Ultrastructural Aspects of Tomato Golden Mosaic Virus Infection in Tobacco

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ABSTRACT

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Azure A-stained tissues of *Nicotiana benthamiana* plants infected with the geminivirus tomato golden mosaic virus (TGMV) were studied by light microscopy. Virion aggregates were readily detected in nuclei of both phloem-associated and mesophyll cells, and this observation was confirmed and extended by transmission electron microscopy. Cytopathic effects typically observed in geminivirus-infected dicotyledonous plants were noted in phloem-associated, mesophyll, and epidermal cells and were most obvious in nuclei and chloroplasts. Nuclear changes in infected cells included dispersal of chromatin to the periphery of the organelle and the appearance of fibrillar rings. Virus particles usually were seen as large, spherical aggregates but also frequently were organized into flat sheets. The cytopathic effects of TGMV infection of *N. benthamiana* in many respects resemble those caused by other geminiviruses in dicotylendonous hosts. TGMV is, however, exceptional in its tissue range and in the novel manner in which virion aggregates are constructed.

Plant viruses that belong to the geminivirus group have genomes of covalently closed circular single-stranded DNA contained within paired, isometric capsids composed of a single major polypeptide (19). Several confirmed members of the group, including maize streak (MSV), wheat dwarf (WDV), chloris striate mosaic (CSMV), and beet curly top virus (BCTV), are transmitted by leafhoppers. Others, such as cassava latent (CLV, synonymous with African cassava mosaic virus), bean golden mosaic (BGMV), mungbean yellow mosaic, and tomato golden mosaic virus (TGMV), are transmitted by whiteflies. With the exception of TGMV, ultrastructural changes induced in plants by each of the above named viruses have been documented (1,2,5-9,14,17,21). Several other leafhopper- or whitefly-transmitted viruses can be tentatively assigned to the geminivirus group on the basis of their particle morphology and cytopathic effects on the host. These include paspalum mosaic (PSMV) (9), tomato yellow leaf curl (TYLCV) (16), tomato yellow mosaic (15), euphorbia mosaic (12,13), and jatropha mosaic virus (11).

Geminivirus infection invariably results in some type of nucleopathy, the details of which vary with the virus and/or host. In dicotyledonous plants, nucleoli may segregate into distinct fibrillar and granular regions (1,12-14), increase in size or number (1,12,14,15,20), or degenerate (5-7,16). Electron-dense fibrillar rings also may appear (1,5,7,11-16,20). With few exceptions (13,15,17), these changes, as well as the presence of virus particles, are confined to phloem parenchyma, companion cells, and developing or mature sieve elements. Similar nuclear inclusions and nucleolar alterations have not been reported for infected monocotyledonous hosts. In these plants, evidence of infection usually is limited to the presence of virus particles. Virus particles have been found in both vascular and nonvascular tissues in two of three monocotyledonous hosts infected with three different viruses (8,19). Regardless of virus, host, or cell type, however, geminivirus particles are invariably found in the nucleus, and in only three cases have cytoplasmic virions been identified in cells that possess an intact nuclear membrane (4,9,20).

In this paper, we describe the cytopathic effects of TGMV infection in *Nicotiana benthamiana* Domin. The study was undertaken to provide the basis for further experiments on the roles of individual viral genes in the replication and systemic spread of TGMV. The effects observed resemble those previously described for other geminivirus-infected dicots. We also show that TGMV infection in tobacco is not restricted to phloem tissue and demonstrate crystalline arrays of TGMV that are organized in a manner that is so far unique to this virus.

MATERIALS AND METHODS

Plant material. *N. benthamiana* plants were grown to the six- to eight-leaf stage. The lower leaves were removed, leaving the upper three leaves, which were dusted with 500-mesh Carborundum and inoculated with TGMV by rubbing with a cotton swab.

Virus. A Brazilian isolate of TGMV was originally supplied by Dr. A. S. Costa, Instituto Agronomico, Sao Paulo, Brazil. Inoculum was prepared by homogenizing TGMV-infected tissue in 0.1 M sodium citrate (pH 7) containing 5 mM disodium ethylenediaminetetraacetic acid and 0.75% (w/v) sodium sulfite. The homogenate was subjected to a high-speed centrifugation (97,000 g) for 2 hr at 4 C, and the pelleted material was resuspended in sterile, distilled, deionized water before use as inoculum. All inocula were stored at −20 C. Systemic symptoms appeared approximately 14 days after inoculation, after which time young leaves displaying the leaf curl and bright yellow mosaic typical of TGMV infection were processed for light and electron microscopy. Inoculated leaves were not examined.

