Tospovirus Replication in Insect Vector Cells: Immunocytochemical Evidence that the Nonstructural Protein Encoded by the S RNA of Tomato Spotted Wilt Tospovirus Is Present in Thrips Vector Cells

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ABSTRACT

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Tomato spotted wilt tospovirus (TSWV) is an insect-transmitted virus that is the type member of the *Tospovirus* genus, which is the only genus in the family Bunyaviridae containing viruses that infect plants. Direct evidence that *Tospoviruses* replicate in their thrips vectors has been difficult to obtain because of limitations to definitively detect replicative intermediates of TSWV or to immunolabel vector tissues. A nonstructural protein is encoded by the small RNA of TSWV, and translation of the NSs protein occurs from a subgenomic RNA formed after transcription of viral sense RNA. This protein is designated as nonstructural because it is found only in TSWV-infected cells and has not been found in assembled virions. Thus, presence of NSs indicates that processes of virus replication have occurred. A specific and sensitive antibody to the NSs has been produced and used to localize NSs in the cells of a thrips vector species, *Frankliniella occidentalis*. The direct, immunocytochemical evidence presented that NSs is present in thrips cells indicates that TSWV, a plant pathogenic virus, replicates in the cells of its invertebrate vector. Replication of TSWV in thrips cells is significant to understanding the pandemic importance and taxonomy of this virus.

Additional keywords: detection, epidemiology, histology, management, serology.

Tomato spotted wilt tospovirus (TSWV) is the type member of the *Tospovirus* genus, the only genus in the family Bunyaviridae containing viruses that infect plants (2,5,8,23,26). Severe pandemics in food, fiber, and ornamental crops have been caused by *Tospoviruses* (10,23). Other members of the Bunyaviridae, a large family of enveloped RNA viruses, cause serious human illness, including mosquito-borne encephalitis and Rift Valley fever (7). Most of the viruses in the family Bunyaviridae that are spread by arthropods to vertebrate hosts have also been shown to replicate in their invertebrate vectors—primarily mosquitoes and ticks (28). The *Tospoviruses* are transmitted from plant to plant by several species of thrips (order: Thysanoptera) (1,14,24,36). While the replicative strategy of TSWV in plants resembles that of viruses in the *Phlebovirus* and the former *Uukuvirus* genera in vertebrate and invertebrate cells (6,11,16–19,33), replication of *Tospoviruses* in thrips vectors or other insect cells has never been demonstrated conclusively (3,10,11,34).

Previous research indicated that TSWV persisted, but did not necessarily replicate, in thrips vectors (24). More recently, enzyme-linked immunosorbent assay (ELISA) of individual thrips, hybridization of cDNA probes specific to viral and viral complementary strands to thrips RNA extracts, and electron microscopic observations of thrips organs have provided indirect evidence that *Tospovirus* replication may occur in thrips (3,10,11). Serological evidence that TSWV increased in populations of individual thrips over time could not be confirmed due to significant variability in mean virus titers detected in individual thrips and between thrips cohorts and because ELISA of individuals lacked sensitivity to consistently detect changes in virus titer in these small insects (0.5 × 1.5 mm) (3,11). Hybridization of cDNA probes specific to viral and complementary sense strands of TSWV large RNA (L RNA) with RNA extracts from viruliferous thrips (10,11; T. L. German and D. E. Ullman, *unpublished*) was inconclusive because both viral and complementary strands of the small (S) and middle (M) RNA components of TSWV are sometimes packaged in virus particles (19). Whether both senses of the L RNA component are packaged during virus assembly is unknown. Finally, in the absence of appropriate immunolabeling, putative viral inclusions observed in thrips cells could not be definitively associated with TSWV presence or replication (11,31). Finally, direct evidence for virus replication in thrips cells by immunocytochemistry has been difficult to obtain because of the insect's minute size and because there have been no methods for maintaining thrips cell ultrastructure while preserving antigenicity of viral structural and nonstructural proteins for immunolabeling (35).

The S RNA of TSWV encodes the viral nucleocapsid protein (N) (a structural protein in assembled virions) and a 52.4 kDa nonstructural protein (NSs) found in fibrous paracrystalline inclusions in infected plant cells but not in assembled virions or healthy plants (4,6,16,17). The N and NSs proteins are encoded in an ambisense fashion in which N is translated from a subgenomic viral complementary sense mRNA and NSs is translated from a subgenomic virus-sense mRNA (6). Because the S RNA segment has to be replicated into a full-length viral-complementary strand before NSs mRNA synthesis can commence and because NSs is a nonstructural protein found only in *Tospovirus*-infected cells and not in assembled virions, the presence of fibrous paracrystalline inclusions composed of NSs protein provides direct evidence of virus replication (16,17,29,33). We recently developed a sensitive

and quantitative immunocytochemical method for assaying the presence of TSWV structural and nonstructural proteins in insect cells using microwave energy for fixation and embedding (35).

In this paper, direct immunocytochemical evidence that TSWV replication occurs in the cells of a thrips vector species, *Frankliniella occidentalis* (Pergande), the western flower thrips (WFT), is presented. The NSs protein was expressed in *Escherichia coli* for use in production of a specific rabbit anti-NSs serum to study the expression of the NSs protein in insect and plant cells. Immunocytochemical evidence indicates that NSs is expressed in thrips cells and localized in association with fibrous paracrystalline arrays in infected thrips and plant cells.

MATERIALS AND METHODS

Plant material and virus isolate. A TSWV isolate collected from infected tomato on the Hawaiian island of Maui (TSWV-MT2) was used for all serological tests and for thrips acquisition. The isolate was maintained by inoculation with the WFT, *Frankliniella occidentalis* Pergande, as previously described (32). Infected *Emilia sonchifolia* (L.) D. C. ex Wight were maintained as thrips acquisition hosts in greenhouses at the University of Hawaii-Manoa campus. The thrips-transmissible isolate TSWV-MT2 was used to mechanically inoculate *Lycopersicon esculentum* Miller 'Celebrity', *Datura stramonium* L., and *Nicotiana benthamiana* L., which were then used for virus purification, extraction of viral proteins for ELISA and Western blotting and for extraction of viral RNA. These plants were maintained in greenhouses at either the University of Wisconsin or Oklahoma State University campuses prior to use.

