Localization of Tomato Spotted Wilt Tospovirus in Larvae and Pupae of the Insect Vector *Thrips setosus*

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ABSTRACT

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Changes in the accumulation of tomato spotted wilt tospovirus (TSWV) nucleocapsid (N) protein in *Thrips setosus*, an insect vector, from eclosion to 17 days were revealed by the double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) system with individual thrips. First-instar larvae were fed on TSWV-infected *Datura stramonium* for 2 h, and then kept on healthy *Vigna susquipedaris*. Individuals were subsampled for 17 days from first-instar larval to adult stage. The N protein concentration increased gradually from the first- to second-instar larval stage after acquisition feeding, peaking on day 5. Viral concentration then declined drastically from the second-instar larval to pupal stage. During the adult stage after ecdysis, persistently low N protein titers were demonstrated by DAS-ELISA, but adult thrips could transmit the virus. N protein was localized in first- and second-instar larvae and pupae by indirect immunofluorescence. On the second to fourth days after acquisition, specific fluorescence signals were detected within the anterior midgut that then spread to the whole midgut during the second-instar larval stage. As time elapsed, N protein was detected throughout the larval midgut and possibly within the salivary glands in pupae at 6 days after acquisition. Fluorescence signals within the pupal midgut were observed, but were not as intense as in larvae.

Additional keywords: detection, histochemistry.

Tomato spotted wilt tospovirus (TSWV) is a member of the genus *Tospovirus* in the family Bunyaviridae (17). TSWV has a very wide host range (4) and is transmitted by several species of thrips (10,18,21). The viral particle is spherical with a diameter of about 85 to 110 nm and spikes on its envelope (8). The genome consists of three species of single-stranded RNA (ssRNA) of approximately 2.9 (small; S RNA), 4.8 (medium; M RNA), and 8.9 (large; L RNA) kb in size (6,7,15). The S RNA is ambisense and encodes the nucleocapsid (N) protein and a nonstructural (NSs) protein. N fractions contain additional RNA molecules that are complementary to each viral RNA species in the virion (25).

The virus is acquired by thrips only during the larval stage, after which it persists and is transmitted to plants. Detection of virus in thrips has been achieved by bioassay using host plants (1,2,11,16,35) and immunological or molecular biological techniques (i.e., enzyme-linked immunosorbent assay [ELISA], dot blotting, and reverse transcription-polymerase chain reaction) (3,5,19,23).

Although a great deal has been learned about thrips/TSWV relationships, many questions remain regarding the acquisition by thrips and the persistence, replication, and location of TSWV within thrips and how these factors influence viral transmission efficiency. In Japan, tospoviruses are mainly transmitted by *Thrips setosus* Moulton, *T. palmi*, *T. tabaci*, and *Frankliniella intonsa* (9,12,13). Recently, several populations of thrips species were compared to assess the transmissibilities of four tospoviruses and the efficiencies with which they are transmitted (34). The TSWV relationship with western flower thrips (*F. occidentalis*) has been best characterized with regard to virus location and replication in the insect (10,29,30,36).

When larval *F. occidentalis* acquired TSWV, viral proteins were detected by serology and electron microscopy during the larval and adult stages (28,36). Furthermore, two viral proteins encoded by the S RNA, N and NSs, were detected in the salivary glands, midgut, and other tissues of thrips (29,33,36), frequently within structures similar to the cytoplasmic inclusions observed in infected plant cells (14,32,33). Recently, four TSWV-encoded proteins, G1, G2, N, and NSs, were detected by immunolocalization in intercellular membranes and possibly in the Golgi complex of *F. occidentalis* (30). This immunocytochemical evidence supports the hypothesis that TSWV replicates in thrips cells.

There is no information on TSWV distribution in thrips other than *F. occidentalis*, and studies of its distribution at different times after acquisition and at different developmental stages have not been carried out. Therefore, we investigated the fate of TSWV in the vector species *T. setosus* using several serological techniques in an attempt to gain further insight into the mechanisms of TSWV transmission by thrips. The goal of this study was to relate TSWV replication and localization to *T. setosus* development.

MATERIALS AND METHODS

Thrips. Thrips used in this study were obtained from laboratory-reared colonies of *T. setosus* as described previously by Tsuda et al. (23). All thrips examined were initially from virus-free colonies maintained on asparagus bean, *Vigna susquipedaris*. Thrips colonies were maintained at 23 to 26°C with a 16-h-light and 8-h-dark cycle.

