Geminivirus-Induced Macrotubules and Their Suggested Role in Cell-to-Cell Movement

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ABSTRACT

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Ultrastructural responses of *Datura stramonium* (an experimental host of Euphorbia mosaic virus, a whitefly-transmitted geminivirus) were studied in situ. Cells of the earliest chlorotic lesions in mechanically inoculated leaves revealed cytopathic effects that were not observed in systemically infected leaves or in advanced lesions of inoculated leaves. These were the occurrence of macrotubules containing geminate viruslike particles in the cytoplasm and continuation of the macrotubules with the plasmodesmata. No viruslike particles occurred in the plasmodesmata of cells that were apparently without macrotubules, suggesting that the presence of the macrotubules is required for the occurrence of viruslike particles in the plasmodesmata. The nuclei of cells that had macrotubules contained characteristic nucleopathic effects of geminivirus infection, indicating that the macrotubules were virus-induced. It is suggested that the macrotubules are involved in cell-to-cell movement of the virus in early stages of infection in this host.

Additional keywords: cytopathology, virus-induced inclusions.

Since the etiological agents of bean golden mosaic (8,9), maize streak, and cassava latent mosaic (10,11) diseases were first discovered to be caused by geminiviruses, more than 20 diseases have been reported to be caused by or associated with geminiviruses.

Geminiviruses have unique particle morphology and are the only plant virus group with a single-stranded DNA genome. They also induce characteristic cytopathic effects that are distinct from those induced by other groups of plant viruses (4,19). These effects, namely nucleolar segregation and occurrence of DNA containing fibrillar bodies, have been helpful in diagnosing many diseases caused by geminiviruses, especially those whitefly-transmitted viruses infecting dicot hosts (13,14,17,19,22,25,26,28).

In all cases of geminivirus infections studied, these cytopathic effects, which precede the accumulation of virus particles, are confined to the nuclei of infected cells. This has led to the assumption that viral DNA replication and viral assembly take place within the nucleus (11). The fact that virus particles are localized only in the nuclei of infected cells (except for sieve elements where the nucleus degenerates naturally) raises a question of how the virus particles are released from the nucleus into the cytoplasm to undergo cell-to-cell spread. This question is especially pertinent in the hosts in which the virus is not limited to the phloem but occurs in all types of cells. No structural features suggestive of cell-to-cell movement of virus particles, such as the occurrence of virus particles in tubules and/or in the plasmodesmata, as are common in infections of comoviruses, nepoviruses, and caulimoviruses (5–7), have been reported to occur in cells of hosts infected with any of the geminiviruses.

This paper reports circumstantial evidence of how cell-to-cell spread may occur by describing the occurrence of tubular structures containing geminate particles in the cytoplasm and their continuation with the plasmodesmata in cells of the experimental host *Datura stramonium* L. infected with a geminivirus, Euphorbia mosaic.

MATERIALS AND METHODS

An isolate of Euphorbia mosaic virus, a whitefly-transmitted geminivirus (17), originally collected from *Euphorbia* spp. near Homestead, FL, was maintained in an experimental host (*D. stramonium*) by mechanical inoculation. An extract of young infected leaves of *D. stramonium* prepared in 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4), pH 7.0, was used as inoculum and wiped onto Carborundum-dusted leaves.

For ultrastructural studies, seedlings of *D. stramonium* with two true leaves were inoculated. The first visible symptom appeared on the inoculated leaves as barely discernible, small, chlorotic lesions 4 days after inoculation. Lesions increased both in size and degree of chlorosis up until 2 wk after inoculation. New leaves that developed after inoculation showed characteristic systemic symptoms, including severe mosaic, distortion, and stunting. One- to two-millimeter^2 pieces of leaf tissue were sampled from lesions of inoculated primary leaves at the first visible occurrence of lesions (4 days after inoculation) and periodically for the next 2 wk as lesions became progressively more chlorotic. Only the lesion and the immediate adjacent areas were sampled. Similar leaf tissue samples were taken from the first leaves that developed after inoculation showing typical systemic symptoms. Samplings were repeated on three different occasions and specimens were taken each time from at least three different plants having typical leaf symptoms. Leaf tissue from uninoculated plants of similar developmental stages were sampled for controls.

Tissue samples were processed for transmission electron microscopy as described previously (13,17). Briefly, the tissues were fixed in a modified Karnovsky's fixative, consisting of 2% glutaraldehyde and 2% paraformaldehyde; postfixed in 1% OsO_4; and embedded in Spurr's low viscosity embedding medium.