Insects. The WFT used in all tests of virus acquisition, replication, and inoculation were maintained on pods of the green bean, *Phaseolus vulgaris* L. 'Green Crop', as previously described (32).

RNA extraction. Purified virus preparations were obtained using previously described procedures (12). RNA was extracted from purified virus preparations in an equal volume of 2× buffer (100 mM TrisHCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid [EDTA], 2% sodium dodecyl sulfate [SDS]) in a 1.5-ml tube at 65 C for 15 min. The extract was then mixed with an equal volume of Tris-buffered phenol and incubated for an additional 5 min at 65 C. The mixture was centrifuged at 12,000 *g* for 5 min and the aqueous layer transferred to a clean tube and then extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The mixture was again centrifuged, and then the aqueous layer was removed. The remaining organic layers were extracted with an equal vol of 1× buffer and clarified by centrifugation. Aqueous fractions were pooled (0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol) and precipitated overnight at −20 C. The RNA was then centrifuged at 12,000 *g* for 20 min, washed with 70% ethanol, and dried in vacuo. The RNA pellet was resuspended in 0.1%-diethylpyrocarbonate treated water and stored at −70 C.

cDNA synthesis. Single-stranded DNA complementary to the sequence encoding the nonstructural (NSs) protein on the S RNA of TSWV was made using the Riboclone cDNA Synthesis System (Promega, Madison, WI). Briefly, 1 μg of TSWV RNA was mixed with 30 pmol NSD1 primer (constructed at the University of Wisconsin Biotechnology Center, 3′-TCCTAGTTTCATTAGAA-CGACGTCGGT-5′ [complementary to bases 1471–1488 of the Brazilian isolate CNPH1 of TSWV (6) with a *Pst*I site at the 5′ end]) and heated to 70 C for 5 min. To the cooled primer-template mixture, first-strand buffer (50 mM Tris-Cl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol), RNasin ribonuclease inhibitor (1 U), Na pyrophosphate (4 mM), and avian myeloblastosis virus reverse transcriptase (12 U) were added and the reaction incubated at 42 C for 60 min. The reaction products were stored at −20 C.

Polymerase chain reaction. The single-stranded cDNA was used as a template for amplification of the NSs gene in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTPs, 50 pmol each of primers NSU2- 3′-GTGAACTTCTGTAATACCGA-CGTCCAT-5′ [bases 75–92 of TSWV-CNPH1 (6) with a *Pst*I site at the 5′ end] and NSD1, and 2.5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in a 100-μl reaction volume. The reaction mixture was overlaid with 100 μl of sterile mineral oil, and amplification was performed for 30 cycles for 1 min each at 94, 45, and 72 C. Reaction products were visualized on a 1% agarose gel run in 0.5× Tris-borate EDTA (TBE) and stained with ethidium bromide.

A 1.4-kb fragment was purified from agarose (Prep-A-Gene, Bio-Rad, Richmond, CA), phosphorylated, and self-ligated with T4 polynucleotide kinase and T4 DNA ligase, respectively (15), and subsequently digested with *Pst*I to clone into the *Pst*I site of pBluescript (Stratagene, La Jolla, CA) (25). This recombinant plasmid, pNS1PST1.4, was used to transform *E. coli* DH5α. Plasmid DNA was isolated using an alkaline lysis procedure (25) and its composition, as compared to the CNPH1 isolate, was verified with restriction enzyme analysis. Additionally, DNA sequencing from both ends was performed on alkali-denatured plasmid templates by the dideoxy termination method (27) using Sequenase reagents (United States Biochemical, Cleveland, OH). Analysis of sequence data was performed using programs provided by the Genetics Computer Group (Madison, WI).

Expression of NSs in *E. coli* and antibody production. The NSs insert from the plasmid pNS1PST1.4 was treated with T4 polymerase, supplied with *Bam*HI linkers, and cloned into the *Bam*HI site of the T7 expression vector, pET-11c (Novagen, Madison, WI) to obtain the plasmid pETNSBM-5. The plasmid was transformed into *E. coli* BL21 (DE3) cells, and DNA was recovered by alkaline lysis. The proper orientation for expression of the insert was determined by restriction enzyme digestion. *E. coli* cells harboring the recombinant expression plasmid were grown in 25 ml of M9ZB media (10 g of tryptone, 5 g of NaCl, 1 g of NH_4Cl, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 4 g of glucose, 1 ml of 1 M $MgSO_4$ per liter) for 3 h and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside to overexpress NSs. Cells were collected after 2.5 h and boiled in SDS loading buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 14 mM β-mercaptoethanol), and proteins were separated on a preparative 12% SDS-polyacrylamide gel (PAGE) using the Laemmli SDS discontinuous buffer system (13). The gel was stained with 0.3 M $CuCl_2$ (20), and the band corresponding to NSs was recovered and the protein eluted from the gel slices in 50 mM NH_4HCO_3, 0.01% SDS with the Bio-Rad model 422 Electroeluter. The NSs protein was dried in vacuo, resuspended in 50 mM Tris, pH 8.0, and its concentration determined with the Bradford dye binding assay (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. To determine the time course of NSs expression, the above procedure was followed except cells were collected at 0, 60, 120, 180, and 240 min in noninduced and induced preparations.

