Cytoplasmic Inclusions in Cells Infected with Isolates of L and I Serogroups of Tomato Spotted Wilt Virus

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ABSTRACT

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A comparative analysis of the cytopathology of *Nicotiana benthamiana* infected with representative isolates of two serogroups of the tospovirus genus of the Bunyaviridae (formerly tomato spotted wilt virus) revealed differences in the accumulation of cytoplasmic inclusions. Transmission electron microscopy of a common isolate (L) of tomato spotted wilt virus (TSWV) from tobacco, TSWV-Lgt, revealed electron-dense areas (viroplasm), single quasispherical particles, and cisternae-bound clusters of mature virions. In addition, filaments arranged in loose bundles were found in a common isolate from lettuce, TSWV-Llet. Tissue infected with an isolate that has a serologically distinct nucleocapsid protein, TSWV-I, showed three differences in cytopathology. TSWV-Igg, from gloxinia, rarely contained particles and had no cisternae-bound mature virions. Filaments were uniquely arranged in layered, paracrystalline arrays. TSWV-Igg also had two forms of electron-dense areas: viroplasm and an area denser and not associated with membranes that we have labeled an amorphous inclusion. Immunogold labeling of tissue infected with TSWV isolates, with antiserum to whole virus, demonstrated that the electron-dense areas previously designated as viroplasm were viral protein. However, viral antibodies did not label paracrystalline arrays of filaments or the nonmembrane-bound amorphous inclusions. The arrangement of filaments and possible amorphous inclusions should be noted as distinguishing characteristics of the I serotype.

The tomato spotted wilt virus (TSWV) group of plant viruses has been considered a monotypic group (8). We have described a second member of the TSWV group that has a serologically distinct nucleocapsid (N) protein and does not produce an abundance of mature enveloped virions (13). This serologically distinct virus (provisionally designated TSWV-I) is the predominant type of TSWV found in greenhouse-grown floral crops in the United States and is also found in Europe (3). In our previous study (13), the cytopathology of only one isolate of TSWV-I was examined in *Nicotiana benthamiana* Domin. The goal of this study was to compare the cytopathology of isolates of TSWV-I and TSWV-L in a common host, *N. benthamiana*.

TSWV is an unusual plant virus that causes diseases in both dicotyledonous and monocotyledonous plants and is one of the two plant viruses reportedly transmitted by thrips (2,11). TSWV is further distinguished from other plant viruses by its quasispherical, enveloped virions, and tripartite RNA genome. These traits most closely resemble the Bunyaviridae family of animal viruses (4,16) than any other taxa of viruses. The virion envelope contains two virus-coded glycoproteins, G1 and G2 (78 and 52 kDa, respectively) and surrounds three ssRNA species encapsidated in a 28-kDa N protein (17,18,20). The three ssRNA species are designated L (8.3 kb), M (5.2 kb) and S (2,916 bases) (4,21). The complete nucleotide sequence has been determined for the S RNA, which revealed an ambisense-coding strategy with two open reading frames (4). The open reading frame at the 5′ end of the viral RNA codes for a nonstructural protein in the viral sense; whereas, the open reading frame at the 3′ end codes for the N protein in the viral complementary sense. In addition to the virions, TSWV-infected cells also contain electron-dense areas, designated viroplasms, and, occasionally, long filaments have been observed (5,9,15).

TSWV-I shares many of the structural characteristics with the common types of TSWV, but was shown to have an N protein that is serologically distinct from other typical isolates (13); isolates of the TSWV-L type all react similarly (23). Although structural proteins and three RNA species of characteristic size were isolated from the I-type isolate in amounts comparable to the typical isolate, enveloped virions were rarely observed and then only by immunosorbent electron microscopy (ISEM). Other more abundant cytoplasmic structures associated with TSWV-I infections included viroplasms and paracrystalline arrays of filaments (13). Differences in cytoplasmic inclusions may have been due to subtle changes in sequence, isolate, or host differences or to examination of tissues at a unique time during the infection cycle.