Light microscopy. Leaves from both infected and healthy *N. benthamiana* were processed as described previously by Christie et al (3). Portions of leaves were abraded on both surfaces with 600-mesh sandpaper, placed in 2-methoxyethanol for 45 min to remove chlorophyll, and stained for 1 hr in 0.1% azure-A (dissolved in 2-methoxyethanol) mixed with 0.2 M dibasic sodium phosphate (nine parts azure-A to one part dibasic sodium phosphate). Stained material was washed for 30 min in 95% ethanol and for 15 min in 2-methoxyethyl acetate, blotted dry, and mounted on glass slides with Euparal.

Electron microscopy. Pieces of leaves from both infected and healthy plants were fixed for approximately 5 hr in one-half strength Karnovsky's fixative (10) in 0.1 M sodium cacodylate buffer, pH 7.3. After several washes in buffer, the material was postfixed for 4 hr in buffered 2% osmium tetroxide, washed several times in buffer, dehydrated in an acetone-propylene oxide series, and embedded in Spurr's resin (18). Thin sections were stained with 1% (w/v) aqueous uranyl acetate followed by Reynolds' lead citrate (22) and examined with a Philips 300 transmission electron microscope.

RESULTS

Light microscopy of mature, infected tobacco leaf tissue treated with azure-A revealed large, darkly stained inclusions in nuclei of infected (Fig. 1), but not healthy (Fig. 2), leaves. The inclusions sometimes appeared as hollow spheres (Fig. 3) but more often appeared to be solid. These inclusions were not restricted to the vascular tissue or its immediate vicinity. Rather, they appeared throughout infected leaves except for the epidermis. However,
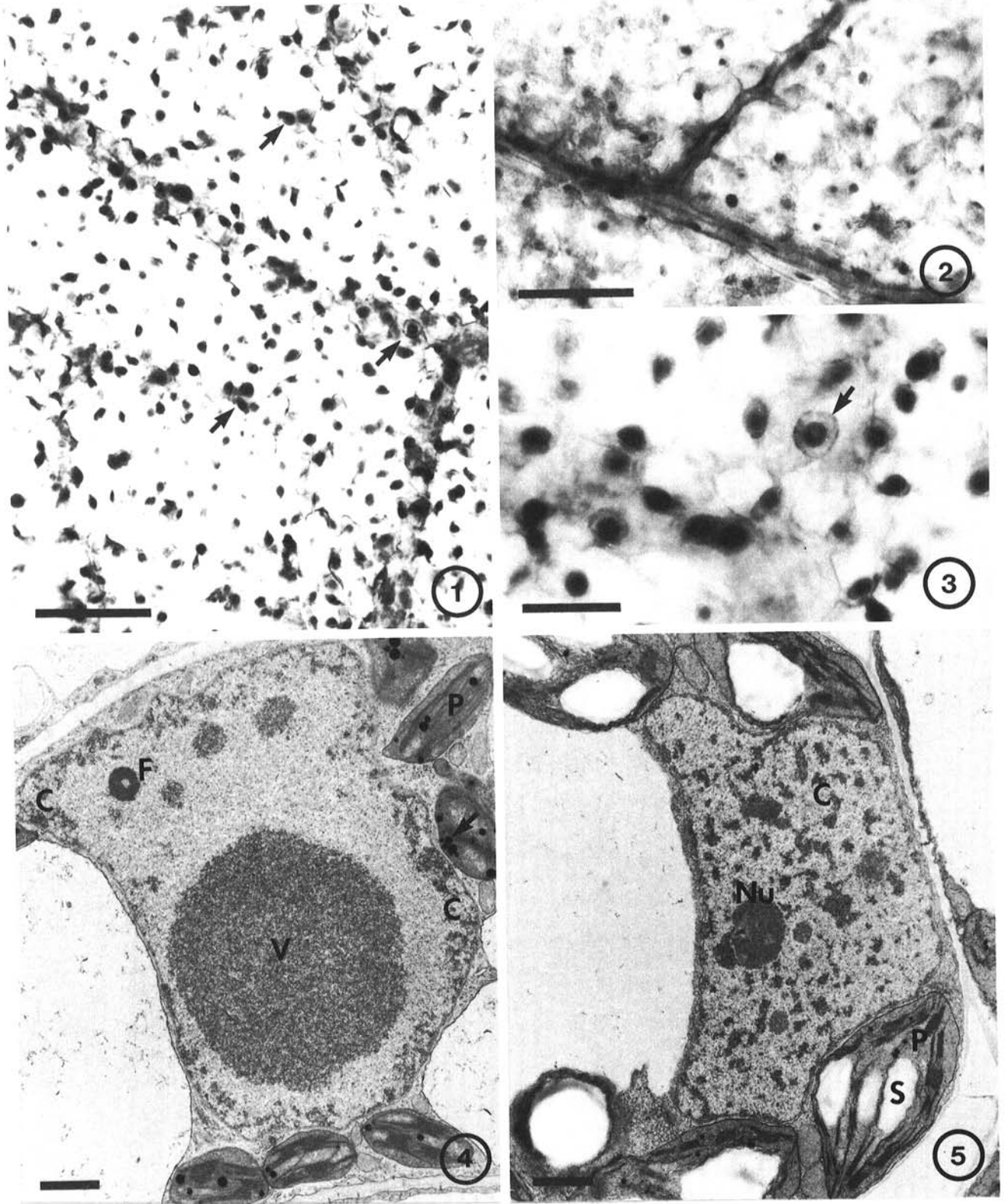
Figs. 1-5. A comparison of nuclei in leaf cells of healthy and tomato golden mosaic virus infected tobacco. **1,** Light micrograph of infected leaf tissue stained with azure-A. Darkly staining nuclear inclusions (see arrows for examples) occur throughout the leaf tissue. Bar = 75 μm. **2,** Light micrograph of healthy leaf tissue stained with azure-A. Nuclei in this tissue do not contain darkly staining nuclear inclusions. Bar = 75 μm. **3,** Higher magnification of infected leaf tissue stained with azure-A. A ring of virus particles (arrow) occupies the nucleus of one cell. Bar = 25 μm. **4,** Electron micrograph of a mesophyll cell from infected leaf tissue. A large inclusion of virus particles (V) fills most of the nucleus. One fibrillar ring (F) is visible and chromatin (C) is restricted to the periphery of the nucleus. Chloroplasts (P) contain numerous osmiophilic inclusions (arrow). Bar = 1 μm. **5,** Electron micrograph of a mesophyll cell of healthy leaf tissue. Chromatin (C) is dispersed throughout the nucleus and a prominent nucleolus (Nu) is visible. Chloroplasts (P) contain abundant starch (S) but only a few small osmiophilic inclusions. Bar = 1 μm.