A female New Zealand white rabbit was injected twice, intramuscularly, 1 wk apart with 100 μl of gel purified NSs protein (120 μg) emulsified in an equal volume of TiterMax (CytRx Corp, Norcross, GA). One month later the animal was injected with 300 μl of protein suspension emulsified in an equal volume of TiterMax. Half the material was injected intramuscularly and the other half injected subdermally. The final bleed from the animal was taken 2 wk later. Antiserum titer was greater than 1:3,000 in nonsandwich enzyme-linked immunosorbent assay against the fusion protein or TSWV-infected plant material. Anti-TSWV antibody was provided by J. J. Cho and M. Wang of the Department of Plant Pathology, University of Hawaii.

Western blotting for detection of NSs protein. TSWV-infected and healthy *N. benthamiana, E. sonchifolia, D. stramonium,* and *L. esculentum* 'Celebrity' were frozen in liquid nitrogen and pulverized with a mortar and pestle. The frozen tissue (500 mg) was suspended in 1 ml of extraction buffer (150 mM NaCl, 50 mM sodium phosphate, pH 7.4, 250 mM sodium ascorbate, 5 mM EDTA, 0.25% SDS, 1.5% (w/v) polyvinylpolypyrrolidine, and 14 mM freshly added β-mercaptoethanol) and solubilized on ice for 30 min. The extracts were clarified by centrifugation at 12,000 *g* for 15 min, and the supernatants were boiled in 4×

SDS loading buffer for 5 min. Approximately 5 mg of tissue equivalents (15 μl) were loaded onto 12% SDS-PAGE gels (mini-Protean II, Bio-Rad, Richmond, CA). The proteins were transferred onto wetted nitrocellulose membranes (BA-S, 0.2 μm, Schleicher & Schuell, Inc., Keene, NH) for 3 h at 400 mA in a TE-42 Transphor electrophoresis cell (Hoefer, San Francisco, CA) using a transfer buffer consisting of 39 mM glycine, 48 mM Tris-Cl, pH 8.3, 0.037% SDS, and 20% methanol (25).

After transfer, membranes were stained in 1× Ponceau S (Sigma, St. Louis, MO) (25) to identify molecular weight markers. The membranes were destained in water and then incubated for 1 h at room temperature in TBS (20 mM Tris-Cl, pH 7.5, 0.5 M NaCl) containing 3% gelatin to block excess protein binding sites (9). Then membranes were washed twice for 10 min each with TBS containing 0.05% Tween-20 (TTBS) and incubated for 1 h at room temperature with antiserum to NSs or N proteins diluted 1:1,500 or 1:2,000, respectively, in 10 ml of TTBS containing 1% gelatin. To remove antibodies in the N antiserum reactive to plant proteins, it was cross-absorbed with 1% (w/v) acetone powder, prepared from healthy *N. benthamiana*, for 30 min at 4 C prior to incubation with the membrane. Membranes were washed twice with TTBS and then incubated for 1 h at room temperature with a 1:2,000 dilution of goat antirabbit IgG coupled with alkaline phosphatase in 10 ml of TTBS-1% gelatin. Then membranes were washed twice with TTBS and then with TBS to remove the Tween-20. Bands were visualized by incubating membranes in 10 ml of alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-Cl, pH 9.5) containing 330 μg/ml nitro blue tetrazolium and 165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO). Membranes were then washed with water.

Electron microscopy of Spurrs-embedded insects. Intact insects processed for embedding in Spurrs resin and viewing with transmission electron microscopy were fixed, embedded, sectioned, and viewed as previously described (30,32). Seven- to ten-day-old adults from cohorts used to inoculate *E. sonchifolia* (see thrips acquisition and transmission of TSWV, below) were employed in these experiments.

Immunocytochemistry. Dissected WFT midguts and infected *D. stramonium* used for immunocytochemical analyses were fixed and embedded as previously described (35). Embedded samples were sectioned at 90–100 nm and placed on nickel or copper Formvar-coated slot grids. Sections were then incubated at room temperature with 5% fetal bovine serum in 0.05 M phosphate buffered saline with 0.02 M glycine (pH 7.4) (FBS-PBS-GLY) for 15 min and reacted with anti-TSWV polyclonal antibody (1:10 in FBS-PBS-GLY) or anti-NSs polyclonal antibody (1:10 in FBS-PBS-GLY) for 45 min. Grids reacted with anti-TSWV antiserum were labeled with 10 nm Protein A-Gold, whereas those reacted with anti-NSs antiserum were labeled with 15-nm gold-conjugated goat-anti-rabbit antiserum. Serum controls included grids incubated with rabbit serum (1:10 in FBS-PBS-GLY) and reacted with both gold conjugates. Finally, grids were fixed with 2% glutaraldehyde, stained with 2% uranyl acetate in water and lead citrate, and viewed with a Zeiss 10A Electron Microscope (Carl Zeiss, Oberkochen, Germany). Results of immunocytochemistry were quantified and statistically analyzed as previously described (35).

Thrips acquisition and transmission of TSWV. First instar larval WFT were placed on groups of excised, TSWV-infected *E. sonchifolia* leaves and allowed to feed for 48–72 h. Noninfected thrips, used as healthy controls in inoculation tests and microscopic analyses, were fed simultaneously on noninfected *E. sonchifolia*. Immunocytochemical analyses were performed on dissected midguts of WFT second instar larvae subsampled after 48 h of acquisition feeding. A separate cohort of larvae were allowed to acquire TSWV as just described and reared to adulthood on noninfected green bean pods replaced daily. The 7- to 10-day-old adults were given a 3-day inoculation access in groups of 25 insects per plant to 10 noninfected *E. sonchifolia* plants to determine presence of infectious TSWV in the thrips. Plants were then held in the greenhouse and observed for symptoms of TSWV. Plants showing symptoms were tested with ELISA as previously described (12). A subsample of nine thrips was then removed from these plants and prepared for embedding in Spurrs resin and viewing by transmission electron microscopy. Spurrs embedded samples were not immunolabeled because antigenicity of TSWV is destroyed by the fixation and embedding processes (32,35).