The specific objectives of this study were to identify the cytoplasmic inclusions in cells infected with TSWV-I, to compare these structures with those that occur in a common host infected with TSWV-L and with those previously reported for TSWV-L isolates, and to identify inclusions that may be composed of structural proteins rather than other viral proteins or of host origin.

MATERIALS AND METHODS

Virus isolates. Isolates from the tomato spotted wilt virus group, representative of two serogroups (13), were selected for comparison in this investigation. All isolates were stable after repeated transfer in *N. benthamiana* with no apparent loss of infectivity. One isolate, designated TSWV-Lgt, originated from tobacco in Georgia and was classified in the L or common serogroup. A second isolate from the L group, TSWV-Llet, isolated from lettuce, was provided by Dennis Gonsalves, Cornell University. This isolate has been used in other studies (23); however, under our conditions it requires 1–2 wk longer to produce symptoms in *N. benthamiana* than do other isolates of either serogroup. The isolate from the I serogroup (13) was obtained from gloxinia (TSWV-Igg, Georgia). Each of the isolates had been continuously cultured in *N. benthamiana* for at least 6 mo before the experiment. Tissue for examination of TSWV-Llet was collected at the peak of symptom expression.

Time-course analysis. TSWV-Lgt and TSWV-Igg were used in the time-course experiment. Three young leaves on *N. benthamiana* plants at the 8–10 leaf stage were inoculated with virus. Inoculum was prepared by triturating infected or healthy leaves in 10 vol of chilled inoculation buffer (10 mM Tris, pH 7.8) and was applied immediately to leaves dusted with carborundum. Samples for western blot analysis were collected daily from systemically infected leaves until the leaves became necrotic (TSWV-Lgt in 16 days; TSWV-Igg in 11 days).

Western blotting. Modifications of the procedure for electrophoresis and transfer of protein samples as described by Gray et al (6) were used. Nonfat dry milk was substituted as a blocking agent. Antibody concentrations were 1:500 for TSWV-I and 1:1,000 for TSWV-L. Blots were developed by using an alkaline-phosphatase system with nitroblue tetrazolium/5-bromo-4-chloro-3-indoylphosphate as the substrate.

Electron microscopy. Leaf tissue pieces (1- × 2-mm) were collected for electron microscopy from areas of the same leaf adjacent to those sampled for serological analysis. Tissue pieces were vacuum-infiltrated with 0.1 M potassium phosphate-citrate buffer (2 hr at 4 C, pH 7.4) and fixed with 1% glutaraldehyde (v/v) and 2.5% paraformaldehyde (w/v) overnight at 4 C (14). Postfixation was in 1% OsO_4, followed by dehydration and embedding in LX-112 (7). The ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a Jeol-100S transmission electron microscope. Tissue was examined from 2, 4, 5, 6, 7, and 11 days postinoculation for TSWV-Igg and from 2, 4, 7, 8, 9, 11, 13, and 16 days postinoculation for TSWV-Lgt. Epidermal, mesophyll, and vascular tissues were specifically examined for the presence of viral structures.

Immunogold labeling. Immunogold labeling was done by the method of Bendayan (1). Nickel grids containing sections of tissue inoculated with buffer or one of the TSWV isolates were dipped in distilled water and floated on drops of saturated sodium metaperiodate for 4 hr. Grids were rinsed three times on successive drops of distilled water and placed on a drop of 1% ovalbumin (w/v) in phosphate-buffered saline (PBS), pH 7.0, for 5 min. Grids were transferred directly to antiserum L or I (1 mg/ml), 1:2 dilution in PBS, and incubated overnight at 4 C in a moist chamber. Grids were rinsed on drops of PBS, blocked with ovalbumin and incubated 1 hr on a drop of Auroprobe EM protein AG 10 (Janssen Biotech N.V., Amersham Corp., Arlington Heights, IL) diluted 1:25 in PBS. Grids were rinsed on drops of PBS and then on distilled water. All solutions were filter-sterilized, except antiserum and gold-labeled protein A. Sections were stained for 30 min in 4% aqueous uranyl acetate, followed by 4 min in Reynold's lead citrate. Controls included heterologous combinations of antiserum to TSWV-Lgt, TSWV-Igg, and preimmunization serum with infected and healthy tissue.

