11). The number of plasmodesmatal connections in cucumber parenchyma was extremely low. In each section, only one connection per 8-10 cells was observed, with no difference (number and shape) between infected cells or healthy controls. The surrounding noninfected cells were normal.

The fine structure of the lesion was similar when sampled 7-8 days after inoculation.

DISCUSSION.—Marked ultrastructural changes were observed in the central cells of the starch lesion. Chloroplasts were extremely swollen and contained large starch grains, displacing the thylakoid system. Swelling of chloroplasts became apparent before accumulation of starch granules. Occasionally, starch grains were fragmented (Fig. 8). The reason for this is unknown. It may be that due to extensive swelling of the chloroplasts, the plastidial envelope is ruptured, giving access to starch-degrading enzymes. More microbodies were also observed in these cells than in noninfected controls, and shrinkage of cells often resulted in the formation of large intercellular spaces.

TMV particles, scattered or in crystalline arrays, were present in the cytoplasm. Virus content seemed to be higher in the central cells of the lesion than in the peripheral cells. However, at the boundary of the lesion many cells were observed that contained virus, but had no apparent ultrastructural alterations. These cells seemed to be connected by plasmodesmata-like structures to neighboring noninfected normal cells.

In previous studies no callose deposition was observed around the starch lesion (7); in the present study there were no indications, that, in the peripheral normal-appearing cells that contain virus, plasmodesmata are blocked, either by paramural bodies or cell wall thickening. Our observation, that in a lesion that had reached its final size, virus-carrying (though otherwise normal-appearing) cells border directly with noninfected cells, suggests therefore that at least in cucumber cotyledons localization of TMV is not due to ultrastructural changes in advance of the infection, or to blocking of plasmodesmata. Apparently the normal cells containing virus do not support virus synthesis.

Aberrant starch metabolism and accumulation of starch is known in infections caused by obligate parasites (3). In viral diseases, accumulation of starch has been observed mainly in deteriorating chloroplasts in necrotizing cells (1, 14), and seldom in nonnecrotic infections (2). Accumulation of starch in TMV-infected cucumber cotyledons, where no necrosis occurs, may indicate that changes associated with the chloroplast, and not cell necrosis per se, are involved in starch accumulation. Several physiological mechanisms have been proposed to explain starch accumulation in diseased plants. It has been hypothesized that a decrease in β-

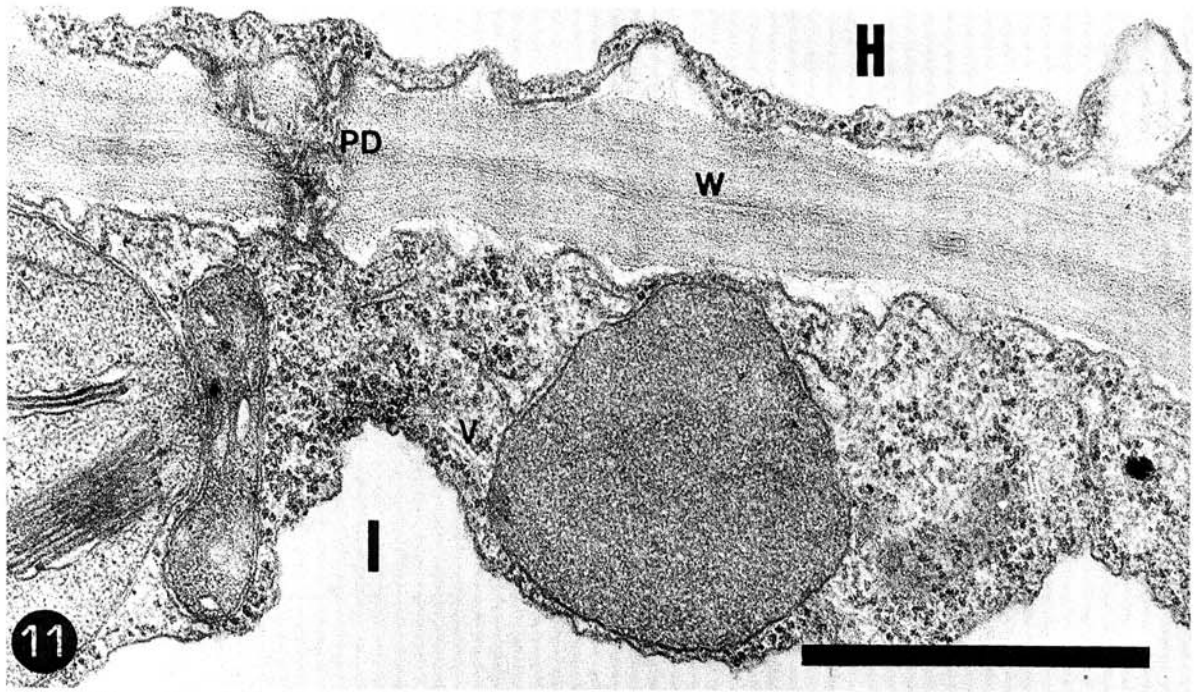


Fig. 11. Plasmodesmata-like structures (PD) connecting infected (I), but otherwise normal, cells with noninfected cells (H); W = cell wall, V = virus; calibration bar = 1 μm.

amylase is responsible for increased starch content (13). Recently, evidence has been presented suggesting that regulation of adenosine diphosphate-glucose pyrophosphorylase by effector molecules controls accumulation of starch in rust-infected wheat leaves (9). It may be worthwhile to verify this suggestion in the TMV-cucumber cotyledon system.

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