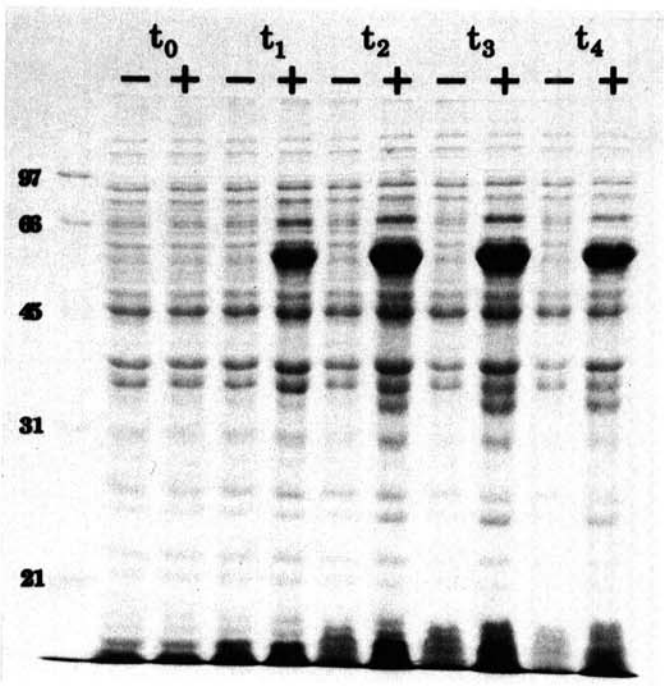

Fig. 1. Time course of NSs protein expression from plasmid pETNSBM-5 in *Escherichia coli* BL21 (DE3) cells. Samples taken from uninduced (−) or induced (+) cultures at 0, 60, 180, and 240 min after induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside were subjected to polyacrylamide gel electrophoresis and stained with Coomassie blue. Molecular weight standards (kDa × 10^3) are shown on the left. The prominent band with a M_r of about 54 kDa in the induced cultures (lanes t_1–t_4) has the expected mobility for the tomato spotted wilt tospovirus nonstructural protein encoded by the small RNA (NSs).

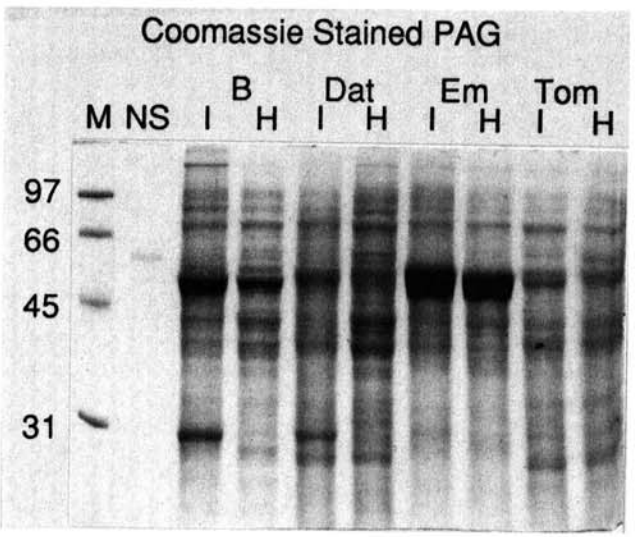

Fig. 2. Polyacrylamide gel stained with Coomassie blue, of total proteins from tomato spotted wilt tospovirus (TSWV)-infected (I) or noninfected (H) *Nicotiana benthamiana* (B), *Datura stramonium* (Dat), *Emilia sonchifolia* (Em), and *Lycopersicon esculentum* 'Celebrity' (Tom) and purified nonstructural protein (NSs) encoded by the NSs gene on the TSWV small RNA expressed in *Escherichia coli* BL21 (DE3) cells. Molecular weights of protein standards (M) are in kDa × 10^3.

RESULTS

Expression of NSs from plasmid pETNSBM-5 in *E. coli* BL21 (DE3) cells and antibody specificity. The time course of NSs protein expression from plasmid pETNSBM-5 in *E. coli* BL21 (DE3) cells can be seen in Figure 1. When cells were not induced, production of protein with the correct molecular weight for TSWV NSs was not detected. In contrast, with induced cells, a recombinant protein of approximately 54 kDa was produced. This protein was the expected size for the TSWV NSs clone inserted, plus an additional 11 amino acids from the vector. Protein production increased with increasing time up to 240 min when protein production decreased slightly. When total proteins from several species of TSWV-infected and noninfected plants were analyzed on a polyacrylamide gel (Fig. 2), bands of expected mobility for the TSWV N structural protein (28.8 kDa) were evident in infected tissue but not in noninfected tissue or in the lane containing purified NSs protein. In constrast, a protein with a mobility expected for expressed NSs (approximately 54 kDa) was evident in the lane containing purified in vitro expressed NSs protein, but was not evident in lanes representing infected or noninfected plants.

In Western blots, polyclonal antisera against the N protein of purified TSWV reacted with a 28.8 kDa protein, the size expected for the N protein of TSWV in infected plants (Fig. 3). This anti-N antibody did not react with bands representing the NSs protein in infected tissue, the 54 kDa in vitro expressed NSs protein, or proteins present in noninfected plants. In contrast, when transferred proteins were probed with polyclonal antisera raised against purified in vitro expressed NSs protein, only proteins representing NSs protein from TSWV-infected plants and the in vitro expressed NSs were detected (Fig. 4). No reaction to the N protein (a TSWV structural protein) or proteins in noninfected plants was detected.