RESULTS

The appearance of cytoplasmic inclusions in TSWV-infected tissues coincided with the accumulation of detectable levels of the three major structural proteins for isolates of both serogroups of TSWV. The N protein was very faintly detectable 3 and 7 days postinoculation for TSWV-Igg and TSWV-Lgt, respectively (Fig. 1). This was approximately 2 days before G1 or G2 were detected in infected tissue from either isolate. The TSWV-Igg antiserum does not detect G1 (13). Cytoplasmic inclusions were first observed when the G1 or G2 proteins were detected. The association of the appearance of cytoplasmic inclusions with the accumulation of G proteins was similar for both isolates even though enveloped virions were relatively rare in tissues infected with TSWV-Igg. The level of all three structural proteins remained relatively constant from the time of first detection until the tissue became necrotic and samples were no longer collected.

Enveloped virions were evident in epidermal cells (Fig. 2A), mesophyll cells (Fig. 2B), and in the vascular parenchyma cells (Fig. 2C) of leaves infected with TSWV-Lgt. The enveloped virions predominantly occur in groups bound by cisternae (15). Virions and/or viroplasms were observed in epidermal, mesophyll, and vascular parenchyma cells in samples collected 9, 11, 13, and 16 days after inoculation. No filamentous structures were observed.

Three predominant structures were found in the cells infected with TSWV-Igg (Fig. 3A–C). Single, enveloped virions were infrequent and no clusters of mature virions in cisternae were found. The diffuse, electron-dense material, previously referred to as viroplasm (5), was observed in varying amounts relative to the other structures (Fig. 3B–C). These areas appear to be limited by a membrane (Fig. 3B); however, the association of a membrane with these structures may not always be apparent (Fig. 3C). A second area (distinct from the viroplasm), consisting of a relatively dark, electron-dense amorphous area, was only observed in mesophyll cells (Fig. 3B). The amorphous inclusion was distinguished ultrastructurally from the viroplasm in that it was more dense and it was not observed to be associated with a membrane. Although the viroplasm type of structure is common among isolates of both serogroups (L and I), the amorphous inclusion was only observed in tissue infected with TSWV-Igg and has not been observed in cells infected with other TSWV-L or TSWV-I isolates.

Filamentous structures arranged in paracrystalline arrays were also present in varying amounts relative to the other structures. Filaments were consistently arranged in rows with adjacent layers at right angles that appeared as alternating rows of rods and spheres (ends). When observed obliquely, they appear as a lattice or Z-structure. Both planes of view reveal a distinct periodicity