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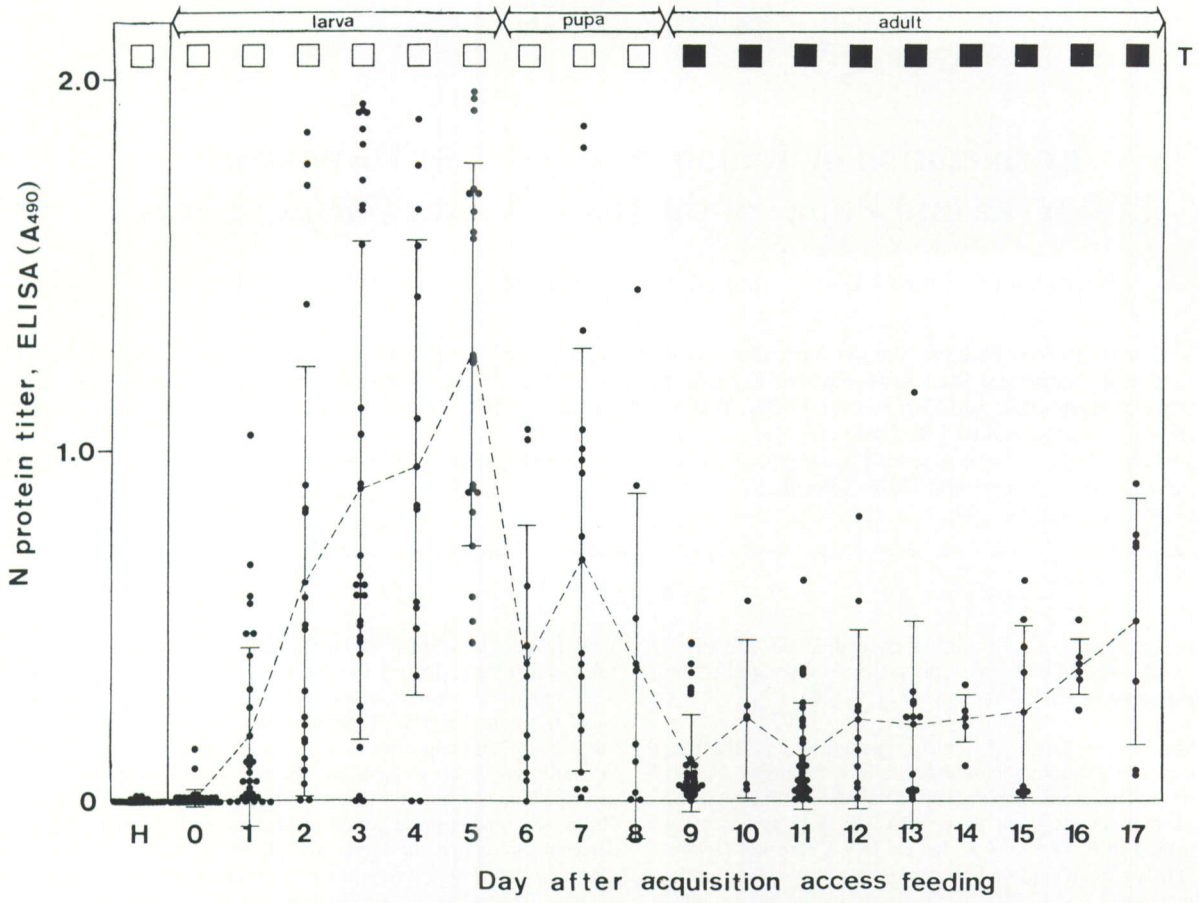

Fig. 1. Time course study using the enzyme-linked immunosorbent assay to detect tomato spotted wilt tospovirus-nucleocapsid (N) protein in individual *Thrips setosus*. (●) indicates the N protein titer in individual thrips and (----) shows mean double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) values obtained at various times. Viral transmissibility by thrips was demonstrated by allowing individual thrips to feed on healthy green pepper seedlings (■ : positive, □ : negative). Hash marks represent standard deviations. H indicates healthy thrips reared on healthy *Datura stramonium*.

