DISSECTING THE MOLECULAR INTERPLAY BETWEEN *TOMATO SPOTTED WILT VIRUS* AND THE INSECT VECTOR, *FRANKLINIELLA OCCIDENTALIS*

by

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B.S., University of Puerto Rico – Mayagüez, 2006
M.S., University of Wisconsin – Madison, 2008

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree of

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Department of Plant Pathology
College of Agriculture

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Abstract

The *Bunyaviridae* is a family of animal and plant viruses that pose a threat to human, animal, and plant health worldwide. In nature, the dissemination of these viruses is dependent on arthropod vectors (genera *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*) or rodent vectors (genus *Hantavirus*). The genus *Tospovirus* is the only one within this virus family that is composed of plant-infecting viruses transmitted by thrips. *Tomato spotted wilt virus* (TSWV), the type species of the *Tospovirus* genus, is one of the ten most devastating plant viruses known. It is most efficiently transmitted by the western flower thrips, *Frankliniella occidentalis* Pergande, in a persistant propagative manner. The insect molecules associated with virus infection and transmission by the thrips vector remain unidentified to date. The aim of this work was to identify *F. occidentalis* larval thrips proteins that are differentially expressed during TSWV infection of the insect vector and those that directly interact with TSWV. To achieve these goals, I used two-dimensional (2-D) gel electrophoresis and mass spectrometry coupled with Mascot searches. I identified 26 protein spots that displayed differential abundances in response to TSWV infection, which contained 37 proteins. Sixty two percent of these proteins were down-regulated by the viral infection demonstrating a complex response. Moreover, 8 and 11 protein spots that directly interacted with purified TSWV virions and a TSWV glycoprotein (G_N), respectively, were identified in overlay assays of larval thrips proteins resolved by 2-D gel electrophoresis. A total of five proteins were identified from these spots. These interacting proteins might play roles in attachment and entry, endocytosis/exocytosis, and escape from different tissues for transmission to occur. Injection of double-stranded RNA (dsRNA) into adult female thrips triggered an RNAi response that resulted in 23% reduction of the target gene transcript level. This significant reduction resulted in increased mortality and decreased fertility compared to insects injected with control dsRNA or water and non-injected insects as well. The work presented here provides new insights on the molecular basis of this virus-vector interaction and describes new tools to conduct functional genomic assays to study gene function and design control strategies of *F. occidentalis*.

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Major Professor
Anna E. Whitfield

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Dedication

"Blessed are those who are able to give both wings and roots to their children."

Arab proverb

I dedicate my doctoral dissertation to my beloved parents who continuously provided me with their encouragement to go after my goals and dreams in life and to fly wherever I needed to, to pursue and conquer them, while offering me their endless love, comprehension, and support so I can always remember where my home really was. Because family like a tree, expands its branches to different directions but have their roots to maintain them strong and always together.

To the amazing human being that is my spiritual and loving inspiration to keep traveling in life:

Ismael Badillo Roldán

MY FATHER

And to the angel that altogether with God provide strength and protection to the traveler:

Luz E. Vargas Plaza

MY MOTHER

Chapter 1 - Introduction and Literature Review

Introduction

The disease known as tomato spotted wilt was first found and described in Australia in 1915 (Brittlebank, 1919). Approximately 12 years later, thrips were implicated as the biological vectors of the disease-causing agent (Pittman, 1927). It was not until 1930 that Samuel and colleagues demonstrated that the disease had a viral etiology and called the causal agent *Tomato spotted wilt virus* (TSWV) (Samuel et al., 1930). In 1984, Milne and Francki suggested that TSWV should be the first member of a monotypic group of plant-infecting viruses in the family *Bunyaviridae* (Milne and Francki, 1984). Finally in 1991, a group of plant viruses were officially recognized and taxonomically placed into a separate genus within the family *Bunyaviridae*, the genus *Tospovirus* (Francki et al., 1991). Currently the *Bunyaviridae* family is comprised of four genera of animal-infecting viruses, *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, and *Phlebovirus*, and the plant-infecting genus *Tospovirus* (reviewed in Gonzalez-Scarano and Nathanson, 1996). TSWV is the prototypic member of the genus *Tospovirus* which currently consists of 8 approved and 21 tentative plant virus species. Recent findings clearly suggest that novel reassortants and new tospoviruses are rapidly emerging and being discovered (Ciuffo et al., 2008; de Oliveira et al., 2011; de Oliveira et al., 2012; Dong et al., 2013; Meng et al., 2013; Tentchev et al., 2011; Webster et al., 2011; Winter et al., 2006; Zhou et al., 2011).

