Tissue tropism related to vector competence of *Frankliniella occidentalis* for tomato spotted wilt tospovirus

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The development of tomato spotted wilt tospovirus (TSWV) infection in the midgut and salivary glands of transmitting and non-transmitting thrips, *Frankliniella occidentalis*, was studied to elucidate tissue tropism and the virus pathway within the body of this vector. Immunohistological techniques used in this study showed that the midgut, foregut and salivary glands were the only organs in which virus accumulated. The first signals of infection, observed as randomly distributed fluorescent granular spots, were found in the epithelial cells of the midgut, mainly restricted to the anterior region. The virus subsequently spread to the circular and longitudinal midgut muscle tissues, a process which occurred late in the larval stage. In the adult stage, the infection occurred in the visceral muscle tissues, covering the whole midgut and foregut, and was abolished in the midgut epithelium. The infection of the salivary glands was first observed 72 h post-acquisition, and simultaneously in the ligaments connecting the midgut with these glands. The salivary glands of transmitting individuals appeared heavily or completely infected, while no or only a low level of infection was found in the glands of non-transmitting individuals. Moreover, the development of an age-dependent midgut barrier against virus infection was observed in second instar larvae and adults. The results show that the establishment of TSWV infection in the various tissues and the potential of transmission seems to be regulated by different barriers and processes related to the metamorphosis of thrips.

**Introduction**

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus*, harbouring the plant-infecting viruses within the family *Bunyaviridae*. Other members of this family include important viruses infecting humans and animals, which are mainly transmitted by mosquitoes, ticks or other blood-sucking arthropods. Tospoviruses are exclusively transmitted by phytophagous thrips in a propagative fashion (Wijkamp et al., 1993; Ullman et al., 1993a). TSWV became one of the most world widely distributed plant viruses (Goldbach & Peters, 1994) due to the recent global expansion of one of the most efficient thrips vectors, *Frankliniella occidentalis* Pergande (Mound & Teulon, 1995; Wijkamp et al., 1995), and its impressive plant host range (Peters, 1998).

The virus replicates and circulates after acquisition in its thrips vector and is transmitted by larvae late in their second stage and by adults (Sakimura, 1962a, b, 1963; Wijkamp & Peters, 1993). An increase of virus antigens in thrips during their development, particularly the nucleocapsid (N) and the non-structural (NSs) protein, is considered to be an indication of the replication of TSWV in thrips (Wijkamp et al., 1993; Ullman et al., 1993a). Replication of this virus has also been shown *in vitro* in cultured thrips cells (Nagata et al., 1997).

Only larvae can acquire virus leading to replication and transmission (Sakimura, 1962a, b, 1963; van de Wetering et al., 1996). Ingestion of virus by adults does not lead to replication and, hence, to the ability to transmit (Ullman et al., 1992a; van de Wetering et al., 1996). The existence of this restriction may be explained by differential expression of barriers during the development of thrips.

Studies on the transmission efficiency and the virus load of individual viruliferous and non-viruliferous adults revealed the existence of three categories of thrips (Wijkamp & Peters, 1993; Wijkamp et al., 1995; van de Wetering et al., 1996). The individuals of the first category could successfully transmit the
virus and possessed high titres of virus antigen in ELISA. The second category was not able to transmit the virus, but was positive in ELISA. The third category was negative in both assays. The existence of these three categories within a single population reflects different physiological potentials to replicate and transmit TSWV among the individual thrips. For vectors of animal-transmitted bunyaviruses, these potentials are often explained by the existence of different barriers (Paulson et al., 1989; Grimstad & Walker, 1991). Such barriers, which determine vector competence, may exist at the level of virus infection or escape from the midgut, dissemination through the insect, infection of the salivary glands and release into the saliva (Hardy et al., 1983; Hardy, 1988). In plant virus vectors, the existence of such barriers has not yet been elucidated.

Most of our knowledge of the tospovirus–thrips relationship has been obtained in transmission studies. Although some histological studies have been made (Ullman et al., 1993a, b, 1995; Wijkamp et al., 1995; Tsuda et al., 1996), little is known about the tissue tropism related to the vector competence of thrips to transmit tospoviruses. This report describes the results of a study on TSWV tissue tropism in the thrips F. occidentalis. For this purpose, an immunohistochemical technique has been developed to study virus infections in plant virus vectors.