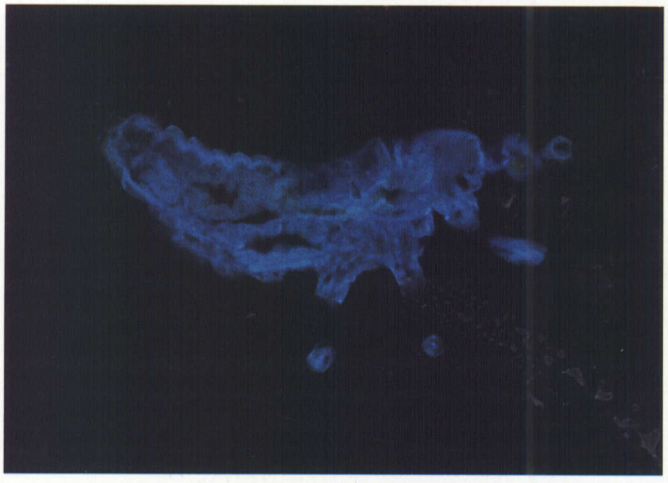

Fig. 2. Immunofluorescence microscopy with longitudinal sections of first-instar larval *Thrips setosus* immediately after acquisition access feeding. No fluorescence signals were evident within the thrips bodies. Ten to 20 insects were tested and six replicate experiments were performed.

Virus isolate. TSWV was isolated from green pepper, *Capsicum annuum* L., in Ibaraki Prefecture, Japan (26). The virus was maintained in *Datura stramonium* L. by mechanical inoculation. These plants were kept in a greenhouse maintained at 23 to 26°C.

Thrips acquisition and transmission of TSWV. Virus-free *T. setosus* were reared on *V. susquipedaris* in a container in our laboratory, and the insects were kept in caged pots in a greenhouse at 23 to 26°C. Infected *D. stramonium* leaves that were developing vein-clearing symptoms were used as the virus source. First-instar larvae were fed on infected leaves for 2 h and then reared on healthy *V. susquipedaris* until they were at the adult stage (17 days after eclosion). To determine whether the thrips were viruliferous, larvae were reared to the adult stage and transferred individually to healthy green pepper seedlings and left for 2 days, after which the plants were kept in the greenhouse for 1 month and examined for TSWV symptoms. Thrips at each developmental stage were removed and stored separately at –70°C until the double-antibody sandwich (DAS)-ELISA test was performed or until placed in a drop of fixative for immediate immunofluorescence microscopic observation.

DAS-ELISA. The DAS-ELISA method was used to detect TSWV N protein in individual thrips as described previously by Tsuda et al. (24). For quantitation of N protein accumulation in individual thrips, viruliferous thrips sampled on each day were homogenized with a mortar and pestle in 50 µl of 0.1 M phosphate buffer (pH 7.0) containing 0.1% (vol/vol) 2-mercaptoethanol, 0.1% (wt/vol) bovine serum albumin (BSA), 10 mM EDTA, and 0.15% (wt/vol) polyvinylpyrrolidone-40. An ELISA reader (MR600; Dynatech Labs Inc., Chantilly, VA) was used to measure the absorbance at 490 nm after incubation at room temperature for 1 h after adding the substrates.

Immunofluorescence microscopic observations. Viruliferous thrips were subsampled as first-instar larvae (immediately after the 2-h acquisition feeding), second-instar larvae, and pupae and then placed in a drop of fixative (ethanol/acetic acid/chloroform = 6:3:1, vol/vol). Antennae and legs were removed under the fixative to enhance its penetration. Thrips were then placed in a vial containing this fixative for 30 to 45 min at 4°C, after which longitudinal sections were prepared by the paraffin-embedding method of Sainte-Marie with modifications (20). Fixed thrips