Viruses belonging to the family *Bunyaviridae* have enveloped virions with two glycoproteins (G_N and G_C), three segmented RNA molecules (S, M, and L) that are coated with nucleocapsid (N) protein, and few copies (10-20) of the RNA-dependent RNA-polymerase (L) protein (Elliot, 1990). Bunyaviruses include important medical, veterinary, and agronomic viral pathogens (Sin et al., 2005a). Interestingly, viruses belonging to all genera are arthropod-borne viruses, vectored by arthropods that include ticks, midges, mosquitoes, sandflies, gnats, and thrips, except for the hantaviruses which are transmitted by rodents (Beaty and and Bishop, 1988). In nature, tospoviruses are transmitted from plant to plant exclusively by thrips, which belong to the insect order Thysanoptera.

In 1927, Pittman determined that *Thrips tabaci* was the biological insect vector of TSWV (Pittman, 1927). However, *Frankliniella occidentalis* is now recognized as the main vector of

TSWV (Gardner et al., 1935). Five other thrips species that include *F. schultzei* (Samuel et al., 1930), *F. fusca* (Sakimura, 1963), *T. setosus* (Kobatake et al., 1984), *F. intonsa* (Wijkamp et al., 1995), and *F. bispinosa* (Webb et al., 1997) have also been reported as vectors of TSWV. The high locomotory activity, high fecundity combined with a short generation time, a strongly female-biased sex ratio, preference for concealed spaces, a wide range of plant hosts, and the habit of piercing and sucking from epidermal and mesophyll plant cells make these insects important and severe pests worldwide (Moritz et al., 2004). The direct and indirect agricultural losses caused by thrips due to their feeding damage and dissemination of tospoviruses, respectively, combined with the low efficiency of control measurements against thrips and the viruses they transmit make these pathosystems a serious threat to food production everywhere they occur.

TSWV is most efficiently transmitted by *F. occidentalis* in a persistent propagative fashion in which it journeys through the thrips' body and replicates in various tissues and organs of the insect vector (Ullman et al., 1993; Wijkamp et al., 1993). Despite the extensive replication of TSWV in thrips' midgut and salivary glands, there is little to no pathogenic effect on *F. occidentalis* (Wijkamp et al., 1996) resulting in efficient virus transmission by this thrips species. In contrast to the extensive knowledge of the functions of TSWV genes and the biological aspects of TSWV-*F. occidentalis* interaction accumulated over the last three decades, very little is known about the molecules from the insect vector that are involved in entry, replication, and spread of the virus within the thrips vector as well as those involved in antiviral defenses during TSWV infection.

The molecular characterization of TSWV-*F. occidentalis* interaction is the focus of my doctoral dissertation. Understanding the initial steps of binding and entry to, infection of, and dissemination within the thrips vector as well as the antiviral defenses deployed by the insect host against the virus will provide novel targets for thrips and tospovirus control. I have begun identifying a suite of candidate proteins from *F. occidentalis* that may play important roles in virus acquisition, infection, spread, and antiviral defenses. Moreover, I have developed RNA interference (RNAi) tools for thrips with the dual purpose of 1) functionally characterizing the specific role of insect proteins during the replication cycle of TSWV within its insect vector and 2) to developing novel control strategies to manage thrips damage and tospovirus transmission. This research laid the groundwork for future studies to further understand the intricate interaction

between TSWV and its most efficient insect vector, *F. occidentalis*, and to explore the potential of several candidate genes in the management of this pathosystem in the field.

This dissertation is divided into five chapters. The first chapter presents a review of the family *Bunyaviridae* with an emphasis on the genus *Tospovirus* and TSWV, the insect order Thysanoptera specifically *F. occidentalis* and other thrips vector species, TSWV-*F. occidentalis* interaction, and the antiviral response of insects to virus infection. The second chapter describes the proteomic analysis of healthy larval thrips and differentially expressed proteins in response to TSWV infection. The third chapter focuses on the identification of larval thrips proteins that directly interact with TSWV particles and/or a specific viral glycoprotein. In the fourth chapter I present data about RNAi tools for thrips using an injection delivery system. The fifth and final chapter summarizes the major findings and contributions accomplished through my doctoral dissertation as well as a discussion and future directions for this research.