For Bernhard's uranyl EDTA lead staining method (1), tissues were fixed in Karnovsky's fixative without postfixation in OsO_4 and processed further for sectioning as above. Thin sections were stained in 5% aqueous uranyl acetate for 15 min. After being washed with distilled water, some sections were floated on a drop of 0.5 M EDTA, pH 7.2, at room temperature for 20–30 min, washed with distilled water, and stained with lead citrate for 3 min. For the controls, the EDTA treatment was omitted.

RESULTS

Cells in all samples of systemically infected leaves and lesions in inoculated primary leaves revealed the consistent presence of

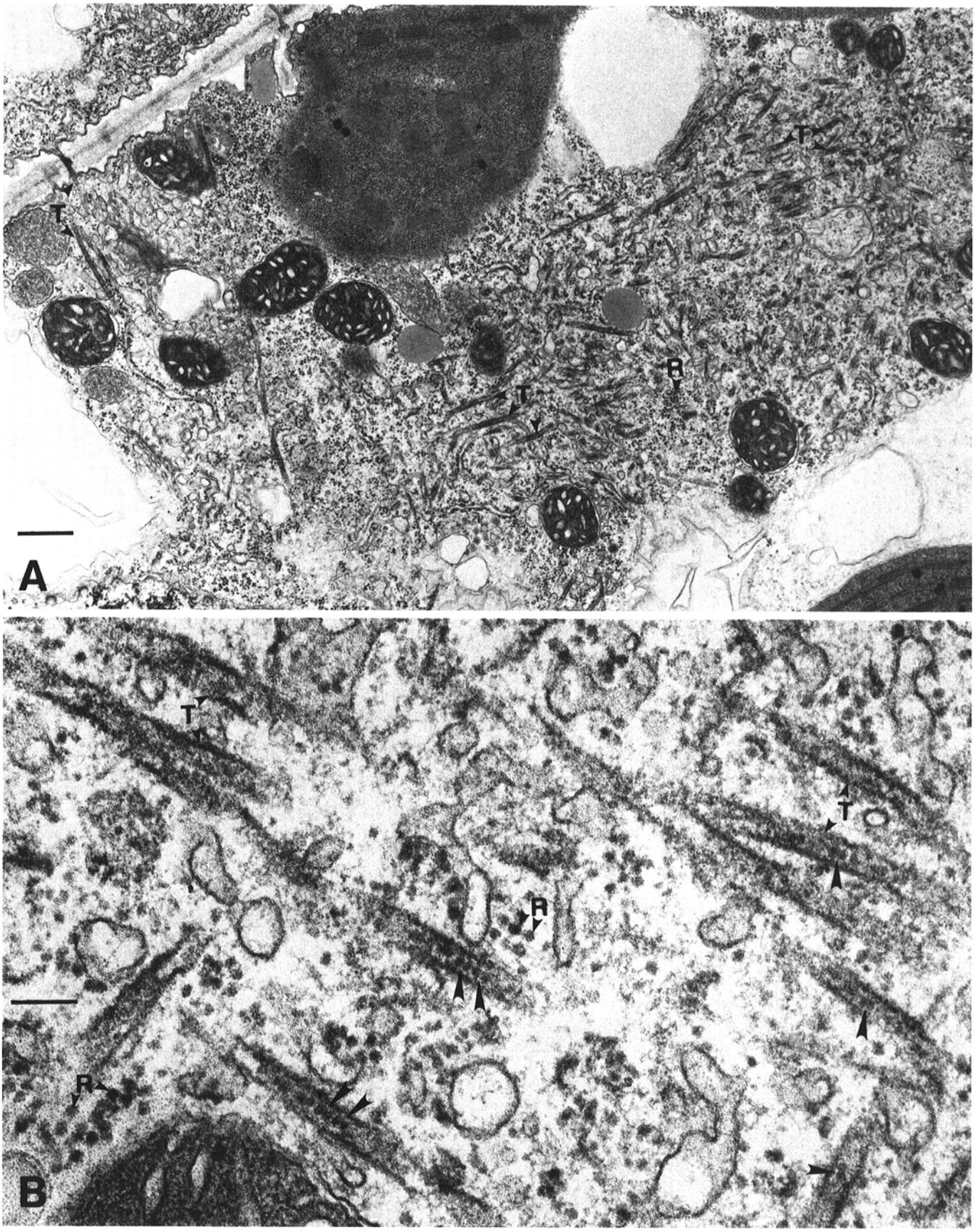

Fig. 1. A palisade mesophyll cell in an early lesion containing the macrotubules. **A,** general view of the cytoplasm occupied by a great number of the macrotubules. A large number of ribosomes (R) are associated with the tubules. Bar represents 500 nm. **B,** a higher magnification of an area of the cytoplasm containing the macrotubules (T). Some tubules, which are sectioned in a longitudinal plane, contain spherical viruslike particles measuring approximately 15 nm in diameter that are in pairs (arrowheads). Note the sizes of ribosomes (R) and viruslike particles. Bar represents 100 nm.

nucleopathic effects characteristic of many whitefly-transmitted geminiviruses, namely the segregation of nucleolar components into discrete granular and fibrillar lesions and the occurrence of fibrillar bodies often associated with virus particles (17,19). Unlike the natural host of Euphorbia mosaic virus, *Euphorbia heterophylla* L. (14), the virus and its cytopathic effects in *D. stramonium* were not limited to the phloem tissue but occurred in all cell types, including mesophyll and epidermal cells.

Some cells in the earliest chlorotic lesions sampled from the inoculated leaves revealed cytopathic effects that were not observed in cells of systemically infected leaves or in the advanced lesions of inoculated leaves. These were the occurrence in the cytoplasm of a great number of tubular structures, which will be referred to hereafter as macrotubules (Fig. 1). The macrotubules in some cells were numerous, filling a large portion of the cytoplasm, but they were random in distribution without forming an orderly arrangement. When the macrotubules were sectioned transversely, they appeared circular in profile (about 45 nm wide), with an external wall (about 13 nm thick) surrounding the central lumen (Fig. 2A). However, the walls of the majority of these circular rings were not continuous throughout their circumference but had an open area of different extent, indicating that the macrotubules are not completely closed cylinders (Fig. 2A). When sectioned longitudinally for a measurable distance, the walls of many macrotubules were alternated by sharply focused and poorly focused or fuzzy segments resulting in a somewhat twisted or