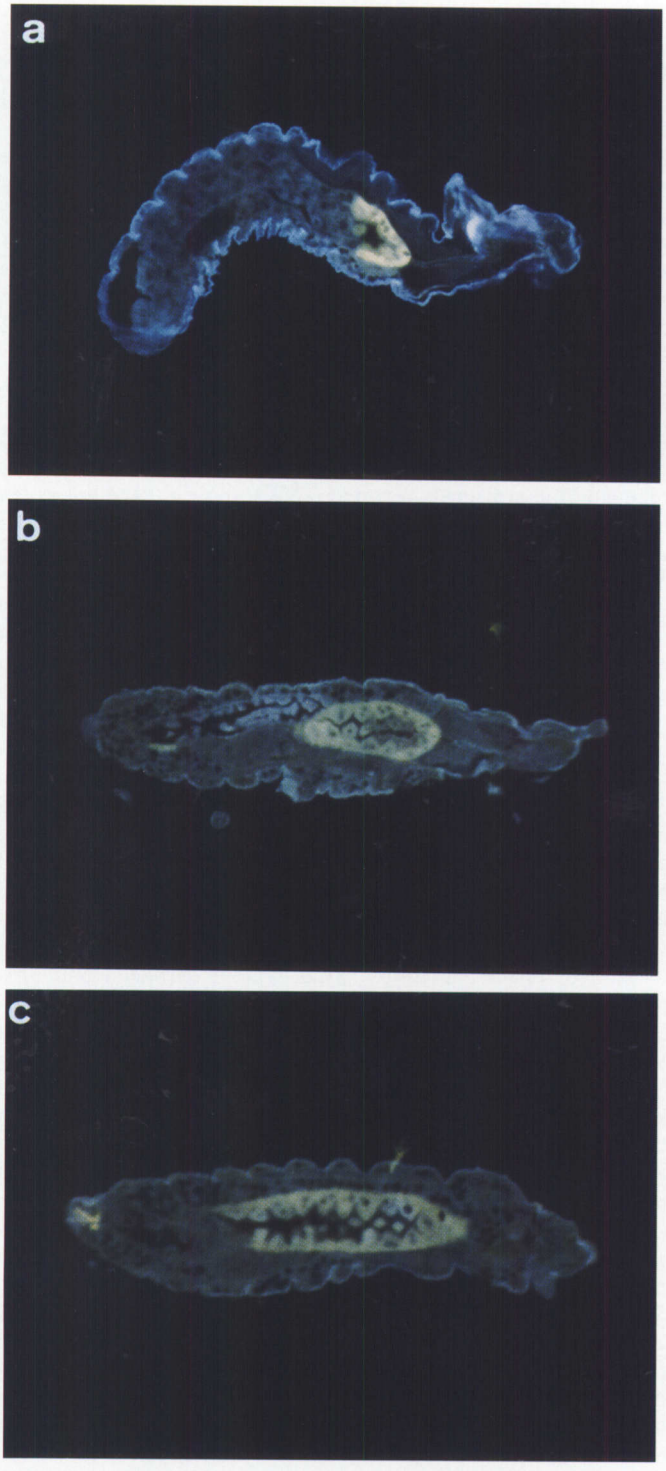

Fig. 3. Longitudinal sections of second-instar larval *Thrips setosus* on the second to fourth days after acquisition access feeding examined by immunofluorescence microscopy. Specific fluorescence signals were observed within **a,** the midgut anterior upper and the esophagus on the second day, and then gradually spread **b,** the third day and **c,** the fourth day from the anterior to the whole midgut as time advanced. Ten to 20 insects were tested and six replicate experiments were performed.

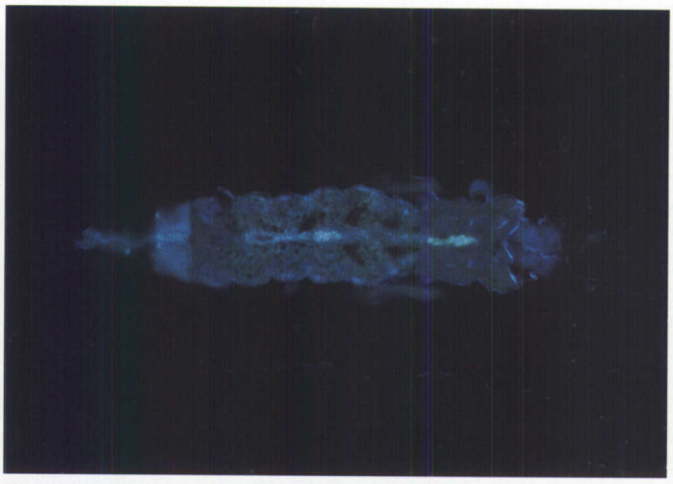

Fig. 4. Longitudinal sections of pupal *Thrips setosus* on the sixth day after acquisition access feeding examined by immunofluorescence microscopy. Specific fluorescence signals were observed within the salivary gland. Tomato spotted wilt tospovirus-specific signals were detected in the salivary gland concomitant with pupation. Ten to 20 insects were tested and six replicate experiments were performed.