Methods

Thrips maintenance and virus acquisition. A Dutch population of F. occidentalis was reared in glass jars on fresh bean pods. The cultures were incubated in a climate chamber at 25 °C with a photoperiod of 16 h. Newborn larvae, up to 4 h old, were given an acquisition access period (AAP) of 16 h on Datura stramonium leaves infected with TSWV strain BR-01 (de Avila et al., 1993) in Tashiro cages and then transferred to non-infected leaves in these cages (Tashiro, 1967; Peters et al., 1997). Samples of thrips were collected at several intervals after ingestion of virus for immunocytochemical studies and to test the virus transmission efficiency when they became adults.

Virus transmission assay and ELISA. Transmission by thrips was tested using the local lesion assay method on petunia leaf disks (Wijkamp & Peters, 1993). Each newly emerged adult was individually placed on a leaf disk in an Eppendorf tube for 2 days at 25 °C. The disks were then transferred to a 24-well plate and incubated for 3 days on water for the development of local lesions. The virus accumulation in these thrips was determined by ELISA using antibodies to the nucleocapsid (N) protein and involved an amplification step as described by Wijkamp et al. (1995). The minimum threshold for positive ELISA values was the average of the readings of ten non-infected thrips plus three times their standard deviation.

Midgut preparation and whole-mount immunofluorescent staining (WMIS). Thrips were dissected in distilled water and the midguts transferred to object glasses coated with poly-L-lysine (0.5% in water) and subsequently air-dried. After 1 h of fixation with cold acetone, the preparations were stored at −20 °C until further processing. After storage, the midguts were incubated in PBS, pH 7.2, containing 10% bovine serum albumin (BSA) for 1 h to block non-specific reactions. Polyclonal antibodies against the viral N protein (2 µg/ml) raised in rabbit were used as a first overlay for 2 h in 10% BSA–PBS. To remove non-specific antibodies, this antiserum was first absorbed with a crude extract from uninfected thrips (10% in PBS, v/v) that was heated at 100 °C for 3 min before adding to the antiserum. After removing the precipitate by microcentrifugation at 10 000 r.p.m. for 10 min, the antibodies were extracted from the supernatant by a two-step method of ammonium sulphate precipitation (33% and 50% saturation). The antibody fraction was dialysed and fractionated on a DEAE–sephacel column (Sigma) with PBS. The midgut preparations were incubated with this pre-absorbed antiserum for 2 h at room temperature in 10% BSA–PBS, washed three times for 10 min with PBS, and then incubated with 10 µg/ml pig anti-rabbit FITC conjugate (Nordic) in 10% BSA–PBS for 1 h. After washing, the specimens were mounted in Citifluor (Agar) and studied by fluorescence microscopy (Leica).

Preparing sections for immunocytochemical studies. The legs and antennae were removed from the body under a stereomicroscope in Bouin’s Hollande sublimate (Smid et al., 1998). After removing the legs, the bodies were incubated in this fixative at room temperature under a vacuum for 1 h and then overnight at 4 °C, dehydrated in a series of 70–100% ethanol solutions, and finally incubated in amyl acetate for 1 h. They were then embedded in Paraplast (Oxford Labware) in 5 µm thick sections which were mounted on an object glass precoated with poly-L-lysine (0.1% in distilled water). The sections were deparaffinized with xylene, rehydrated in a series of 100–70% ethanol solutions and incubated in PBS. After this treatment, they were incubated with 10% normal pig serum in PBS for 30 min, then with pre-absorbed anti-N protein antibodies (1 µg/ml PBS) for 2 h, washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated pig anti-rabbit antibodies (5 µg/ml PBS containing 10% normal serum) for 1 h (Dako). After washing, the sections were incubated for 5 min with a substrate solution consisting of 0.05% (w/v) diaminobenzidin (DAB) and 0.01% (w/v) hydrogen peroxide in 50 mM Tris–HCl, pH 7.6. This reaction was stopped by washing with distilled water. The sections were then stained with Mayer’s haematoxylin (Sigma), dehydrated, mounted with DPX (Fluka) and studied by light microscopy (Leica).