most of the epidermis had been removed by abrasion with sandpaper.

Electron microscopic comparisons of infected and healthy leaf tissues also revealed significant differences. Large, spherical aggregates of virions were found in nuclei of infected, but not healthy, leaf cells (Figs. 4 and 5). In some cases the aggregates appeared to be hollow spheres (Fig. 6). Associated with these large inclusions were smaller fibrillar rings (Figs. 4, 6–8), which
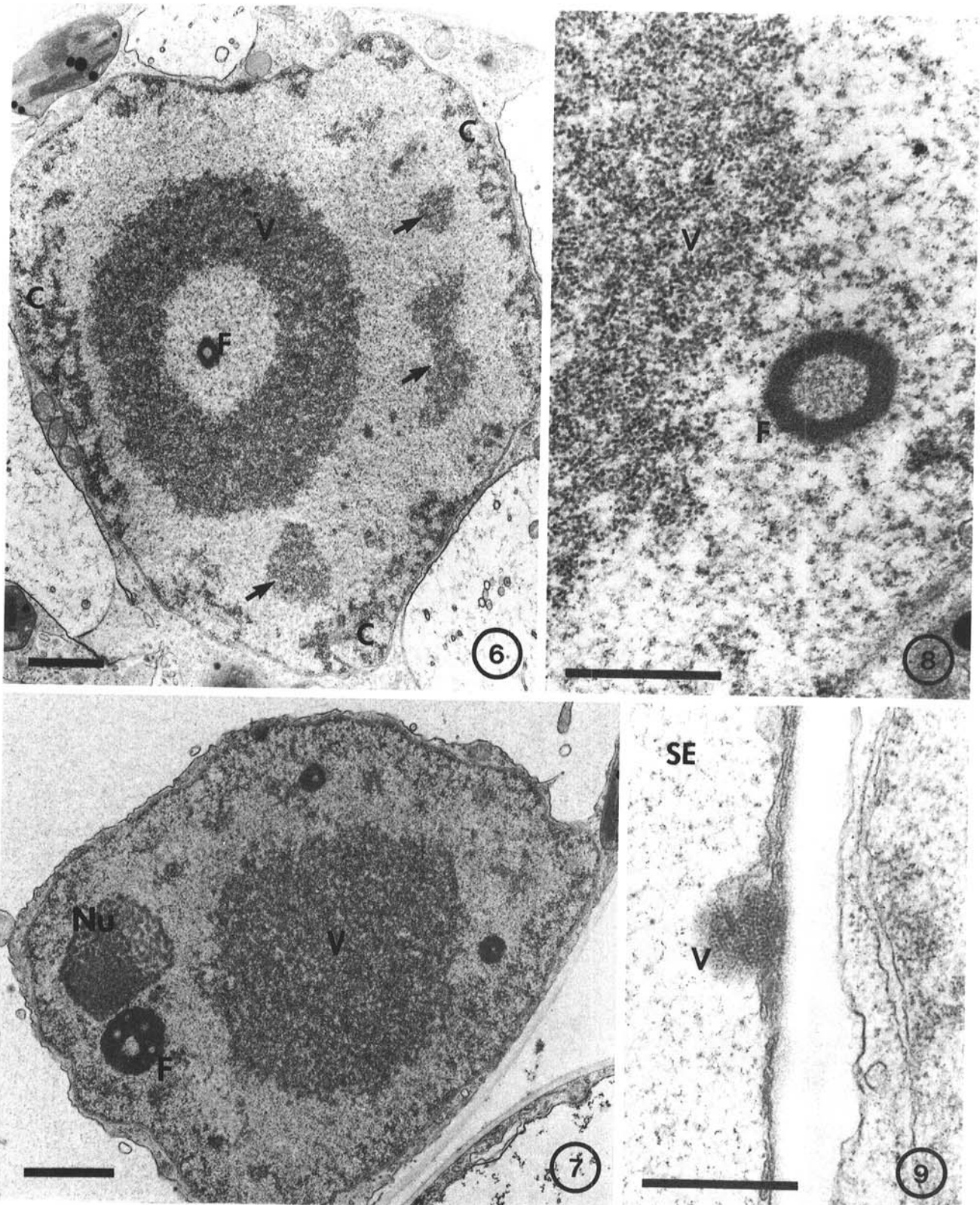
Figs. 6–9. Electron micrographs of different types of tobacco leaf cells infected with tomato golden mosaic virus. **6,** Infected nucleus of a vascular parenchyma cell showing a ring of virus particles (V) and one small fibrillar ring (F). Several smaller clusters of virus particles (arrows) are located between the large ring and the peripherally located