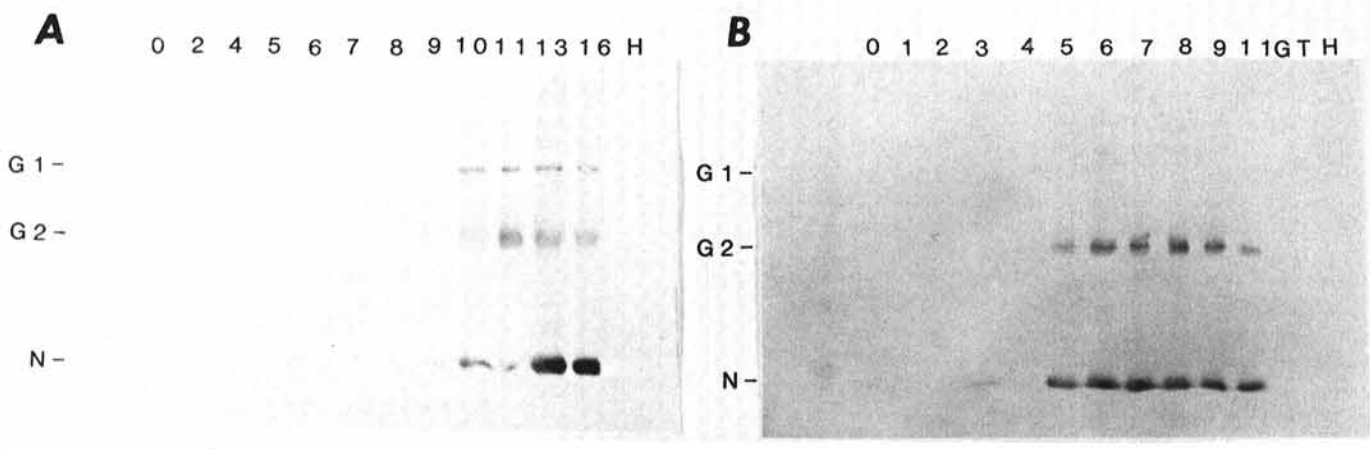

Fig. 1. Accumulation of N, G1, and G2 tomato spotted wilt virus (TSWV) structural proteins in systemically infected *Nicotiana benthamiana* leaves. **A,** Samples from plants inoculated with TSWV-Lgt and probed with anti-TSWV-L antiserum. Numbers at the top of the lanes refer to days postinoculation. A positive sample at days 7 and 8 was visible with the eye. **B,** Samples from plants inoculated with TSWV-Igg and probed with anti-TSWV-I antiserum. Numbers refer to days postinoculation. H = healthy control, GT = TSWV-Lgt.

to the arrangement of filaments. In the later stages of disease, the cytoplasmic space of some epidermal cells may be packed with the paracrystalline structures (Fig. 3A).

Filamentous structures were also observed in cells infected with TSWV-Llet (Fig. 4). These filaments occur in infected cells that contain enveloped virions (Fig. 4A) and in cells that contain viroplasm. These filaments are arranged in loose bundles rather than the distinct paracrystalline arrays observed in TSWV-I.