Electron microscopic observations. Electron microscopic observations of larval WFT fed on TSWV-infected plants revealed spherical membrane-bound virions in the lumen of the digestive tract immediately following acquisition feeding (6:6 insects sectioned). In infected larvae ($n = 6$) and adults ($n = 9$), two morphologically distinct viral inclusions, viroplasms (VP), and fibrous paracrystalline arrays (F), sensu Urban (33), were observed (Figs. 5–7). VP consisted of an amorphous, matrix material with embedded aggregates of more electron-dense material, whereas F consisted of aggregates of fibrous material in paracrystalline arrays of various shapes and sizes (Figs. 5–7). Among larvae, where observations were limited to midgut cells, VP were seen in the midgut epithelia of five of six insects sectioned and F were seen in four of six insects sectioned (Figs. 5,6). Among adults reared on healthy beans following TSWV acquisition, VP were observed in muscle cells surrounding the midgut of four of nine insects sectioned (data not shown) and VP, F, and assembled virions in the salivary gland of three of nine insects (Fig. 7). Virus particles and inclusions were morphologically similar to those observed in infected plant material (Figs. 8,9). Furthermore, the cohort from which these adults were subsampled successfully transferred TSWV to eight of 10 *E. sonchifolia* plants.

Immunocytochemistry. Immunocytochemical analysis of serial ultrathin sections of larval midguts embedded in LR White revealed that anti-TSWV polyclonal antibody followed by Protein A-Gold label (10 nm) reacted with virus particles in the WFT digestive tract (data not shown), VP in midgut epithelial cells (Fig. 5), and virions in infected plants (Fig. 8). However, anti-TSWV antibodies did not react with F in the same WFT epithelial cells and infected plant cells (Figs. 5,8). In contrast, F in WFT epithelial cells and infected plant cells were specifically tagged by anti-NSs polyclonal antibodies followed by goat-anti-rabbit-gold label (15 nm), whereas VP were not immunostained (Figs. 5,8). Accuracy and specificity of our immunolabeling is demonstrated by highly significant differences in gold label/cm^2 on morphologically distinct inclusions, surrounding cytoplasm, and serum controls (Table 1). Virions and inclusions were absent and immunolabel negligible (less than serum controls) in negative controls consisting of insects of the same age fed on noninfected plants.

DISCUSSION

Significant immunolabeling of F in the midgut epithelial cells of larval WFT with anti-NSs provides direct immunocytochemical evidence that the 52.4-kDa NSs protein encoded by TSWV S RNA is present in these inclusions. To our knowledge, this is the first direct evidence that TSWV replication occurs in thrips vectors. Western blot analysis and immunocytochemical analysis demonstrate the specificity of anti-NSs and provide further evidence that NSs protein is not present in virions or VP. TSWV

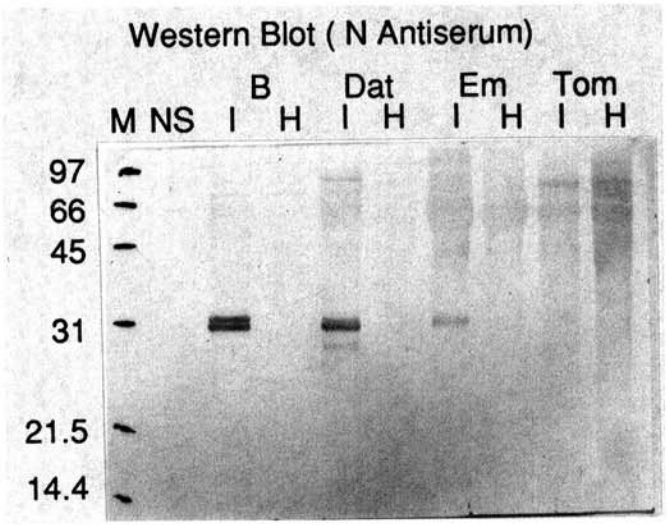

Fig. 3. Western blot analysis of proteins transferred from a gel identical to that shown in Figure 2. Transferred proteins were probed with a polyclonal antiserum to the nucleocapsid protein (N) from purified tomato spotted wilt tospovirus (TSWV) (N antiserum). A protein of 28.8 kDa, the size expected for the TSWV N protein, a structural protein, is evident in the lanes containing transferred proteins from TSWV-infected tissues. No reaction was observed to noninfected plant proteins or to bands representing the TSWV nonstructural protein encoded by the small RNA (NSs).

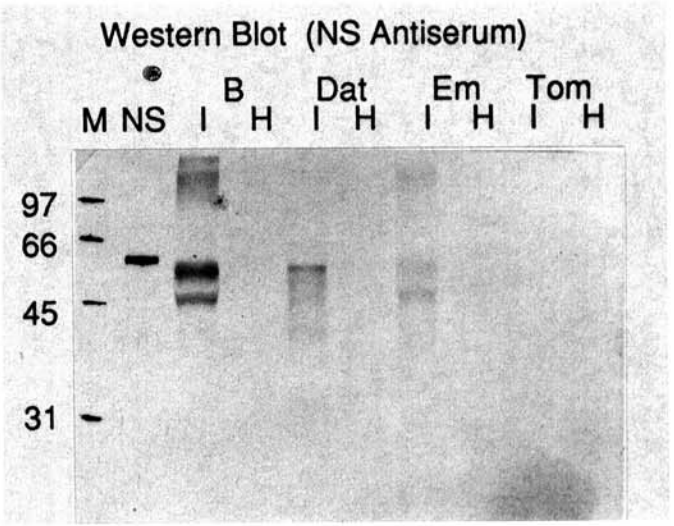

Fig. 4. Western blot analysis of proteins transferred from a gel identical to that shown in Figure 2. Transferred proteins were probed with polyclonal antiserum against the nonstructural protein encoded by the small RNA (NSs) of tomato spotted wilt tospovirus (TSWV) expressed in *Escherichia coli* (NSs antiserum). A reaction was observed to purified in vitro expressed NSs protein (lane NS) and to a protein of approximately 53 kDa, the expected size of NSs in infected tissues, in lanes containing transferred proteins from infected plants. No reaction was observed to noninfected plant proteins or to TSWV structural proteins.