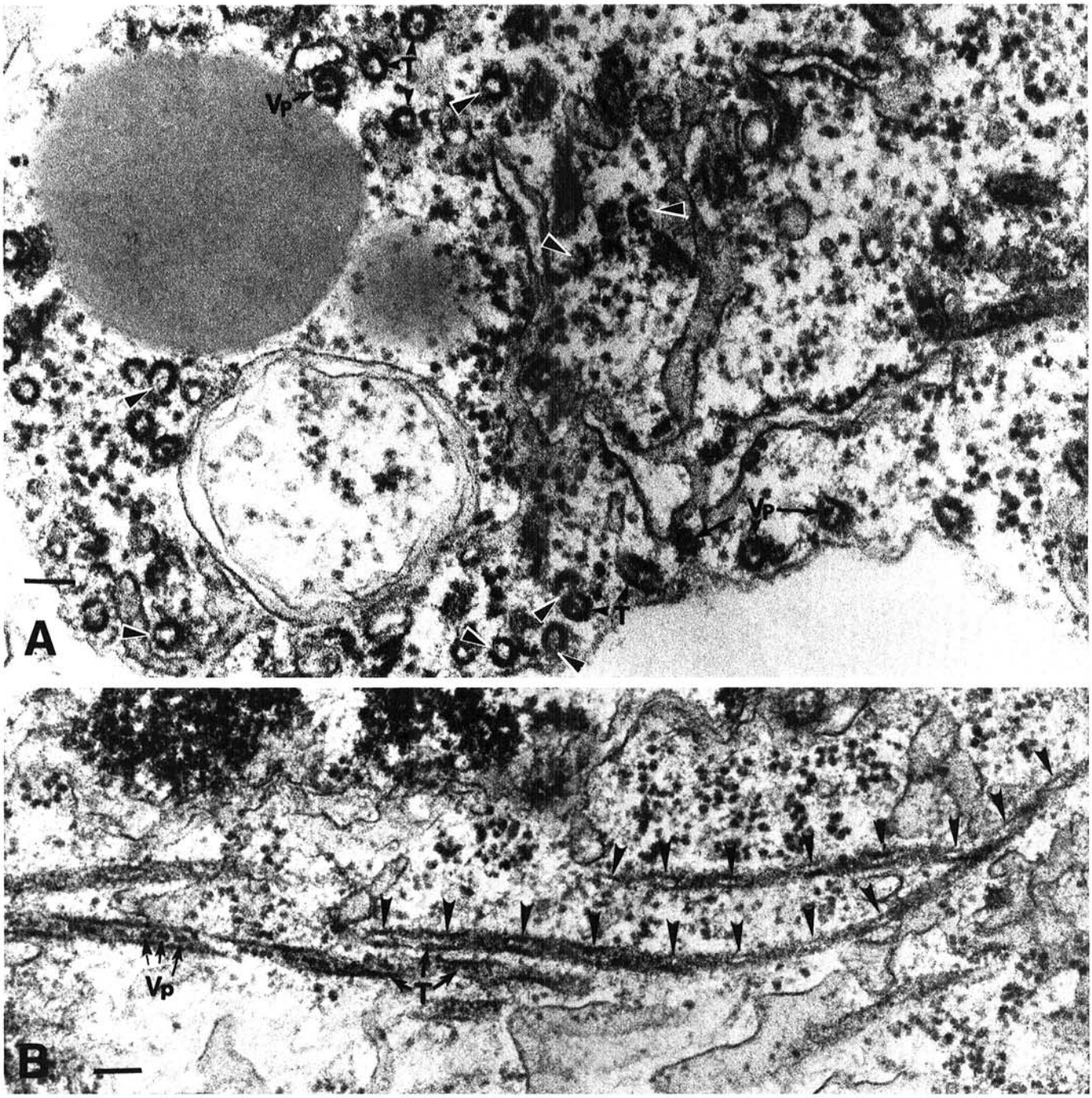

Fig. 2. Transverse and longitudinal sections of macrotubules. **A,** transverse sections of many macrotubules (T), having an average diameter of 45 nm, appear circular in profile, with the external wall (about 13 nm thick) surrounding the central lumen often containing viruslike particles (Vp). The walls of the majority of spheres have an open area (unlabeled arrowheads). Bar represents 100 nm. **B,** longitudinal sections of macrotubules (T) alternated by sharply focused (arrowheads) and fuzzy segments, suggesting that they are not completely closed cylinders. Some macrotubules contain paired particles (Vp). Bar represents 100 nm.

spiral, ropelike appearance (Fig. 2B). This agrees with the profile of transverse sections and suggests that the wall of macrotubules is not a solid cylinder but that it has openings (Fig. 2A and B).

Many of the macrotubules, especially those sectioned through a longitudinal plane, showed the presence of spherical particles measuring approximately 15 nm in diameter, many of which were in pairs, suggesting that they are the geminate virus particles of Euphorbia mosaic virus (Figs. 1B, 2B, and 3). When cells contained a large number of macrotubules, some of the macrotubules near the cell wall were often continuous with the plasmodesmata (Fig. 3). It appears that the macrotubule is continuous with the internal tubular structure of the plasmodesmata (modified desmotubule) but not with the plasmalemma of the plasmodesmata (Fig. 3). When the macrotubule and desmotubule were continuous, no structural distinction between the two could be made. When the macrotubules containing virus particles were continuous with the plasmodesmata, the virus particles also occurred in the plasmodesmata (Fig. 3). In cells that appeared not to contain macrotubules, no virus particles were detected in plasmodesmata. Furthermore, no plasmodesmata contained virus particles unless they were continuous with the macrotubules with virus particles, suggesting that the presence of macrotubules in the cytoplasm is prerequisite for the occurrence of virus particles in the plasmodesmata.

Nuclei of the cells that had macrotubules in the cytoplasm contained fibrillar bodies (Fig. 4A), indicating that the cells were infected by the virus. Unlike the cells of systemically infected leaves, many of these fibrillar bodies were not associated with aggregates of virus particles, suggesting that the cells were in an early stage of infection (Fig. 4A). When serial sections of the same nucleus containing such fibrillar bodies (Fig. 4A and B) were prepared by Bernhard's staining technique for nucleic acids, EDTA treatment clearly destained the fibrillar bodies, suggesting that they are DNA-containing structures like those associated with bean golden mosaic and Jatropha mosaic viruses, two whitefly-transmitted geminiviruses (13,19).