Literature Review

Family *Bunyaviridae*

The family *Bunyaviridae* is one of the largest groups of animal RNA viruses, including more than 350 virus species (Schmaljohn and Nichol, 2007). It was established in 1975 to encompass a large group of arthropod-borne viruses (Porterfield et al., 1973; Porterfield et al., 1976). This family of viruses is composed of five genera, *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (reviewed in Gonzalez-Scarano and Nathanson, 1996). The genus *Tospovirus* is the only genus within this family whose members are plant pathogens. Bunyaviruses are arthropod-borne viruses with the exception of members of the genus *Hantavirus* which are rodent-borne (Beaty and and Bishop, 1988). However, within each genus viruses are transmitted by a specific and limited range of arthropod/rodent vectors. Viruses belonging to this family cause persistent, nonlethal, lifelong infection in their vectors in which they also replicate (German et al., 1992). Furthermore, some bunyaviruses are perpetuated by sexual (Thompson and Beaty, 1977) and/or transovarial (Watts et al., 1973) transmission between their arthropod vectors.

All the viruses belonging to the *Bunyaviridae* family share similar morphology with pleomorphic virions ranging from 80 to 120 nanometers in size (Gonzalez-Scarano and

Nathanson, 1996). They are all membrane-bound viruses and have two glycoproteins decorating their surface (Shope, 1985). All bunyaviruses have a segmented genome consisting of three negative or ambisense, single-stranded RNA molecules (S, M and L RNAs) coated by nucleocapsid (N) protein. Their three genomic segments share eight nucleotide consensus sequence at the 3' and 5' termini, which give the viral ribonucleoproteins (RNPs) a pseudocircular or panhandle configuration (de Haan et al., 1989; Kellmann et al., 2001; Pardigon et al., 1982). Ten to twenty copies of the viral RNA-dependent RNA-polymerase (L) protein are attached to the RNPs (Adkins, 2000). The entire replication cycle of all bunyaviruses take place in the cytoplasm of an infected cell, which finally mature by budding in or near Golgi vesicles (Bishop et al., 1980). However, bunyaviruses differ with respect to the patterns of genomic RNA, genome segment coding strategy, size of viral proteins, and the coding of nonstructural (NS) proteins (viral proteins that are not found in mature virions).

Some viruses in the family *Bunyaviridae* have been listed as emerging or reemerging viruses and are considered as potential bioterrorism weapons (Sidwell and Smee, 2003). Several bunyaviruses are serious pathogens of humans and animals causing acute debilitating disease (Oropouche virus), encephalitis (La Crosse virus), acute respiratory disease (Sin Nombre virus), or hemorrhagic fever (Rift Valley fever virus) (Elliot, 1990). Moreover, members of the genus *Tospovirus* are serious plant pathogens that cause agricultural losses in a wide range of cultivated plants worldwide (Culbreath et al., 2003; Goldbach and Peters, 1994; Pappu et al., 2009). TSWV, the type member of the *Tospovirus* genus, is considered to be one of the ten most devastating plant viruses known (Scholthof et al., 2011).

Genus *Tospovirus*

The genus *Tospovirus* is the only genus within the family *Bunyaviridae* that contains plant-infecting viruses. Tospoviruses pose an enormous threat to agricultural production worldwide due to their extreme broad host range that includes hundreds of plant species in more than 110 distinct botanical families (Campbell et al., 2008). The host range of plant viruses belonging to this genus includes agronomic, vegetable, fruit, fiber, and ornamental crops as well as several weed species. Viral diseases caused by tospoviruses have been gaining importance since the 1980s. For example, the Hawaiian vegetable industry experienced extensive damage in

lettuce with losses of 50-90% caused by TSWV (Cho et al., 1987). Bezerra and colleagues reported the occurrence of Iris yellow spot virus (IYSV) as a major problem in onion crops at the São Francisco River basing resulting in yield losses of up to 100% (Bezerra et al., 1999). Furthermore, the ornamental industry worldwide has suffered devastating tospovirus epidemics with entire crops being destroyed in some cases (Daughtrey et al., 1997). In the last few years economic losses caused by tospoviruses have been estimated to reach millions of US dollars per year (Culbreath et al., 2003; Goldbach and Peters, 1994; Mumford et al., 1996; Pappu et al., 2009; Persley et al., 2006). Unfortunately, there are not effective management strategies for the control of thrips-transmitted tospoviruses.