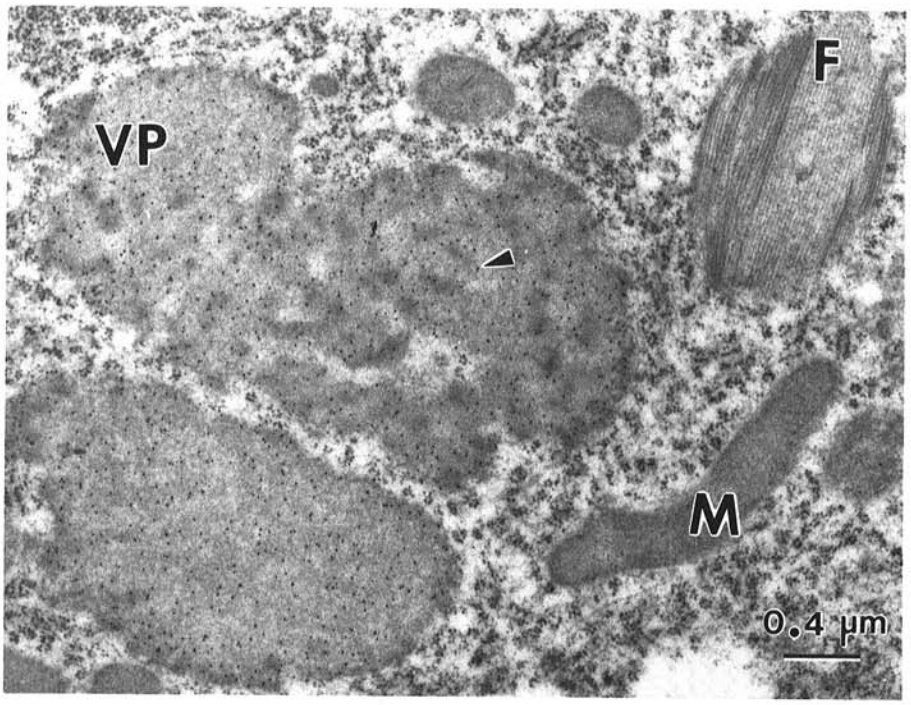

Fig. 5. Micrograph showing morphologically distinct cytoplasmic inclusions in a larval *Frankliniella occidentalis* midgut epithelial cell following 72 h of feeding on tomato spotted wilt tospovirus (TSWV)-infected plants. Viroplasms (VP) consisting of formations of amorphous, matrix material in which aggregates of more electron-dense material were sometimes embedded reacted specifically with anti-TSWV polyclonal antibody. Aggregates of fibrous material in paracrystalline arrays (F), cellular organelles such as mitochondria (M), and cytoplasmic regions were not immunostained.

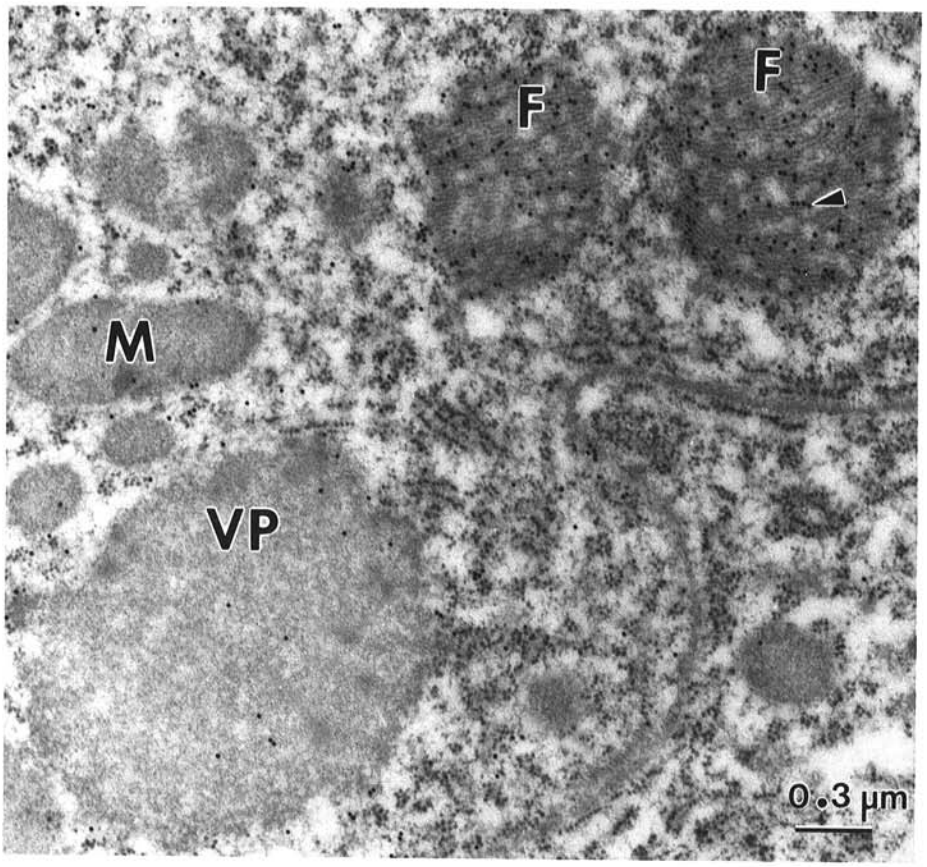

Fig. 6. Micrograph of a midgut epithelial cell serial to that shown in Figure 5 reveals that fibrous paracrystalline arrays (F) reacted specifically with polyclonal antibody against the nonstructural protein encoded by the small RNA segment of tomato spotted wilt tospovirus. In contrast, viroplasms (VP), cellular organelles such as mitochondria (M), and cytoplasmic regions were not immunolabeled.

infection is apparently widespread in thrips vectors, as comparative cytopathology of 10-day-old viruliferous adults also revealed VP in muscle cells surrounding the midgut (data not shown) and VP, F, and assembled virions in salivary glands. Results from inoculation of *E. sonchifolia* with TSWV by insects subsampled from the same cohort further support the hypothesis that the cellular events we observe lead to production of infective TSWV particles and do not represent abortive or abnormal replicative processes.