Electron microscopy. Thrips midguts were dissected and fixed in a 3% paraformaldehyde–2% glutaraldehyde solution for 30 min, dehydrated in a series of 50–100% ethanol solutions, and embedded in LR-Gold (London Resin). The 60–70 nm thick sections were incubated with 0.8 µg/ml pre-absorbed antibodies to the N protein for 2 h, washed with 30 droplets of PBS, and incubated with gold-conjugated protein A for 1 h. After washing with PBS, the sections were post-fixed in 1% glutaraldehyde, contrasted with uranyl acetate and lead citrate, and studied with an electron microscope (Zeiss EM 109).

Results

Thrips midgut infection in different developmental stages

The midgut of F. occidentalis consists of two loops (Ullman et al., 1989, 1992 b; Moritz, 1997), which divide the midgut into three distinct regions, designated Mg1, Mg2 and Mg3 (Fig. 1). The foregut ends in the anterior Mg1 region, which ends at a constricted bending where the next region (Mg2) starts. This part runs in the direction of the head and continues after a second loop into the third region (Mg3), which goes towards the hindgut.

The development of TSWV infection in the midgut was studied by the WMIS technique (see Methods) using antiserum...
Correlation of midgut infection and transmissibility

To understand the mechanism underlying the vector competence of *F. occidentalis*, the effect of the AAP on the infection in the tissues and the transmission rate was studied using the same cohorts of thrips. First instar larvae (up to 4 h old) were given an AAP of either 3 h or 16 h. Some of these thrips were analysed by WMIS for midgut infection 24 h p.a., 96 h p.a. or a few days after becoming adult (Table 1). To test the transmission rate, larvae were grown to adults and individually tested for their ability to transmit. The virus content of these adults was analysed by amplification ELISA or immunohistologically for the infection of their tissues.

The WMIS results revealed that all thrips became infected when they were given AAPs of either 3 h or 16 h. Infection was mainly found in the epithelial cells of Mg1 and occasionally also in other midgut regions 24 h after the AAP of 3 h. (Table 1). The percentage of larvae with infected midguts (epithelia and muscle cells) increased from 83% (n = 60) 24 h p.a. to

The symptoms of TSWV infection in thrips started to be discernible at 72 h p.a. (Fig. 2D, E). This infection was usually discernible as a lattice pattern, but, in some cases, restricted to a few rows of longitudinal muscle cells in the posterior midgut (Mg3). The presence of the virus in the Mg2 and Mg3 muscle tissues in the adult stage and its almost absence in the epithelium of these regions during larval development suggests a lateral cell-to-cell migration of the virus in visceral muscle cells from the Mg1 to those of other midgut regions.

Besides infection of the muscle cells lining the midgut of the adult, the infection was also observed in the foregut (Fig. 2D). During larval development, this organ did not show any detectable infection, suggesting that the ingested virus cannot infect the foregut epithelium from the intestinal tract lumen. Infection of this organ, late in the infection process, represents a novel aspect of tissue tropism for TSWV infection in thrips.

In cells of the salivary glands, the first infection signals were at 72–92 h p.a. as spots in the border region of the salivary glands (Fig. 2F, G). The salivary glands in a large percentage of adults appeared to be completely infected (Fig. 2H). No or limited infection in one or few cells could be detected in the salivary glands of a small group of adults. Furthermore, infection in the ligament connecting the salivary glands with the anterior part of Mg1 was often observed at 72 h p.a. or later (Fig. 2F–H).

Signals of infection were never encountered in the tubular salivary glands or in the Malpighian tubes, which were dissected with the midgut.

The infection of the midgut of larvae 72 h p.a. could further be confirmed by electron microscopy. Large aggregates of N protein were observed in the cytoplasm of the epithelial cells of the midgut (Fig. 3A) and also in the circular and longitudinal muscle cells (Fig. 3B). Infection was observed only in Mg1 (two out of three of the larvae tested), confirming the results obtained by WMIS.
Fig. 2. For legend see facing page.
Tissue tropism in TSWV–thrips interaction

Fig. 3. Electron micrograph of an infected epithelial (A) and a muscle cell (B) of the Mg1 72 h p.a. The gold particles were labelled with antibodies to the N protein. Bar, 300 nm.