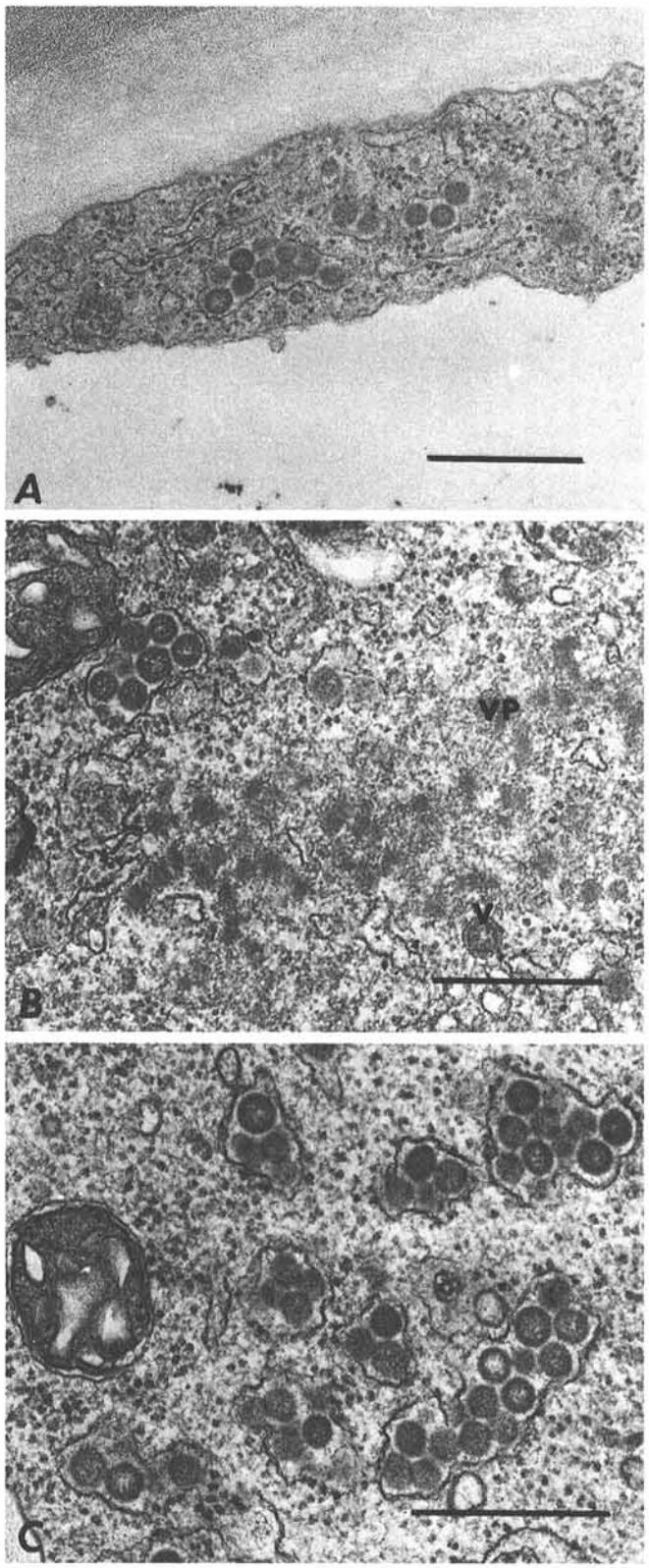

Fig. 2. Ultrathin sections of *Nicotiana benthamiana* leaf tissue infected with a common isolate (L serogroup) of tomato spotted wilt virus (TSWV), TSWV-Lgt, from tobacco. Bar = 0.5 μm. **A,** Epidermal cell with cisternae-bound mature virions (11 days postinoculation). **B,** Mesophyll cell showing typical viroplasm (VP), single quasispherical virions (V) and mature, cisternae-bound virions (16 days postinoculation). **C,** Mature, cisternae-bound virions found in vascular tissue (11 days postinoculation).