While the function of the NSs and the purpose of its association and accumulation in fibers remains unknown (16,17,29), the presence of NSs in inclusions in thrips cells denotes virus replication for the following reasons. First, analysis of viral proteins by our laboratories and others (6,17) demonstrates that NSs protein only occurs in infected cells and is not present in virions or healthy plant tissue. Second, the replicative strategy of TSWV S RNA is ambisense, whereby N is translated from a subgenomic viral complementary sense mRNA and NSs is translated from a subgenomic virus-sense mRNA (6,29). Thus, the S RNA segment has to be replicated into a full-length viral-complementary strand before NSs mRNA synthesis can commence. For these reasons, presence of fibrous paracrystalline inclusions composed of NSs provide direct evidence of virus replication (11,29).

The antibodies used in our immunocytochemical analysis are specific and sensitive as demonstrated by western blot analysis and quantification of immunolabeling of virions and inclusions in plant and insect cells. In vitro expressed NSs protein from *E. coli* was present in preparations from induced cells only and was of the size predicted for the inserted NSs clone insert plus 11 amino acids from the vector (approximately 54 kDa). These data show that we have expressed NSs and not some portion of the bacterial plasmid. Furthermore, comparison of total proteins from infected and noninfected plants demonstrates that a protein of approximately 53 kDa is present in infected plants but not in noninfected plants. Western blot analysis with anti-N antisera and anti-NSs antisera (the serum we raised against purified in vitro expressed NSs protein) shows that purified in vitro expressed NSs protein and NSs protein in infected plants reacts with anti-NSs antisera but not with anti-N antisera. In contrast, anti-N antisera reacted with the TSWV N protein and

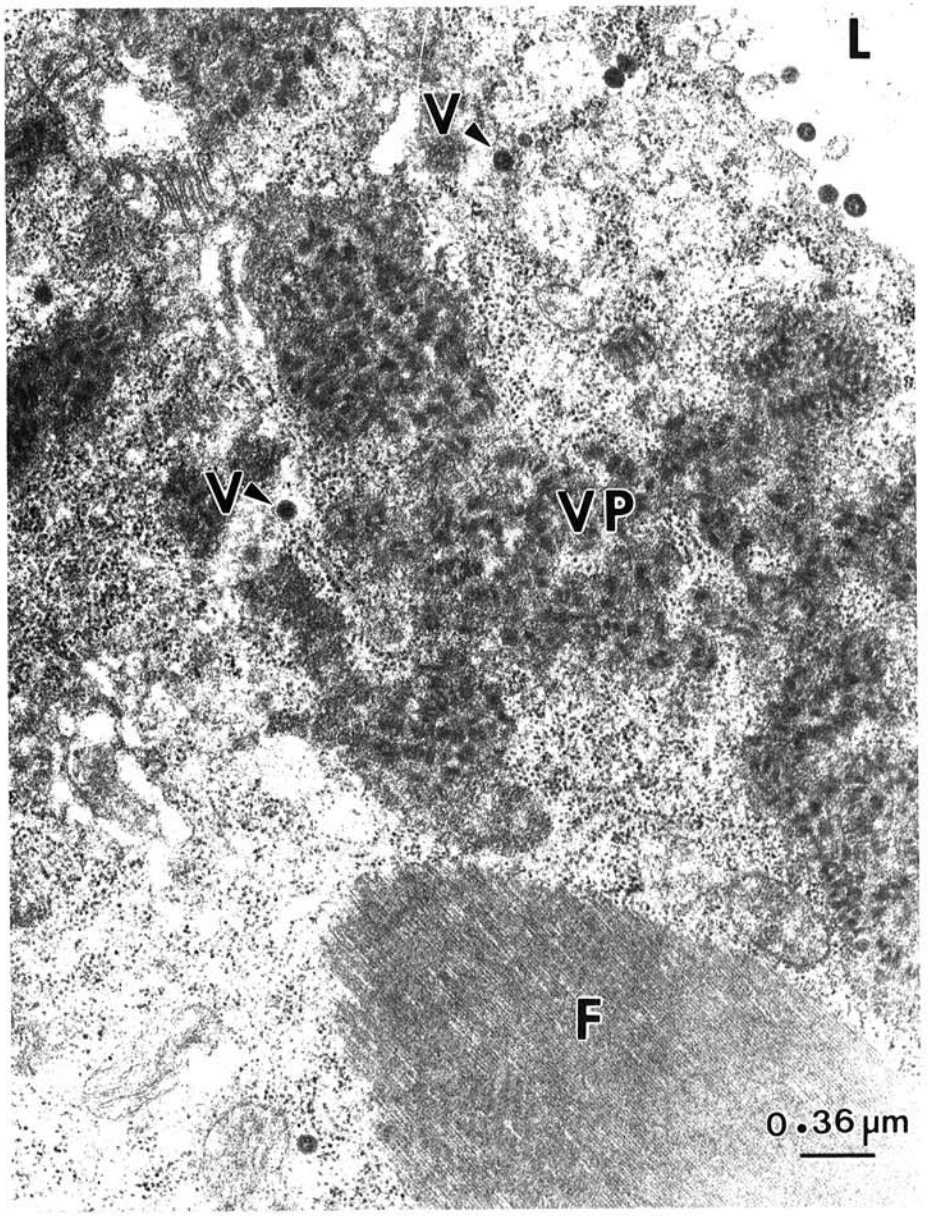

Fig. 7. Micrograph of tomato spotted wilt virions (V), viroplasms (VP), and fibrous paracrystalline inclusions (F) in the cytoplasm of the salivary gland of a 10-day adult *Frankliniella occidentalis* subsampled from an insect cohort that inoculated plants with tomato spotted wilt tospovirus. Following larval acquisition, the insect was reared on healthy bean pods and embedded in Spurrs resin. Virions (V) are also present in the lumen (L) of the gland leading to the salivary ducts.

