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Salivary gland morphology, tissue tropism and the progression of tospovirus infection in *Frankliniella occidentalis*

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ABSTRACT

Tomato spotted wilt virus (TSWV) is transmitted by thrips in a propagative manner; however, progression of virus infection in the insect is not fully understood. The goal of this work was to study the morphology and infection of thrips salivary glands. The primary salivary glands (PSG) are complex, with three distinct regions that may have unique functions. Analysis of TSWV progression in thrips revealed the presence of viral proteins in the foregut, midgut, ligaments, tubular salivary glands (TSG), and efferent duct and filament structures connecting the TSG and PSG of first and second instar larvae. The primary site of virus infection shifted from the midgut and TSG in the larvae to the PSG in adults, suggesting that tissue tropism changes with insect development. TSG infection was detected in advance of PSG infection. These findings support the hypothesis that the TSG are involved in trafficking of TSWV to the PSG.

1. Introduction

Outbreaks of known and emerging arthropod-vectored viruses are increasing in frequency and importance due to factors associated with climate change and human demographics (Cincotta et al., 2000; Pappu et al., 2009; Walter and Barr, 2011). This situation is driving interest in using knowledge of virus-vector interactions to develop strategies for virus management (Whitfield and Rotenberg, 2015). Circulatively transmitted viruses, such as *Tomato spotted wilt virus* (TSWV), are transmitted via saliva during the feeding of insect vectors (Whitfield et al., 2015). Thus, insect vector salivary glands are of great interest in revealing mechanisms of virus transmission. In spite of their importance, salivary gland morphology, physiology and interactions with viruses remain poorly studied, although efforts are underway to study salivary gland-virus interactions using transcriptomics and proteomics (Djegbe et al., 2011; Girard et al., 2010; Sim et al., 2012; Stafford-Banks et al., 2014; Su et al., 2012).

Tomato spotted wilt virus (TSWV) is the type member of the genus *Tospovirus*, the only plant-infecting genus within the family *Bunyaviridae*. Like the mammal-infecting bunyaviruses, tospoviruses have a circulative-propagative relationship with their vectors, replicating in multiple organs (Whitfield et al., 2005). Tospoviruses must be acquired by larval thrips in order for adults to successfully inoculate plants (Sakimura, 1962). The most efficient acquisition is by first instar larvae (L1s) (van de Wetering et al., 1996) and the virus replicates in midgut epithelia, intestinal muscles and primary salivary glands (Ullman et al., 1993). Infection of the principal salivary glands (PSG) is thought to be required for transmission (Kritzman et al., 2002); however, the route by which TSWV reaches these glands is unknown. Several hypotheses have been proposed: (i) TSWV moves from the midgut to the salivary gland by direct contact between these tissues during early larval development (Moritz et al., 2004); (ii) TSWV moves from the midgut to the salivary gland through the ligament-like structures that connect each PSG to the anterior region of the midgut (frequently referred to MG1) (Assis Filho et al., 2002, Nagata et al., 1999); and, (iii) that the tubular salivary glands (TSG) may enable TSWV to move directly from the midgut to the primary salivary glands (Ullman et al., 1989). While these hypotheses were proposed separately, it is also possible that virus infection of the salivary glands occurs by multiple means including several of these possibilities.

The morphology and biology of thrips salivary glands and the tospovirus dissemination pathway in vectors are not fully understood. Thus, the objectives of this study were to: 1) describe the morphology of *Frankliniella occidentalis* salivary glands; and, 2) study progression of TSWV infection by localizing the structural viral proteins glycoprotein G_N (G_N) and nucleocapsid (N) in the

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midgut and salivary glands of *F. occidentalis* at specific times in the insect life cycle. Our work shows that virus infection occurs in the midgut, ligaments tethering the PSG to the midgut, in the efferent duct and filament-like structure connecting the tubular salivary glands (TSG) and primary salivary glands (PSG), as well as in the TSG and PSG. Furthermore, our results suggest that tissue tropism changes with thrips development such that the primary site of infection shifts from the midgut and TSGs in larvae to the PSGs when the insect proceeds to adulthood. Our results reveal the internal morphology of the salivary glands and support the hypothesis that the TSG and associated structures (e.g. ligaments, efferent duct, filament-like structure) serve as a conduit for virus infection to progress from the midgut to the PSG.

2. Materials and methods

2.1. Plant materials and TSWV inoculum

Emilia sonchifolia and *Datura stramonium* plants were kept in a growth chamber set at 24°C and a photoperiod of 14 h light/10 h dark. TSWV (isolate TSWV-MT2)-infected plant tissue for acquisition access period (AAP) was obtained by mechanical inoculation of two-week-old *E. sonchifolia* plants or three-week-old *D. stramonium*. Mechanical inoculation was done using *E. sonchifolia* TSWV-symptomatic tissue from thrips-inoculated plants kept in the greenhouse and no older than two months after exposure to thrips. Infected tissue was ground in a chilled mortar and pestle in 10 ml of cold 100 mM Na_2SO_3 buffer. Plants to be inoculated were dusted with carborundum and gently rubbed with a cotton swab wet with inoculum. Twelve days after mechanical inoculation, symptomatic leaves were harvested, assembled into bouquets, and placed into plastic deli cups used for AAP. Similar harvesting of leaf tissue was done for non-inoculated plants of the same age as the control. *Datura stramonium* leaf discs were used to test virus transmission by thrips. The third youngest leaf from the apex of four-week-old *D. stramonium* plants was used to obtain 1.5-cm-diameter discs for the inoculation access period (IAP) in transmission assays.