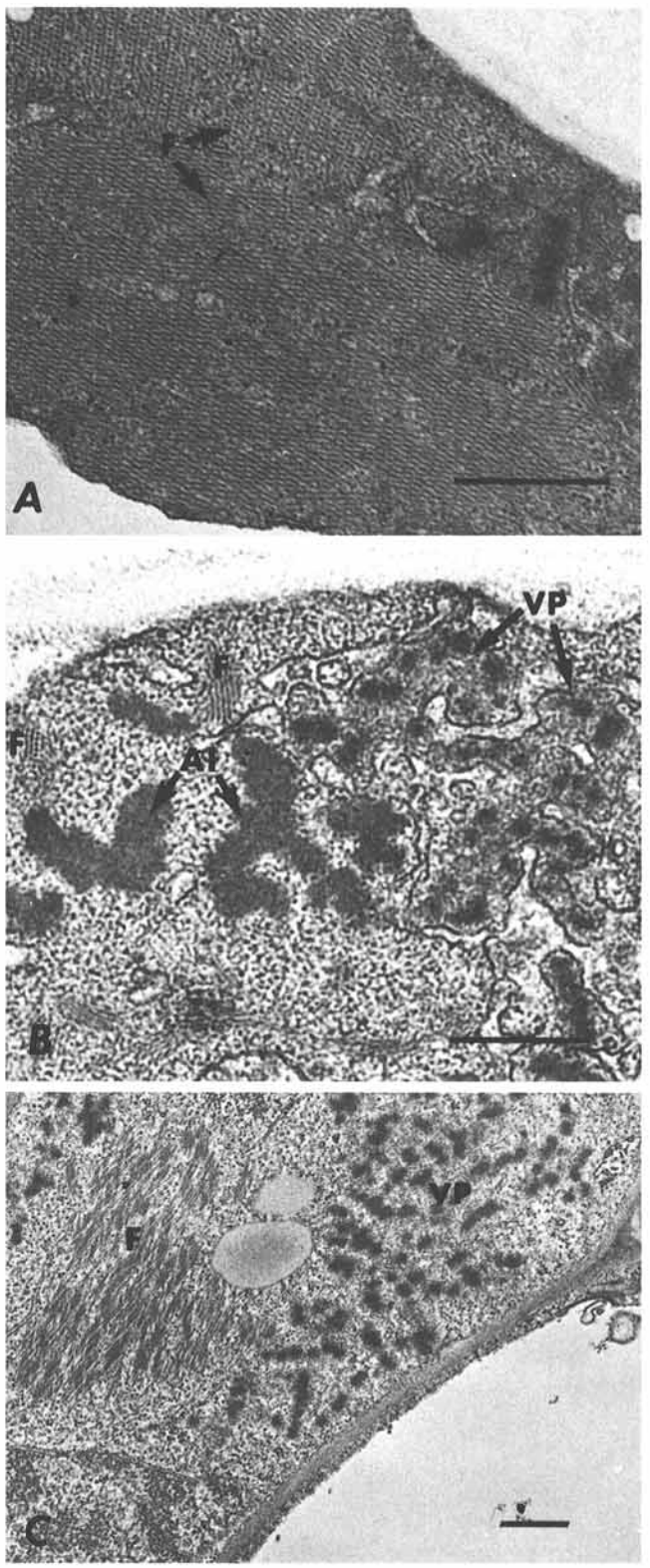

Fig. 3. Ultrathin sections of *Nicotiana benthamiana* leaf tissue infected with an I serogroup isolate of tomato spotted wilt virus, TSWV-Igg, from gloxinia. Bar = 0.5 μm. **A,** Epidermal cell, 7 days postinoculation, filled with filaments (F) arranged in paracrystalline arrays. **B,** Two different inclusions found in mesophyll cells: viroplasm (VP) and amorphous inclusions (AI) (6 days postinoculation). Filaments are also visible. **C,** A vascular cell with filaments and viroplasm. Amorphous inclusions were not observed in these cells (11 days postinoculation).

TSWV-Llet also had a very distinctive arrangement of viroplasm that appeared as a chain of electron-dense areas (Fig. 4B).

Immunogold labeling of tissue infected with TSWV-Igg revealed that only the viroplasm areas bound antibodies to TSWV-I. Neither the filamentous paracrystalline structures nor the amorphous inclusions bound viral antibodies (Fig. 5A–B). The viroplasm areas in tissue infected with TSWV-Lgt also bound homologous viral antibodies. Viroplasms incubated with heterologous combinations of antibodies were not labeled (data not shown).

Evidence was presented for the existence of two types of electron-dense masses, only one of which binds antibodies to viral structural proteins. In addition, we provided evidence for the absence of viral structural proteins in the filamentous inclusions. We also demonstrated the existence of distinct morphological types of filamentous inclusions in the two serotypes.

In general, we observed that viral-related structures were relatively stable and tended to increase in abundance as the post-inoculation period increased. Although we did not specifically investigate the effects of TSWV infection on host ultrastructure, we did observe that chloroplasts maintained their structural integrity even though the virus infection caused distinct chlorotic symptoms. However, we are uncertain if the chloroplasts show diminished function.

DISCUSSION

Three differences were observed between the cytopathology of cells infected with TSWV-L and with TSWV-I isolates. The two major differences were the absence of clusters of cisternae-bound mature virions (15) and the highly ordered paracrystalline array of filaments in tissues infected with the TSWV-I isolates. The third difference was the presence of electron-dense masses that did not bind viral antibodies. We also provided serological evidence to support the hypothesis that the filaments are not composed of the major structural proteins found in the virion and thus are not aggregated nucleocapsids.

In this and previous investigations (13), we examined tissues infected with two isolates of TSWV-I, and in each tissue individual virions were rarely observed. Membrane-bound clusters of mature virions were never observed in any tissues. Observation of individual virions by ISEM (13) suggested that uncoupling occurs after virion assembly and before maturation into membrane-bound clusters. Milne has described this process as the outer membrane of several virions coalescing to form a cisterna, while the inner membrane and core are released into the center as mature particles (15). It is unlikely that the inhibition of virion maturation is a host-directed event because the maturation of virions in *N. benthamiana* infected with TSWV-L isolates was similar to that reported in other hosts (5,9,15).