chromatin (C). Bar = 1 μm. **7,** Infected nucleus of a mesophyll cell containing a large inclusion of virus particles (V), three fibrillar rings (F), and a nucleolus (Nu). Bar = 1μm. **8,** Higher magnification of the nucleus of a vascular parenchyma cell showing virus particles (V) and a fibrillar ring (F). The fibrillar ring is electron-dense abrad contains finely granular material at its center. The virus particles are densely packed. The paired nature of the geminivirus is not readily apparent in this view. Bar = 0.5 μm. **9,** Sieve element (SE) containing a small cluster of virus particles (V) near the periphery of the lumen. Bar = 0.5 μm.
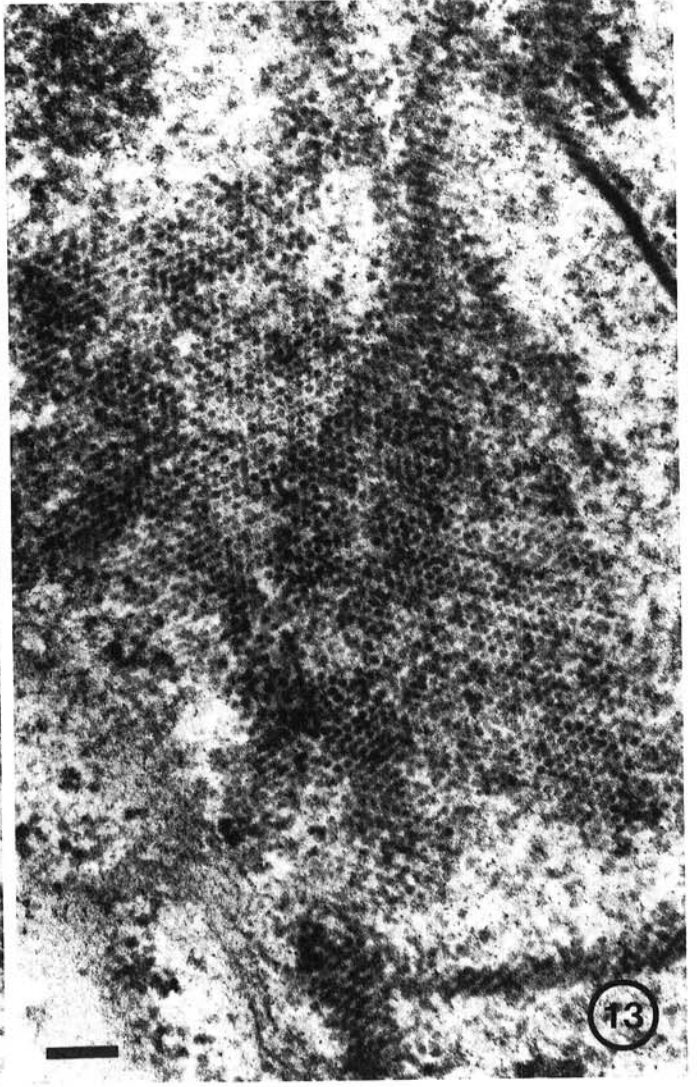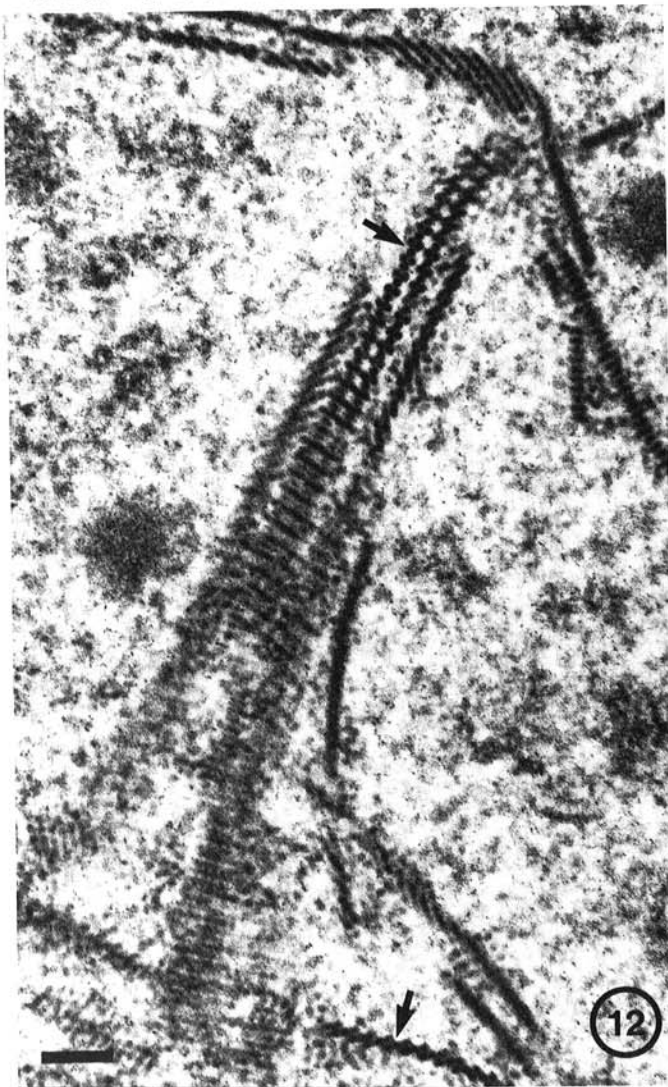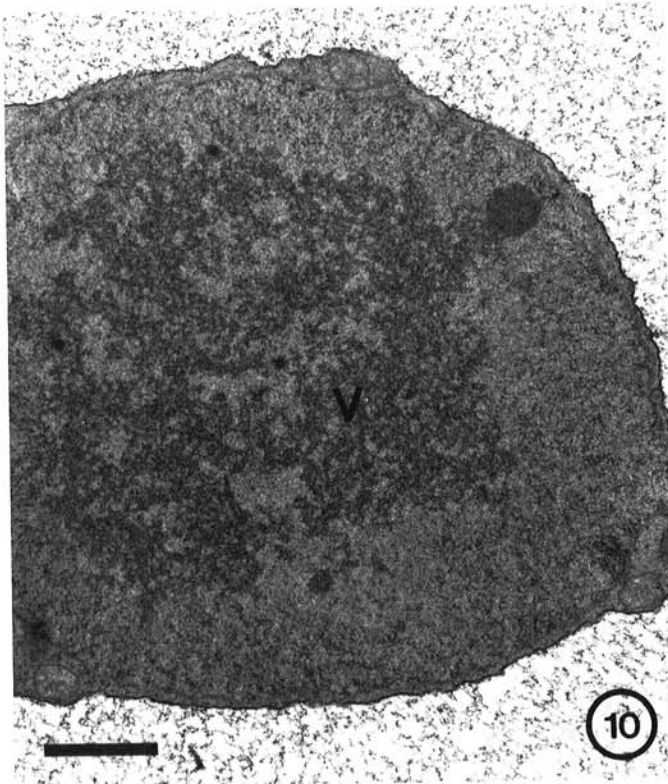
occasionally appeared as solid spheres depending on the angle of the section. These fibrillar rings stained more darkly than other nuclear components and typically contained a fine granular material in the center of the ring (Fig. 8). On occasion the rings appeared vacuolated and frequently more than one was observed in a nucleus (Fig. 7). Fibrillar rings also were found in nuclei containing only small clusters of virus particles. The chromatin of infected nuclei was displaced to the periphery of the nucleus, adjacent to the nuclear membrane (Figs. 4 and 6), in contrast to the evenly dispersed chromatin of healthy cell nuclei (Fig. 5). Likewise, nucleoli, when observed in infected cells, were also located near the periphery (Fig. 7). Compared with chloroplasts of healthy leaf cells, those of infected cells generally contained a reduced amount of starch and a considerable number of osmiophilic inclusions (Figs. 4 and 5).