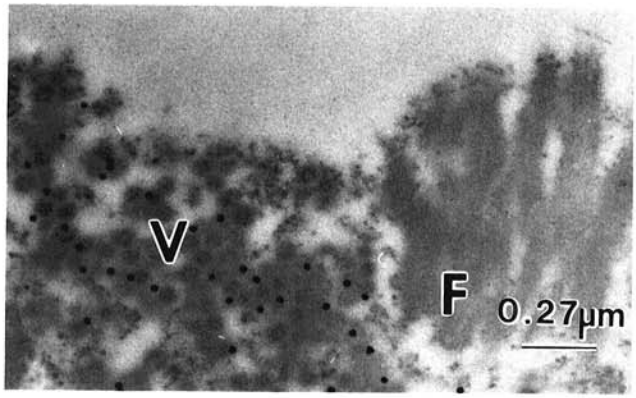

Fig. 8. Micrograph of tomato spotted wilt virions (V) and fibrous paracrystalline inclusions (F) in *Emilia sonchifolia* reveals that V reacted specifically with polyclonal antibody against tomato spotted wilt tospovirus, whereas F was not immunolabeled.

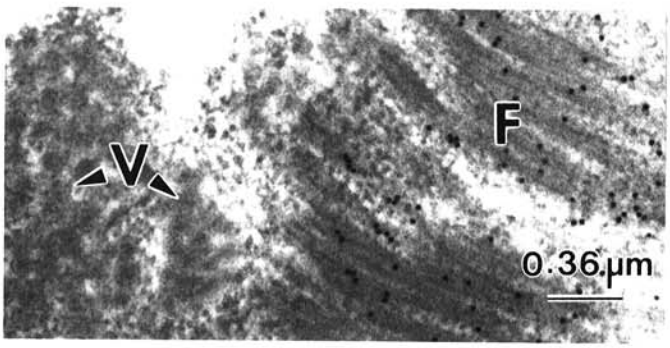

Fig. 9. Micrograph of a section serial to that shown in Figure 8 reveals that fibrous paracrystalline inclusions (F) reacted specifically with polyclonal antibody against the nonstructural protein encoded by the small RNA segment of tomato spotted wilt tospovirus, whereas virions and cytoplasmic regions were not immunolabeled.

not with purified in vitro expressed NSs, NSs in infected plants, or proteins from noninfected plants. Furthermore, immunocytochemical analysis of infected plants shows that anti-TSWV antisera reacts with virions and VP, but not with F.

Replication of TSWV in thrips vectors has important epidemiological implications. First, virus propagation in thrips vectors allows insects to remain infective throughout their life span (25–30 days) (24). Second, replication of the virus in vector thrips allows TSWV retention by insects during dispersal. Hence, a single dispersing individual may infect many plants. Finally, thrips may serve as an alternate host for TSWV, maintaining the virus in the environment even in the absence of appropriate TSWV plant hosts. This may be of particular importance in areas where adult thrips can survive between cropping seasons in the absence of a food source that is infected by TSWV. These insects may then infect TSWV plant hosts as soon as they become available. Replication of TSWV in thrips vectors makes quarantine procedures regulating entry of plants that may be infested with potentially viruliferous thrips of even greater importance in preventing the spread of TSWV. Replication of TSWV in thrips may underly the pandemic of TSWV that has developed on a number of food and horticultural crops. To date, TSWV is not thought to be transovarially passed from infected adult thrips to their offspring; however, this possibility warrants more investigation, as many viruses that replicate in their arthropod vectors are transovarially passed (22).

Most of the viruses in the family Bunyaviridae that are spread by arthropods to vertebrate hosts have also been shown to replicate in their invertebrate vectors (28). Replication of TSWV in the WFT adds support to placement of the *Tospoviruses* in this family of animal viruses. Replication of TSWV in animal cells also raises

TABLE 1. Comparison of mean gold label/cm^2 on viroplasms, fibrous paracrystalline arrays, and cytoplasmic regions in thin sections of larval *Frankliniella occidentalis* (Pergande) fed on tomato spotted wilt tospovirus (TSWV)-infected plants for 72 h. Measurements were made in midgut epithelial cells reacted with polyclonal antibodies raised against TSWV (anti-TSWV) or the nonstructural protein (NSs) encoded by the small RNA segment of TSWV (anti-NSs)

	Treatments			
Cell regions	Anti-TSWV	RS[a]/PA-Gold[b]	Anti-NSs	RS/GAR[c]
Viroplasms	9.83	0.73	0.70	1.23
Paracrystalline arrays	0.17	0.80	17.00	1.20
Cytoplasm	0.13	0.50	1.37	0.73

[a] RS = rabbit serum.
[b] PA-Gold = Protein A-Gold.
[c] GAR = goat-anti-rabbit serum.

important questions with regard to TSWV origin and plant virus taxonomy. Like the phytoreoviruses and phytorhabdoviruses that infect invertebrates (22), TSWV replication in animal cells raises questions with regard to whether TSWV infections first occurred in a plant or animal host and why these viruses are able to infect such disparate hosts. Plant viral taxonomy has long used only two hierarchical levels (group and virus) instead of family, genus, and species used in classification of viruses infecting other organisms (22). Replication of TSWV in invertebrate cells adds support to the concept of unity in virology and reinforces recent proposals to reconsider the systems used for taxonomic classification of plant viruses (21,22).

Finally, our results demonstrate that immunocytochemical analysis can be successfully used to investigate processes associated with TSWV replication and cell-to-cell spread in thrips and plants. Information developed in continuing studies will undoubtably provide information critical to designing novel therapies and understanding the continuously expanding *Tospovirus* plant and insect host ranges (11).

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