The TSWV-I isolates remained highly infectious throughout repeated transfers, and characterization of TSWV-Iinc (13) has not revealed any detectable deletions in the M RNA. Deletions in the M RNA have been associated with a defective isolate of

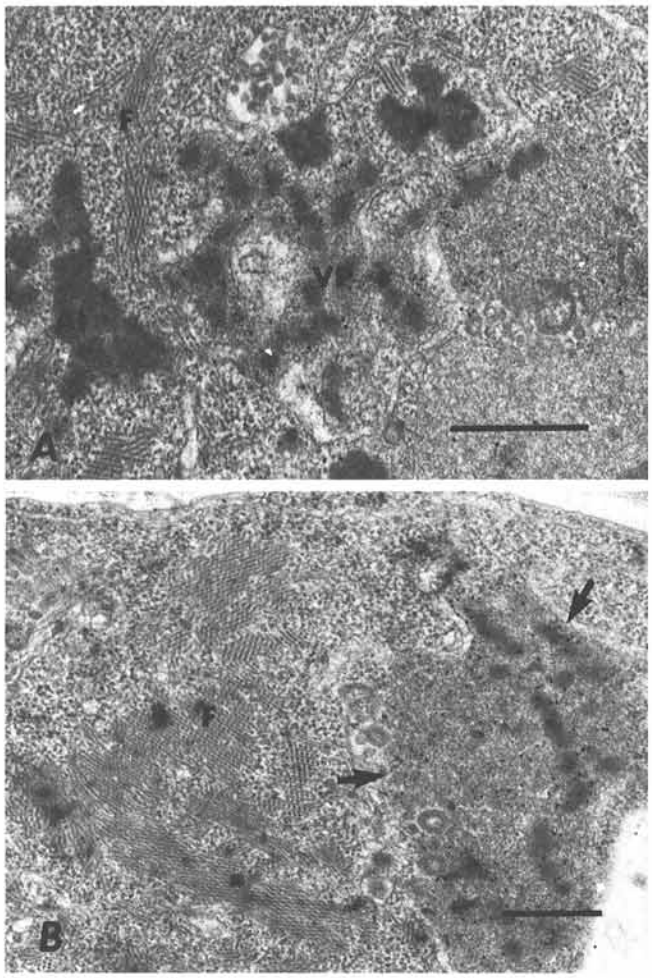

Fig. 4. Ultrathin sections of a lettuce isolate, tomato spotted wilt virus (TSWV)-Llet, in *Nicotiana benthamiana* leaf tissue. Bar = 0.5 μm. **A,** Mature, cisternae-bound virions, found only in vascular tissue. **B,** The distinct, segmented appearance of TSWV-Llet viroplasm (VP) in an infected mesophyll cell. Also, lateral and end views of filaments (F).