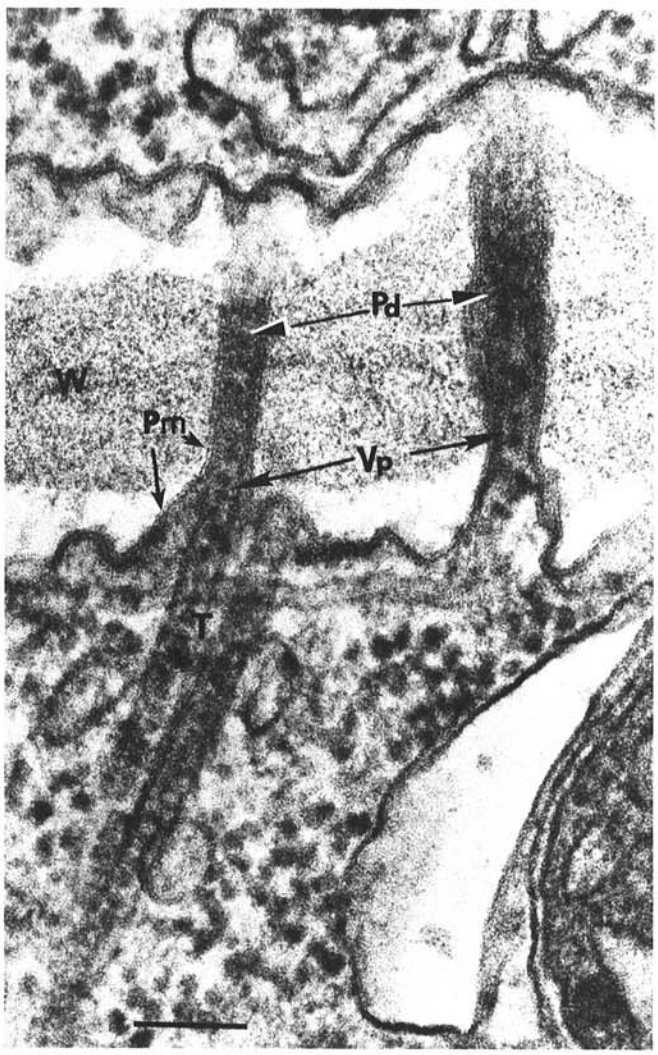

Fig. 3. A macrotubule containing virus particles (Vp) is continuous with the desmotubule of the plasmodesma (Pd). Pm = plasma membrane; W = cell wall. Bar represents 100 nm.

DISCUSSION

When a virus enters a susceptible plant host either by insect transmission or mechanical inoculation by rubbing, the virus moves from the initially infected cells to neighboring healthy cells in order to establish a successful infection. In local lesion hosts, cell-to-cell movement of the virus is limited to a short distance from the initially infected cell, whereas in systemic hosts, the virus moves to distant parts of the plant and infects different cell types. In some systemic hosts, however, the invading viruses are limited to the vascular system, especially the phloem (24).

The macrotubules described here are apparently virus-induced structures not only because they were absent in uninfected cells, but also because they contained geminate particles that are believed to be the particles of Euphorbia mosaic virus based on morphology, size, and associated cytopathic effects characteristic of whitefly-transmitted geminiviruses, to which Euphorbia mosaic virus belongs (13,14,17,19,25). The presence of paired particles in the macrotubules and their continuation with the plasmodesmata strongly suggest that the tubules are involved in intracellular as well as intercellular movement of the virus, as in the case of comoviruses, nepoviruses, and caulimoviruses, which induce similar structures in infected cells (2,5–7,15,16,18,20,21,30). Unlike these viruses, however, the macrotubules observed in this study appeared to be transient and present only in cells of very early lesions from inoculated leaves. No macrotubules were observed in cells of late lesions from inoculated leaves or in the systemically infected leaves of the same plant, although these cells contained characteristic nuclear inclusions and aggregates of virus particles (17). Furthermore, no virus particles were observed in the plasmodesmata of cells that lacked macrotubules, suggesting that the presence of macrotubules in the cytoplasm is prerequisite for the occurrence of virus particles in the plasmodesmata. Tubules with virus particles and virus particles in the plasmodesmata in comovirus, nepovirus, and caulimovirus infections are common throughout the infection stages in inoculated and systemically infected leaves (2,5–7,15,16,21,30).