Table 1. Infection of the midgut as determined with anti-N serum at several intervals in thrips after acquisition of TSWV by 0–4 h old F. occidentalis larvae

<table>
<thead>
<tr>
<th>AAP (h)</th>
<th>Time p.a. (h)</th>
<th>Midgut infected (%)</th>
<th>Infected midgut regions* (%)</th>
<th>No. of individuals tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Mg1</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>16.7</td>
<td>83.3‡</td>
<td>63.3</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>2.9</td>
<td>97.1‡</td>
<td>78.3</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>0.0</td>
<td>100.0§</td>
<td>38.9</td>
</tr>
<tr>
<td>16</td>
<td>24</td>
<td>14.1</td>
<td>85.9‡</td>
<td>56.2</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.0</td>
<td>100.0§</td>
<td>71.6</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>0.0</td>
<td>100.0§</td>
<td>26.7</td>
</tr>
</tbody>
</table>

*Mg1, infection restricted to region Mg1; Mg2, infection in regions Mg1 and Mg2; Mg3, infection in regions Mg1, Mg2 and Mg3.
† Infection restricted to the epithelium.
§ Infection in the epithelium and muscle cells of midgut.
§§ Infection only in midgut muscle cells.

97% (n = 69) 96 h p.a. and to 100% when they became adults (n = 18). These percentages were slightly higher for larvae that acquired the virus in an AAP of 16 h, indicating that almost no quantitative differences existed in the development of virus infection in the midguts of larvae when given AAPs of 3 h or 16 h. However, significant differences were observed

Fig. 2. Infection of the midgut and salivary glands of thrips at different developmental stages after ingesting the virus in a 16 h AAP given to 0–4 h old larvae. (A) Mg1 at 24 h p.a.; (B) Mg1 at 72 h p.a. with a discernible infection in the longitudinal muscle; (C) healthy thrips (3 days old); (D) infected Mg1, Mg2 and foregut of adult transmitter thrips; (E) Mg1 of adult transmitter thrips; (F)–(G) part of Mg1 and of a salivary gland at 72 h p.a. also showing positive signals in the ligament; (H) part of Mg1 and a salivary gland of adult transmitter thrips showing positive signals in the ligament; (I) thin section of adult transmitter thrips; (J) thin section of a healthy control. (A), (D), (E) and (H) were made using a I2/3 filter (Leica), and (B), (F) and (G) using an HTC filter (Leica). Mg, midgut; LM, longitudinal muscle; Fg, foregut; Lg, ligament; SG, salivary gland. Bar, 50 µm.
The infection in both salivary glands was often unevenly distributed (Table 2). Virus antigen was readily detected in the salivary glands of all transmitter thrips ($n = 20$). On the other hand, infection of the salivary glands was completely absent in 17 out of the 40 non-transmitter thrips and very restricted (one or few cells in one gland) in 20 out of the 40 non-transmitters (Table 2). These results show that a limited infection of the salivary glands is not sufficient for a thrips to become a transmitter.

### Discussion

The pathway of TSWV in the tissues of thrips and the intrinsic factors related to the transmission of the virus are of major interest for understanding the relationship between this virus and its vector. Evidence of infection of midgut epithelial cells, visceral muscle cells, and salivary glands of *F. occidentalis* by TSWV has already been obtained by electron microscopic studies (Ullman et al., 1993a, 1995; Wijkamp et al., 1995). However, these studies did not provide information on the development of the infection in the individual thrips at different periods after acquisition of the virus, or in thrips populations with any degree of age variation.
The infection in the posterior midgut remained restricted to small isolated foci of epithelial cells of the Mg1 region, followed by infection of the visceral and longitudinal muscle cells of Mg2 and Mg3. The infection was restricted to the midgut muscle cells of the Mg2 and Mg3 regions. The infection was restricted to the midgut muscle cells of the non-transmitters that were ELISA negative. These results suggest that the inability of thrips to transmit the virus after an early acquisition was not due to a failure to infect thrips, but to different quantitative effects of some barriers operating in different tissues or to partial abortion of the infection in some cells.

A virus, circulative and propagative in its vector, has to pass several tissues, the barriers of which will finally determine the vector competence (Hardy et al., 1983; Hardy, 1988).