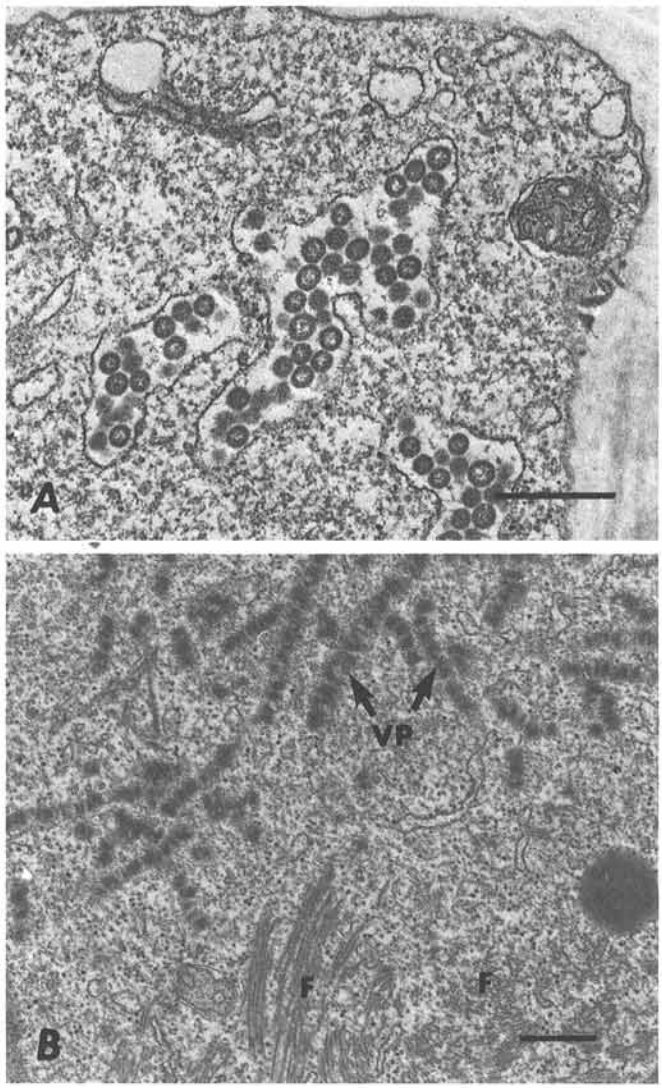

Fig. 5. Immunogold-labeled sections from *Nicotiana benthamiana* leaf tissue infected with tomato spotted wilt virus (TSWV-Igg). Bar = 0.5 μm. **A,** Cell containing amorphous inclusions (AI), filaments (F), and viroplasm (VP). Gold-labeled protein A bound mainly to viroplasm. **B,** Arrows indicate areas of viroplasm labeled with 10 nm protein A-gold.

TSWV-L (22). Ie reported that in serial passage in *N. tabaccum* of a defective isolate from amaryllis, particle formation decreases and viroplasm increases (10). Virions have been found in lobelia infected with a TSWV-I isolate, but at a very low frequency (T. Allen, *personal communication*). Thus, the relatively low abundance of virions in TSWV-I isolates is probably a stable character of these isolates in *N. benthamiana*.

Filamentous structures have been observed in other hosts infected with common-type isolates (L) of TSWV (5), and we observed similar structures in our isolate TSWV-Llet. Although previous reports were on different isolates in different hosts, the filaments were arranged in loose bundles of parallel filaments similar to our TSWV-Llet isolate. Filamentous structures in cells infected with TSWV-Igg and TSWV-Iinc (13) were arranged in paracrystalline arrays with alternating layers of filaments aligned at right angles. We also observed that these paracrystalline arrays did not bind viral antibodies, which suggests that they consisted of either nonstructural viral proteins and/or host proteins. Preliminary information presented by D. Peters at the international TSWV workshop indicated that bundles of filaments produced in cells infected with TSWV-L type isolates were composed of a nonstructural viral protein (19).

Viroplasms observed in cells infected with isolates from both TSWV-L and TSWV-I were structurally similar to those reported previously for isolates that produced mature virions and were probably of the L serogroup (5,12,15). The electron-dense areas of infected cells have been shown to be proteinaceous (9) and were designated as viroplasm (15). Ie digested amorphous masses with pronase and determined they were protein, but did not determine their origin (9). We provided evidence that these structures are involved in the synthesis of viral proteins through labeling with antibodies to viral proteins. The immunogold labeling of cells infected with TSWV-Igg also revealed a second type of electron-dense area that we have provisionally designated as amorphous inclusions. The amorphous inclusions are distinguished from the viroplasms by the absence of binding of viral antibodies. They also stain more densely with conventional stains and are not associated with membranes as are the viroplasms. We have observed some electron-dense areas that appeared to be partially differentiated into filaments (data not shown), but have not determined if these areas are viroplasms or amorphous inclusions.

These investigations demonstrated that the unusual cytopathology of TSWV-I, consisting of the absence of mature clusters of virions and the paracrystalline arrays of filaments, is shared by at least two isolates, TSWV-Igg and TSWV-Iinc. The paracrystalline arrays of filaments should be considered as one of the distinguishing factors of the I serogroup of TSWV in addition to the N protein, which is serologically distinct from that in the L serogroup. We also suggested that the presence of particles should not be used as a diagnostic tool. The presence or low abundance of particles seems to have no effect on the infectious nature of TSWV-I, and may vary by host. Although there were distinct differences in cytopathology between the L and I groups, they induced similar macroscopic symptoms in *N. benthamiana*; they were observed in vascular parenchyma, mesophyll, and epidermal cells; and structural proteins had a similar pattern of accumulation in plants infected by both types.

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