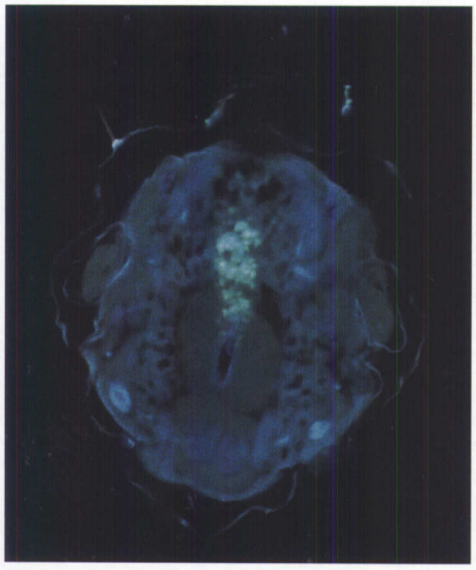

Fig. 5. Cross section of the thoracic region of *Thrips setosus* on the sixth day after acquisition access feeding examined by immunofluorescence microscopy. Specific fluorescence signals were observed within the salivary gland. Tomato spotted wilt tospovirus-specific signals were detected in the salivary gland concomitant with pupation. Ten to 20 insects were tested and six replicate experiments were performed.

were dehydrated in ice-cold, 99.5% (vol/vol) ethanol for 4 h at 4°C, soaked in xylene overnight at 4°C, and infiltrated with low-melting-point paraffin (Paraffin Blockform; E. Merck AG, Darmstadt, Germany) for 8 h at 46 to 48°C. The paraffin blocks were sectioned serially (4 to 7 μm thick) using a microtome (PR-50; Yamato Kohki Co., Tokyo). Each section was smeared onto a poly-L-lysine-coated glass slide, air-dried, and stored at 4°C until required for immunostaining. Sections were deparaffinized with xylene, hydrated with an ethanol series (99.5 to 30%, vol/vol), and rinsed with phosphate-buffered saline (PBS). They were then treated with an anti-N protein polyclonal rabbit antibody (26,27) in PBS containing 1% (wt/vol) BSA (PBS-BSA), followed by washing with PBS and incubation with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Cappel, Durham, NC) in PBS-BSA. After washing with PBS twice, the preparations were observed under a fluorescence microscope (AH3-RFC; Olympus, Tokyo). Ten to 20 insects at each stage were tested, and six replicate experiments were carried out to ensure precision and reproducibility.

RESULTS

Changes of N protein within individual *T. setosus* at different developmental stages. The TSWV N protein titer was found

to increase in individual thrips over time after viral acquisition (Fig. 1). The virus was seldom detected in first-instar larvae assayed immediately after acquisition feeding. The N protein was detected in some larvae after 24 h and in about half the larval population on the second day. The peak of N protein accumulation occurred on day 5, at the second-instar stage. The concentration of N protein in *T. setosus* decreased drastically when second-instar larvae became pupae (immediately after metamorphosis, 6 days). For the adults, the optical density at 490 nm (OD_{490}) varied from 0.1 to 0.5 after 9 days following emergence, when most are capable of transmitting TSWV to plants. These values by DAS-ELISA were significantly higher ($P < 0.01$) than those of negative control thrips (50 virus-free *T. setosus* reared in the laboratory that showed negligible DAS-ELISA values), which ranged from 0.001 to 0.015. The increasing accumulation of the N protein during the larval stages of *T. setosus* was consistent with the findings reported by Ullman et al. (29) and Wijkamp et al. (36) regarding TSWV replication in *F. occidentalis*. The increase in N protein titer during *T. setosus* development provided evidence that TSWV also multiplies in this vector. The reason for the decrease in the N protein levels during the pupal and adult stages is still unknown.

Immunolocalization of TSWV in *T. setosus* at various developmental stages. The internal anatomy and morphology of *T. setosus* have never been reported, but, by analogy with *F. occidentalis* (10,31), the positions and structures of organs and tissues of *T. setosus* were identified.

We found no FITC-fluorescence signals specific to the N protein in first-instar larvae immediately after acquisition feeding (Fig. 2). On the second day (48 h) after acquisition, specific fluorescence signals were often observed within the anterior midgut, just distal to the esophagus (Fig. 3A). As time elapsed, fluorescence signals were observed in increasingly large portions of the larval midgut (Fig. 3B and C). These observations were consistent with our DAS-ELISA data showing that N protein titers increased over time, peaking during the second-instar stage. The high level of N protein accumulation at this stage was probably because of viral replication in the midgut.