The presence of macrotubules with paired particles in the cytoplasm and their continuation with the plasmodesmata may address one of the two questions raised in this paper about how the virus particles undergo cell-to-cell spread. However, the other question of how the virus particles are released from the nucleus into the cytoplasm remains unanswered, since no structural features suggestive of involvement in the movement of virus particles from the nucleus to the cytoplasm were encountered. No evidence was found of an association between the macrotubules with the perinuclear space or the rough endoplasmic reticulum. The possibility that the nuclei of infected cells might degenerate, causing the spillage of virus particles into the cytoplasm through the damaged nuclear envelope, appears not to be the case because the nuclei of cells that contain the macrotubules with virus particles in the cytoplasm appeared to be structurally intact.

Recently, there is increasing evidence that cell-to-cell movement of plant viruses is mediated by specific virus-encoded proteins (12), which have been referred to as M proteins (3). It has been demonstrated that the 30 kDa (3), 32 kDa (27), 48/58 kDa (R. Hull, *personal communication*), and 38 kDa (23) proteins encoded by tobacco mosaic virus (TMV), alfalfa mosaic virus (AMV), cowpea mosaic virus (CPMV), and cauliflower mosaic virus (CaMV), respectively, are involved in cell-to-cell movement of their respective viruses. Immunogold labeling of infected tissue revealed that the M proteins occurred either near or on the inside

of the plasmodesmata (23,27,29), suggesting that the M proteins act on the plasmodesmata to enable the virus to pass to the next cell. In addition, M proteins were also located in or near the cytopathic tubular structures induced by comoviruses and caulimoviruses, suggesting that these structures are indeed involved in cell-to-cell movement of virus particles (23, R. Hull, *personal communication*).

With regard to cytopathology, plant viruses can be divided into two categories, depending on whether or not the structural features suggestive of cell-to-cell movement of virus particles are present. Viruses in the comovirus, nepovirus, and caulimovirus groups may belong to one category of viruses that induce cytoplasmic tubular structures that are continuous with the plasmodesmata and contain virus particles (2,15,16,21,30). These virus-induced structures are very common in all stages of infection. The M proteins of viruses in this category are associated with the plasmodesmata along with tubular structures containing virus particles (23, R. Hull, *personal communication*). These findings have led to an assumption that the form of virus involved in cell-to-cell movement is assembled particles rather than unassembled forms such as viral nucleic acid or virus specific nucleoprotein (12). Viruses in the other category may include the remaining groups of viruses that do not induce any structural features comparable to those induced by the viruses in the first category, such