2.2. Thrips rearing and virus acquisition access periods (AAP)

A Western flower thrips (*F. occidentalis*) colony was maintained on green pods (*Phaseolus vulgaris*) as previously described (Ullman et al., 1992). First instar larval thrips (L1s) 0–8 h old were collected from green beans obtained from the colony where adult thrips had previously fed and laid eggs. The beans were brushed to remove adult thrips, placed in plastic cups, and incubated for 8 h to allow eggs to hatch. Collected L1s were allowed to feed on TSWV-infected and healthy *E. sonchifolia* leaf tissue in separate plastic cups for an acquisition access period (AAP) of 18 h in an incubator at 25°C with a photoperiod of 12 h light and 12 h dark. After the AAP, thrips were transferred to cups with green beans and kept in the incubator at 25 °C with a photoperiod of 12 h light and 12 h dark.

2.3. Sampling points for thrips immunolabeling time-course experiment

Individual thrips were sampled for dissecting and immunolabeling at four times in their life cycle. The times sampled were selected to study the progression of virus infection and to reveal any changes in viral tissue tropism. The times sampled were larval thrips at a relatively early time in the infection cycle (L1 24 h post acquisition, or 45–53-h-post egg eclosion), followed by key later points in the infection process: just prior to pupation (L2 72 h post acquisition, or 93–101-h-post egg eclosion) and then during two times as adults (24 h and 96 h post pupal eclosion). Two adult time points were sampled to evaluate the viral infection in the tissues as adult thrips aged. This experiment was replicated twice. Approximately 40 individuals were selected per time point and treatment for each replication of the experiment (TSWV-exposed and non-exposed). A fine paint brush was used to place the thrips in a plastic cup with double layer of parafilm housing one hundred microliters of 7% sucrose solution. Thrips fed on this solution for three hours which cleared their guts of plant material before harvesting for immunolabeling.

2.4. Dissecting and immunolabeling of thrips guts and salivary glands

Thrips were dissected by decapitation to obtain the gut and salivary glands, and these tissues were fixed and immunolabeled (Bressan and Watanabe, 2011; Whitfield et al., 2004). Individual thrips were placed on a glass slide with ice-cold phosphate buffer-saline (PBS) and decapitated using Teflon-coated razor blades under a dissecting stereoscope. The thrips were moved to a Tissue tack slide (Polysciences Inc., Warrington, PA) and placed on a drop of ice-cold PBS surrounded by an incubation chamber (Electron Microscopy Sciences, Hatfield, PA). The gut was removed from the remaining thrips body with a cut at the hindgut region of the thrips. After processing the guts, PBS was removed and the slide was left to sit at room temperature until dry. The incubation chamber on the slide was filled with 4% paraformaldehyde in 50 mM sodium phosphate (pH 7.0) and incubated for 1 h at room temperature. All slides were placed inside a humid box to prevent desiccation of samples throughout the immunolabeling procedure. The slide was rinsed once with PBS and left overnight at 4 °C with PBS-1% Triton X-100. All post-fixation and permeabilization incubations were done at room temperature on a rocker with slight movement. The slides were washed three times with PBS and incubated in blocking buffer (PBS, 0.1% Triton X-100 and 10% normal goat serum (NGS)) for 30 min. After blocking, the guts and salivary glands were incubated with peptide rabbit antibodies against TSWV glycoprotein G_N at a concentration of 50 μg/ml in PBS supplemented with 0.1% Triton X-100 and 1% NGS for 2.5 h. The remaining incubations were done in a humid box wrapped in aluminum foil to protect the slides from the light. The slides were washed three times with PBS and incubated with chicken anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA) at 10 μg/ml in PBS supplemented with 0.1% Triton X-100 and 1% NGS for 2.5 h. The slides were washed three times with PBS and incubated for 2 h with phalloidin conjugated to Alexa Fluor 594 and with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) at 4 units/ml and 125 μg/ml, respectively (Invitrogen). The slides were washed three times with PBS, and then rinsed three times with distilled water. The incubation chamber was removed and the slides air-dried. Thirty-five microliters of PBS-50% glycerol were added on the area where the guts were placed, covered with a cover slip, and sealed with nail polish. Slides were kept at 4 °C and protected from light.

2.5. Confocal fluorescent microscopy

The slides of immunolabeled thrips guts and salivary tissues from the time-course experiments were observed by confocal fluorescence microscopy with a Zeiss Axiovert 200M confocal microscope equipped with a Zeiss Laser Scanning System LSM 510 META for image acquisition. Images were observed and handled with Zeiss LSM Image Browser Version 4.2.0.121. The confocal microscope settings were a 405-nm laser diode at 5% transmission for detecting DAPI fluorescence with a bandpass (BP) filter 420–