Since the initial description of TSWV in Australia, plant virologists have identified several tospovirus species. Currently, there are 8 approved and 21 tentative tospovirus species (Table 1.1). Most importantly, the emergence and identification of new tospoviruses or reassortant variants has significantly increased during the last two decades. In 2006, Winter and colleagues found a new plant virus with properties characteristic of tospoviruses infecting tomatoes in Iran, for which the name Tomato fruit yellow ring virus (TFYRV) was proposed (Winter et al., 2006). Moreover, Dong *et al.* characterized a distinct tospovirus from tomatoes in China, for which the name Tomato zonate spot virus (TZSV) was suggested (Dong et al., 2008). Polygonum ring spot virus (PRSV) was identified as a novel tospovirus species infecting knotgrass in Italy (Ciuffo et al., 2008). Generally, tospoviruses cluster into the American (New World) or the Eurasian (Old World) groups. However, Soybean vein necrosis virus (SVNV) and Bean necrotic mosaic virus (BNMV), two new bean-infecting tospoviruses recently found in USA and Brazil, respectively, form their own phylogenetic group based on their L protein sequences (de Oliveira et al., 2011; de Oliveira et al., 2012; Zhou et al., 2011). More recently, a new tospovirus temporarily named Mulberry vein banding virus (MuVBV) has been identified from mullberries in China (Meng et al., 2013) and Hippeastrum chlorotic ringspot virus (HCRV) was isolated from amaryllis also in China (Dong et al., 2013). In 2011, a naturally-occurring reassortant between *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) was found in Florida (Webster et al., 2011). The resurgence and emergence of tospoviruses is due in great part to the evolution of these viruses through the reassortment of genome segments during co-infection of a plant host. Thus, exchange of entire genome segments can result in the occurrence of tospoviruses with new biological characteristics.

Viruses within this genus have spherical particles ranging between 80 to 120 nanometers in diameter (Fig. 1.1) (Vankamme.a et al., 1966). Electron microscopy studies identified tospoviruses as the only plant-infecting viruses with a spherical structure and an envelope membrane (Black et al., 1963; Ie, 1971; Kitajima, 1965; Milne, 1970). In general, plant viruses belonging to the genus *Tospovirus* cause ring spot patterns, chlorosis, necrosis, mottling, silvering, vein banding, mosaics, rugosity, and stunting of their plant hosts (German et al., 1992). However, symptoms might vary depending on the virus, isolate, plant host, environmental conditions, and time of the year in which infection takes place (Best and Gallus, 1953; Francki and Hatta, 1981). Tospoviruses are not seed-borne (Reddy and Weightman, 1988) and in nature they are exclusively transmitted from plant to plant by thrips vectors (Pittman, 1927). Interestingly, the genetic information of viruses in the genus *Tospovirus* must be expressed in the phylogenetically and physiologically disparate biological systems of the plant host and the insect vector.

Tomato spotted wilt virus

Tomato spotted wilt virus (TSWV), the prototype member of the *Tospovirus* genus, is considered to be one of the ten most devastating plant viruses known (Scholthof et al., 2011). Its worldwide distribution throughout tropical, subtropical, and temperate zones combined with its broad host range increases the difficulty of controlling the spread of this plant virus. TSWV infects over 1,050 different plant species in more than 70 distinct botanical families and it is responsible for substantial losses in agronomic, fruit, vegetable, ornamental, and fiber crops worldwide (Pappu et al., 2009; Sether and and DeAngelis, 1992). Crop losses from severe disease epidemics of TSWV in Hawaii have reached 60-70% in tomato, pepper, and lettuce (Cho et al., 1987; Cho et al., 1989). Moreover, TSWV incidence has reached 60% in commercial fields in Louisiana (Greenough et al., 1985), while devastating epidemics have occurred on peanuts in Texas (Stewart et al., 1989). Furthermore, Bertrand reported that annual losses in Georgia due to TSWV are estimated at a $100 million US dollars (Bertrand, 1998). Symptoms of TSWV are characteristic of other tospoviruses, being the chlorotic ring spots and wilting symptoms which led to the naming of the disease tomato spotted wilt. However, the virus has

been detected and isolated from plants showing symptoms as well as from asymptomatic plants (Antignus et al., 1997).