Table 3. Midgut infection in thrips after ingesting of virus by first and second instar larvae and adults

<table>
<thead>
<tr>
<th>Age when fed on virus source</th>
<th>Transmission efficiency (%)</th>
<th>Midgut analysed</th>
<th>No. analysed</th>
<th>Midgut infection*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Larvae 0–4 h old</td>
<td>47.3</td>
<td>24 h p.a.</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transmitters†</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transmitters†</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Larvae 72–76 h old</td>
<td>12.4</td>
<td>24 h p.a.</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transmitters†</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transmitters†</td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td>Adults 1–2 days old</td>
<td>0</td>
<td>1 day p.a‡</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 days p.a.</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 days p.a.</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 days p.a.</td>
<td>37</td>
<td>36</td>
</tr>
</tbody>
</table>

* No, no infection discernible; faint, reaction not discernible using 100 × magnification but visible using 400 × magnification; Mg1, infection restricted to region Mg1; Mg2, infection found in the muscle cells of Mg1 and Mg2; Mg3, infection seen in the muscle cells of the whole midgut.
† Transmission was evaluated after adult emergence by leaf-disk assay.
‡ Post-acquisition incubation time (days) before midgut dissection. All adult thrips in this category did not transmit.
These barriers for TSWV may include a midgut infection, midgut escape, dissemination, salivary gland infection and salivary gland escape barriers. In this study, two important barriers were recognized. The midgut escape barrier in adults of *F. occidentalis* was believed to be the first barrier (Ammar, 1994; Wang *et al.*, 1994). Our results showed that the first effective barrier is the midgut infection barrier. This barrier becomes increasingly effective during the development of larvae and is almost complete in adult thrips. A similar barrier may be present in the leafhopper *Agaria constricta*, the vector of wound tumour reovirus and potato yellow dwarf rhabdovirus. The efficiency by which this vector transmits these viruses also decreases with its age (Sinha, 1963), again suggesting the development of an infection in the midgut. However, this type of barrier is not common or may even be lacking in mosquito–arbovirus interactions, as acquisition and transmission of arboviruses is restricted to the adult stage except when the virus is transovarially passed from one generation to another.

To reach and infect salivary glands, TSWV has to pass three more barriers, i.e. the midgut escape, midgut to salivary gland dissemination, and salivary gland infection. Since escape from the midgut and dissemination cannot be determined and the pathway of TSWV to the salivary gland is not known, the factors which limit the infection of the salivary glands remain to be elucidated.

In our study, evidence was found for the existence of a salivary gland escape barrier since transmission seems to be dose-dependent. The observation that a low infection level of the salivary glands was associated with an inability to transmit suggests a failure of the virus to spread to other cells or to be released in sufficiently large amounts in the saliva. The immunohistological studies of transmitters showed that the salivary glands of such individuals become completely infected. A dose-dependent barrier is the most common model of the salivary gland escape barrier for the arbovirus–mosquito relationship (Hardy *et al.*, 1983). Similar observations have also been made for JE virus in the salivary glands of *C. triaeniorhynchus* (Takahashi & Suzuki, 1979; Takahashi, 1982). Their results suggest that heavily infected salivary glands are required to transmit the virus, indicating that a successful transmission is a dose-dependent phenomenon.

It has been suggested (Ullman *et al.*, 1992b) that TSWV can be transported from the midgut through the tubular salivary glands to the salivary glands. The former was thought to form a channel-like structure between the midgut and the salivary glands. This possible role in virus transport is not confirmed by the present studies as no virus antigen was detected in this organ. Rather, this study supports the absence of any direct connection between the midgut and salivary glands, as has been suggested by Del Bene *et al.* (1991). A possible new pathway by which the salivary glands become infected could be deduced by detecting infection of the ligaments which connect the Mg1 region with the salivary glands (Fig. 1). These positive signals were always found in the late larval stage (72 h p.a. or later) and in adults, associated with infected salivary glands (Fig. 2F–H).

In conclusion, using immunohistological techniques, infection by TSWV can be followed in thrips during their development. The development of infection in all young larvae which acquired virus showed that the susceptibility to virus entry and midgut infection are stable characters of the thrips population used in this study. Thus, it can be concluded that inability to transmit (often observed) does not originate from a lack of virus entry, but is largely dependent on existing barriers which must be passed through during infection. The ability to transmit TSWV by thrips is therefore the outcome of successful virus replication, spread and passage through several barriers resulting in a release of virus in the saliva.

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**References**


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