At the pupal stage (6 days after acquisition), fluorescence signals were detected in tissues of the central thorax, while the signals within the midgut became less intense than those observed in larvae (Fig. 4). The decline of fluorescence-signal intensity in the midgut occurred at the same time that DAS-ELISA analysis revealed that the N protein titer of pupal thrips decreased. Examination of cross sections of thoracic regions of pupal thrips at the same stage revealed fluorescence signals within a confined area of the upper esophagus (Fig. 5). This area is probably the salivary gland because hematoxylin-eosin stained the cross and longitudinal sections of *T. setosus* at this stage (data not shown) and because of the close similarities of the results to previously reported data for *F. occidentalis* (10,31).

No FITC-fluorescence signals were detected in any *T. setosus* fed on healthy *D. stramonium* plants (0 of 10 to 20 insects at each stage).

DISCUSSION

In this paper, the accumulation of TSWV N protein over time and throughout the developmental stages of *T. setosus* following the 2-h acquisition feeding on infected plants is described. The data demonstrates that the virus first accumulates within the anterior midgut and gradually spreads to the entire midgut by the end of the second-instar larval stage. The spread to other organs, possibly the salivary gland, occurred during the pupal stages, when viral accumulation in the midgut became less intense.

Accumulation of TSWV in *F. occidentalis* has been reported. An increase in the concentrations of two TSWV-encoded proteins, N and NSs, indicated multiplication of TSWV from the first- to second-instar larval stages (29,36). Our findings in *T. setosus* by ELISA and histochemistry with immunofluorescence are consistent with these studies and show that TSWV also multiplies in *T. setosus*.

Our results also suggest that the midgut is the first site of viral multiplication in *T. setosus*, as has been shown with *F. occidentalis* (29,30). The specific immunofluorescence signals within the larval midgut became fainter with the development of the pupal stage, when the specific signal appeared in the salivary gland within the pupal thorax. Wijkamp and Peters (35) have demonstrated that *F. occidentalis* could transmit the virus even in its second-instar larval stage. However, the efficiency of transmission by thrips, both larvae and adult, was influenced by the difference of temperature from 20 to 27°C (35). This suggests that the salivary gland of these second instars was already infected; therefore, movement of the virus was before pupation. On the other hand, since, in our study, detection of the virus in the salivary gland coincided with molting to the pupal stage under a different environment from previously reported, TSWV movement to this organ in *T. setosus* may be associated with developmental processes or the progress of infectivity in thrips might occur at pupation under our conditions.

Furthermore, our results demonstrate that the salivary gland is the more important reservoir site of TSWV in the vector at pupal and adult stages. The fact that DAS-ELISA titers for N protein remain relatively low (OD_{490} values of 0.1 to 0.5) in adults is probably attributable to TSWV replication in a small organ, the salivary gland, that is responsible for viral transmissibility. Published biological and histochemical data on *F. occidentalis* (10,28, 31) prompted us to hypothesize that TSWV virions, which flowed into the hemocoel from the midgut, could gather in the salivary gland of *T. setosus*. If TSWV is maintained in this tissue, it would be transported through this gland to the salivarium and excreted into a host plant when the thrips feed (10,28,31). Thus, the salivary gland is an epidemiologically significant site for virus transmission to plants via thrips. Viral protein derived from virus replication has also been found within salivary glands of the viruliferous adult *F. occidentalis* by Ullman et al. (29,30) and Wijkamp et al. (36).

TSWV can only be transmitted by thrips when the virus was acquired at the larval stages. The adults then can transmit it throughout their lives after their emergence (10,21,22). The midgut of *T. setosus* is a major site for virus entry and infection in larvae. Similar findings were also reported for *F. occidentalis* (29,36). The specific immunofluorescent signals within the midgut at the larval stage after viral acquisition became faint in the pupal stage. This specific signal, however, dramatically appeared on a salivary gland at the pupal stage. The midgut was no longer an important site of infection, and the salivary gland became the reservoir for the virus. The loss of TSWV from the midgut immediately after metamorphosis to the pupal stage may be associated with the process of ecdysis and changes occurring during pupation. Future studies of regulatory processes of either viral or those related to insect physiology should reveal the mechanisms underlying TSWV replication and movement between thrips organs.

It is likely that viral movement between organs during the development of thrips, including pupation, is involved in TSWV transmission by thrips. The mechanism responsible for viral movement between organs and the route from the midgut to the salivary gland during development remain to be determined. Histochemical examination of adult thrips that can transmit TSWV should provide additional information on the interactions between thrips and TSWV.

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