TSWV particles have been observed in epidermal and mesophyll cells, stomata, tracheids, sieve tubes, phloem parenchyma, and even among the chromosomes whose nuclear membrane had been broken during mitosis (Francki and Grivell, 1970). Different biological variants of TSWV exist which have altered host ranges and symptoms (Norris, 1946). This plant pathogenic virus is characterized by having enormous genetic heterogeneity (Best and Gallus, 1953; Norris, 1946). TSWV can evolve by reassortment of its genome segments which occurs during mixed infections, exploiting the genetic variability in its population (Hoffmann et al., 2001; Qiu et al., 1998; Qiu and Moyer, 1999; Sin et al., 2005b; Tentchev et al., 2011; Webster et al., 2011). Importantly, TSWV has the property to infect and replicate in both the plant host and the insect vector (Ullman et al., 1993; Wijkamp et al., 1993). Resende *et al.* found that during a series of mechanical passages of TSWV in plants, morphologically defective isolates and defective interfering (DI) RNAs were produced (Resende et al., 1991). TSWV isolates carrying DI RNAs have deletions in diverse parts of their genome, however, the defective molecules retain the sequences required and necessary for virus replication, genomic RNA encapsidation by nucleocapsid proteins, and packaging of the RNP complexes into virus particles in plant cells (Hillman et al., 1987; Huang, 1973; Verkleij and Peters, 1983). Interestingly, an envelope deficient isolate of TSWV failed to infect the thrips midgut epithelial cells after ingestion from virus-infected plant tissue (Nagata et al., 2000b). Also a DI isolate of TSWV containing a truncated L RNA was no longer transmitted by thrips (Nagata et al., 2000b). Moreover, a nonsynonymous mutation (C1375A) in the TSWV glycoprotein open reading frame (ORF) resulted in the loss of thrips transmissibility without compromising virion assembly in plant cells (Sin et al., 2005b).

TSWV has a tripartite genome that consists of ambisense or negative-sense, single-stranded RNAs (Fig. 1.2) (Gonzalez-Scarano and Nathanson, 1996). Its virions are membrane-bound and pleomorphic in shape with a diameter that ranges from 80 to 120 nanometers in size (Gonzalez-Scarano and Nathanson, 1996; Shope, 1985). TSWV virion composition is 5% nucleic acid, 70% protein, 5% carbohydrate, and 20% lipid (Adkins, 2000). Sequence and translational analyses of TSWV has shown that the S (de Haan et al., 1990) and M (Kormelink et al., 1992) RNAs are ambisense, while the L RNA is negative-sense only (de Haan et al., 1991).

Kikkert and colleagues reported that the TSWV genome encodes a total of 6 proteins from 5 ORFs (Kikkert et al., 1997). Every viral protein is expressed by translation of a subgenomic RNA (sgRNA) species with exception of the L protein which is expressed from a messenger RNA (mRNA) species (de Haan et al., 1991; German et al., 1992). RNA terminal sequences share eight nucleotides at the 3' and 5' ends which are thought to contain recognition sites for the viral polymerase within important regulatory signals such as replication promoters (Strauss and Strauss, 1988). Genomic RNAs encapsidated by nucleocapsid (N) proteins are immediately transcribed into mRNAs or sgRNAs by the viral polymerase. TSWV mRNAs/sgRNAs are not polyadenylated and have eukaryotic cap structures of host origin at their 5' ends that are generated by cap-snatching, a process that occurs when the viral polymerase cleaves 10 to 20 nucleotides from the cap structure of host mRNAs and incorporate them into the 5' terminus of the newly synthesized viral mRNA/sgRNA (Duijsings et al., 2001; Duijsings et al., 1999; vanPoelwijk et al., 1996). Then the viral mRNAs/sgRNAs are translated to produce the viral proteins by the plant or insect host cell's machinery. After enough viral proteins are produced, the viral polymerase switches from transcription to replication in order to generate more full length genomic RNA for virion assembly. The panhandle structures are thought to be important for encapsidation of the three full length genomic RNAs by the N protein and finally for packaging of the RNPs into virus particles (Elliot, 1990).