Virus particles were observed by electron microscopy in all cell types of the leaf except for the tracheary elements. Infected vascular parenchyma and mesophyll cells typically contained large numbers of aggregated particles (Figs. 4 and 6). At higher magnification, the densely packed nature of these viral aggregates was evident (Fig. 8). Loosely packed aggregates of virions were frequently observed in epidermal cells (Fig. 10). In mature sieve elements, virus particles clustered at the periphery of the lumen, separated from the wall by an intact plasmalemma. Some sieve-element clusters contained particles in ordered arrays (Fig. 9), whereas others possessed particles having a random arrangement similar to that shown in the nucleus of the parenchyma cell of

Figure 8. Sieve-element clusters also differed in that some were enclosed by one or more membranes, whereas others were free in the lumen. Immature sieve elements, in which nuclear aggregates might be expected, were not examined. In no instance were virions observed in the cytoplasm of any cell type where the nuclear membrane of the cell was intact.

Virus particles approximately 15 to 16 nm in diameter frequently were found in elaborate, apparently crystalline, arrays (Figs. 11–13). These arrays were composed of sheets of particles either alone or in stacks (Fig. 12). In surface view, the sheets appeared to consist of rows of evenly spaced particles showing hexagonal packing (Fig. 13). One arrangement commonly observed when these sheets were cut perpendicular to the surface consisted of single particles alternating with geminate particles set at right angles to the plane of the sheet (Fig. 12). In the case of two or more stacked sheets, the geminate particles and single particles of all sheets were in register (Fig. 12). It seemed as if an increase of crystallization was associated with a decrease in the numbers of free virus particles within the nucleus (cf. Figs. 4 and 11).

Tissue showing extreme chlorosis and leaf curl contained numerous cells in a state of degeneration (Figs. 14 and 15). Such cells typically contained large, unorganized masses of virions that were not delimited by nuclear membranes.