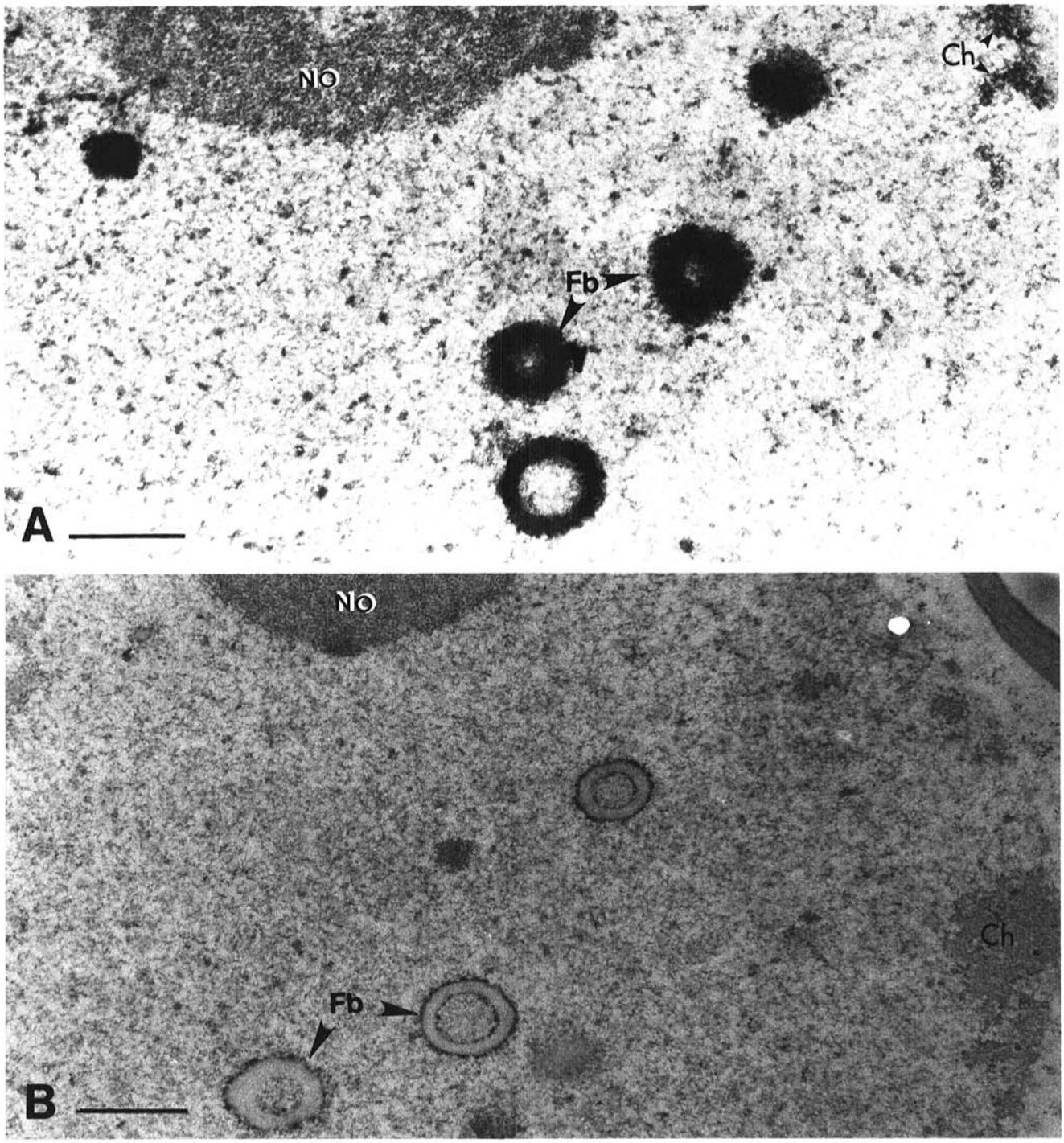

Fig. 4. Intranuclear fibrillar bodies (Fb) characteristic of whitefly-transmitted geminiviruses in a cell that contains macrotubules in the cytoplasm. The specimen was prepared for Bernhard's preferential staining technique for nucleic acids. **A,** fibrillar bodies (Fb) after fixation with aldehyde alone and staining with 5% uranyl acetate and lead citrate appear electron-dense. No = nucleolus; Ch = Chromatin. Bar represents 500 nm. **B,** a serial section of the nucleus in Fig. 4A treated with EDTA. The fibrillar bodies (Fb) and chromatin (Ch) are clearly destained by the treatment, suggesting that they are DNA-containing structures. No nucleolus. Bar represents 500 nm.

as tobamoviruses, cucumoviruses, and alfalfa mosaic viruses. Particles of these viruses have not been reported to occur in the plasmodesmata, and, although the M proteins of these viruses, such as 30 kDa of TMV and 32 kDa of AMV, are associated with the plasmodesmata, they occur transiently only in the early stages of infection (3,27,29). The form in which these viruses move is, therefore, suggested to be something other than assembled virus particles (12).

Based on the presence of macrotubules with geminate particles and their association with the plasmodesmata, Euphorbia mosaic virus probably belongs to the first category with the comoviruses, nepoviruses, and caulimoviruses except for the transient nature of the macrotubules. The transient nature of macrotubules observed in this study raises a few questions as to their absence in cells of advanced lesions, in systemically infected cells of the same host, and in any cells of the naturally infected original host. It may be that the cells in advanced lesions on inoculated leaves and those in systemically infected leaves were all in well-advanced stages of infection and no longer active in cell-to-cell movement of virus particles. Macrotubules formed in an earlier stage of infection might have been degraded or depolymerized into a nonstructural form, as in the case of mitotic spindle microtubules. In the natural host of Euphorbia mosaic, *E. heterophylla*, the virus is phloem-limited, whereas in *D. stramonium* used here, the virus is not phloem-limited and infects all cell types. It is possible that the mechanisms of cell-to-cell spread between the two hosts may be different. If this is the case, then macrotubules may not be formed in the natural host, in which the virus is phloem-limited. Geminiviruses are phloem-limited in most natural hosts, and studies on cytopathology have been carried out in such hosts. The use of an unnatural host may explain why no macrotubules or similar structures described in this paper have been reported to occur in any geminivirus infection in spite of numerous ultrastructural studies.

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