DISCUSSION

Examination of azure-A stained plant tissue by light microscopy
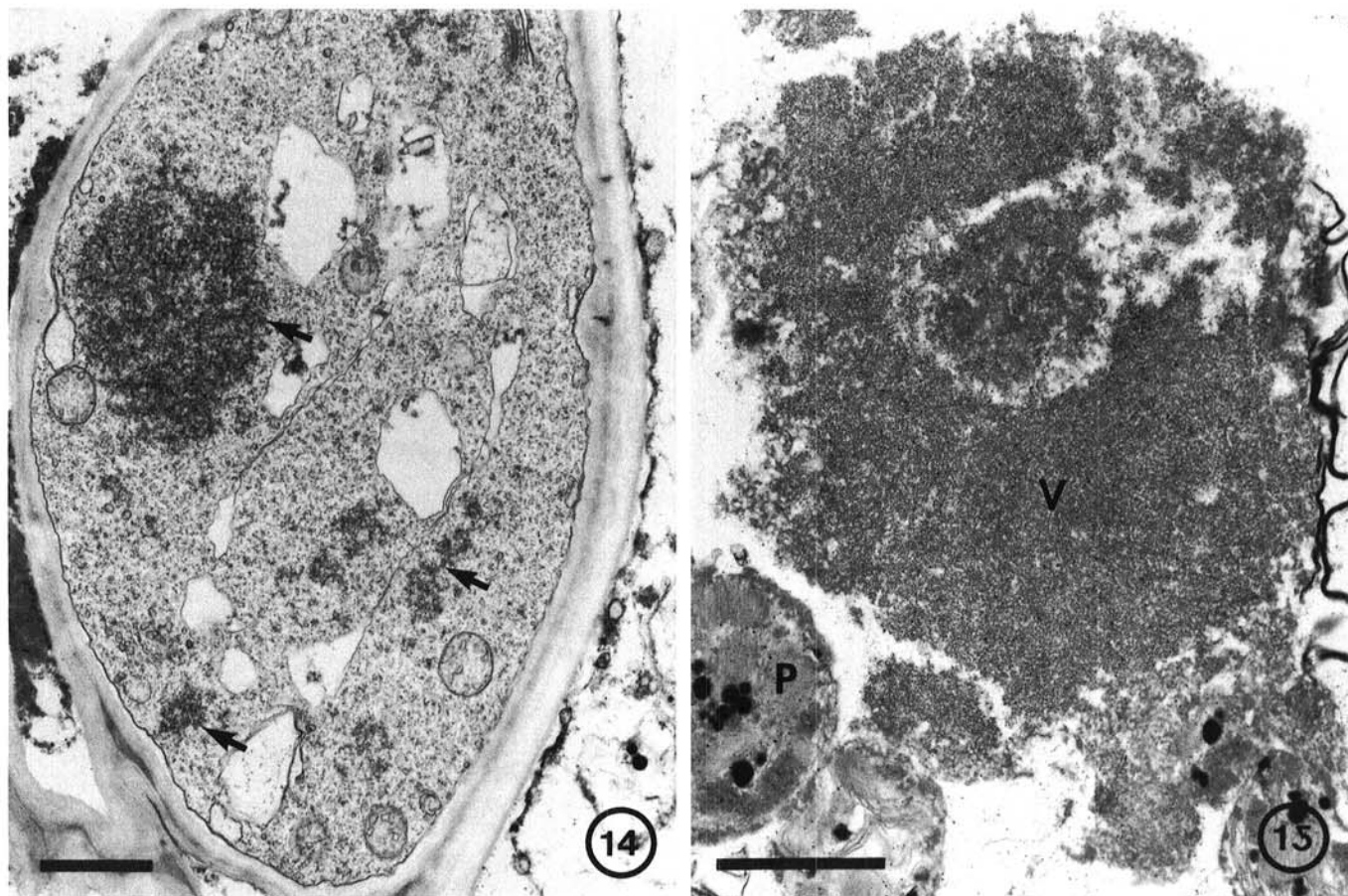
Figs. 14 and 15. Electron micrographs of degenerating tobacco leaf cells infected with TGMV. **14,** Vascular parenchyma cell showing virus particles distributed in the cytoplasm (arrows). The nuclear envelope of this cell is no longer intact. Bar = 1 μm. **15,** A vascular parenchyma cell at a later stage of degeneration than that in Figure 13. Although the nucleus is no longer present, the virus particles (V) still remain in clumps. The nearby chloroplasts (P) have also degenerated. Bar = 1 μm.

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Figs. 10–13. (Facing page.) Electron micrographs of virus particle arrangement in infected tobacco leaf cells. **10,** Nucleus of an epidermal cell containing virus particles (V) scattered throughout its center. Bar = 1 μm. **11,** An overall view of crystalline arrays in a mesophyll nucleus. Bar = 1 μm. **12,** A detailed view of crystalline arrays sectioned perpendicular to the surface (arrows). Bar = 0.1 μm. **13,** High magnification of a crystalline array as seen in surface view. The virus particles exhibit a hexagonally packed structure. Bar = 0.1 μm.

is a useful tool for detection of virion aggregates and fibrillar rings characteristic of geminivirus-infected cell nuclei (3). Our studies have verified the usefulness of this technique for localizing the sites of viral infection and have demonstrated that TGMV infection of tobacco is not limited to phloem cells as is the case with many other geminiviruses. We have confirmed and extended this observation with ultrastructural analysis by demonstrating that inclusions of the size and morphology of those stained with azure-A represent viral aggregates.

Nucleopathic effects typical of geminivirus infection in dicotyledonous hosts were seen in TGMV-infected tobacco. Most obvious of these were the presence in nuclei of fibrillar rings and the restriction of chromatin as well as nucleoli to the periphery of the nuclei.

Nearly all previous ultrastructural studies of geminivirus infection have revealed some form of viral aggregate, though the degree of organization varies among viruses (9). Several types of arrays have been reported, and unorganized clumps are generally observed whether or not arrays are found. Flat sheet arrays have been observed in cells of plants infected with PSMV (9), BGMV (14), CSMV (8), and MSV (2). We likewise have found that TGMV particles also can be arranged in flat sheets, although the arrangement of individual virions within the sheets appears to be unique. We cannot rule out the possibility that geminate and single capsids alternate to produce the pattern evident when sheets are observed in transverse view. However, single (nongeminate) geminivirus capsids are generally believed to be degradation products (9). Therefore, we suggest that TGMV sheets consist of rows of virions arranged in such a way that the axis of each row is rotated 90° with respect to that of its nearest neighbor. Such an alternating arrangement, with virions at right angles to each other, has not been demonstrated for any other geminivirus. The significance of this unusual packing is unknown.

It is interesting to compare the cytopathology of TGMV infection in *N. benthamiana* with that reported by Adejare and Coutts (1) for CLV infection of the same host. While there are many similarities, there are some obvious differences. Particles of CLV do not appear to aggregate extensively. Further, CLV infection is mainly limited to phloem parenchyma cells (1), although virus particles have been reported to occur occasionally in the cortex, mesophyll, and epidermis (17). These differences suggest that certain aspects of the geminivirus-host interaction, namely tissue affinity and degree of virion aggregation, are determined by the virus. It therefore may be possible to identify viral genes or regulatory sequences responsible for these phenomena.

LITERATURE CITED

1. Adejare, G. O., and Coutts, R. H. A. 1982. Ultrastructural studies on *Nicotiana benthamiana* tissue following infection with a virus transmitted from mosaic-diseased Nigerian cassava. Phytopathol. Z. 103:87-92.

2. Bock, K. R. 1974. Maize streak virus. No. 133 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England.

3. Christie, R. C., Ko, N.-J., Falk, B. W., Hiebert, E., Lastra, R., Bird, J., and Kim, K. S. 1986. Light microscopy of geminivirus-induced nuclear inclusion bodies. Phytopathology 76:124-126.

4. Dollet, M., Accotto, G. P., Lisa, V., Menissier, J., and Boccardo, G. 1986. A geminivirus, serologically related to maize streak virus, from *Digitaria sanguinalis* from Banuatu. J. Gen. Virol. 67:933-937.

5. Esau, K. 1977. Virus-like particles in nuclei of phloem cells in spinach leaves infected with the curly top virus. J. Ultrastruct. Res. 61:78-88.

6. Esau, K., and Hoefert, L. L. 1973. Particles and associated inclusions in sugarbeet infected with the curly top virus. Virology 56:454-464.

7. Esau, K., and Magyarosy, A. C. 1979. Nuclear abnormalities and cytoplasmic inclusions in *Amsinckia* infected with the curly top virus. J. Ultrastruct. Res. 66:11-21.

8. Francki, R. I. B., Hatta, T., Grylls, N. E., and Grivell, C. J. 1979. The particle morphology and some other properties of chloris striate mosaic virus. Ann. Appl. Biol. 91:51-59.

9. Francki, R. I. B., Milne, R. G., and Hatta, T. 1985. Atlas of Plant Viruses, Vol. I. CRC Press, Inc., Boca Raton, FL.

10. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27:137A.

11. Kim, K. S., Bird, J., Rodriguez, R. L., Martin, E. M., and Escudero, J. 1986. Ultrastructural studies of *Jatropha gossypifolia* infected with jatropha mosaic virus, a whitefly-transmitted geminivirus. Phytopathology 76:80-85.

12. Kim, K. S., and Flores, E. M. 1979. Nuclear changes associated with Euphorbia mosaic virus transmitted by the whitefly. Phytopathology 69:980-984.

13. Kim, K. S., and Fulton, R. W. 1984. Ultrastructure of *Datura stramonium* infected with an euphorbia virus suggestive of a whitefly-transmitted geminivirus. Phytopathology 74:236-241.

14. Kim, K. S., Shock, T. L., and Goodman, R. M. 1978. Infection of *Phaseolus vulgaris* by bean golden mosaic virus: Ultrastructural aspects. Virology 89:22-23.

15. Lastra, R., and Gil, F. 1981. Ultrastructural host cell changes associated with tomato yellow mosaic. Phytopathology 71:524-528.

16. Russo, M., Cohen, S., and Martelli, G. P. 1980. Virus-like particles in tomato plants affected by the yellow leaf curl disease. J. Gen. Virol. 49:209-213.

17. Sequeira, J. C., and Harrison, B. D. 1982. Serological studies on cassava latent virus. Ann. Appl. Biol. 101:33-42.

18. Spurr, A. R. 1969. A low viscosity resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.

19. Stanley, J. 1985. The molecular biology of geminiviruses. Adv. Virus Res. 30:139-177.

20. Thongmeearkom, P., Honda, Y., Saito, Y., and Syamananda, R. 1981. Nuclear ultrastructural changes and aggregates of viruslike particles in mungbean cells infected with mungbean yellow mosaic disease. Phytopathology 71:41-44.

21. Tomenius, K., and Oxelfelt, P. 1981. Preliminary observations of virus-like particles in nuclei in cells of wheat infected with the wheat dwarf disease. Phytopathol. Z. 101:163-167.

22. Reynolds, E. E. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208.