The S RNA segment of TSWV is 2,916 nucleotides in length and does not have significant homology with any animal-infecting bunyavirus (de Haan et al., 1990). It encodes a 52.4 kDa nonstructural small (NSs) protein in the viral (v) sense and a 29 kDa nucleocapsid (N) protein in the viral complementary (vc) sense (de Haan et al., 1990). The NSs protein from TSWV and other tospoviruses have been shown to be necessary and sufficient for the silencing suppressor activity in plants by binding double-stranded and short interfering RNAs (dsRNAs and siRNAs, respectively) (Bucher et al., 2003; Schnettler et al., 2010; Takeda et al., 2002). Furthermore, NSs have been shown to act as a silencing suppressor in heterologous insect and arthropod systems (Garcia et al., 2006; Oliveira et al., 2011). This protein is highly expressed in the cytoplasm of infected plants cells (Kormelink et al., 1991) and also accumulates to high levels in the salivary glands of thrips (Kitajima et al., 1992; Wijkamp et al., 1993). The N protein binds to single-stranded RNA (ssRNA) and other N protein units to form the RNP complexes (Richmond et al., 1998) by a "head-to-tail" interaction of the N terminus with the C

terminus to form a multimeric chain (Uhrig et al., 1999). It is hypothesized that this homotypic interaction and multimerization of the N protein leads to the initiation of viral RNA transcription and replication in a structural and regulatory manner.

The M RNA segment of TSWV is 4,821 nucleotides long (Kormelink et al., 1992). The v sense encodes a nonstructural medium (NSm) protein of 33.6 kDa, while the vc sense encodes the polyprotein precursor to the G_N and G_C glycoproteins with a predicted size of 127.4 kDa (Kormelink et al., 1992); the mature G_N and G_C glycoproteins are predicted to be 50 kDa and 77 kDa, respectively. The NSm protein is involved in virus movement of TSWV RNPs in plants (Kormelink et al., 1994). It has been shown that NSm targets the plasmodesmata, alters the size exclusion limit of plasmodesmata, and subsequently forms aggregated tubules to aid in the virus cell-to-cell and long-distance movement (Lewandowski and Adkins, 2005; Li et al., 2009; Storms et al., 1998; Storms et al., 1995). The G_N/G_C polyprotein precursor is cleaved by a yet unknown host protease in the absence of other viral proteins to generate the mature viral glycoproteins, G_N and G_C, which are involved in virion assembly and transmission by thrips vectors (Adkins et al., 1996; Whitfield et al., 2004; Whitfield et al., 2008; Whitfield et al., 2005b). TSWV particle morphogenesis occurs in the Golgi system, in which G_N and G_C accumulates prior to a process of wrapping, by which the viral RNPs obtain a double membrane (Kikkert et al., 1999). In a later stage of virus maturation, the membranes of these double enveloped particles fuse to each other and to the endoplasmic reticulum to form single enveloped particles clustered in membranes (Kikkert et al., 1999).

Both S and M RNA segments of TSWV possess an intergenic region (IGR) between the v and vc sense ORFs with a high A-U content capable of forming a stable hairpin structure that contains a conserved sequence at the top of the hairpin which has been proposed to act as a signal for transcription termination (Goldbach and Peters, 1996). The IGR of the S RNA of TSWV was also correlated with its competitiveness in virus reassortment during mixed infections (Qiu et al., 1998).

The L RNA, which is of negative polarity, is 8,625 nucleotides in length and it encodes a 331.5 kDA protein in the vc sense (Vanpoelwijk et al., 1993). Adkins *et al.* found that purified L protein contained the RNA-dependent RNA-polymerase (RdRp) activity, as is the case with other animal-infecting bunyaviruses, which synthesized both the v sense and vc sense strands (Adkins et al., 1995). Furthermore, TSWV L protein contains a highly conserved serine-aspartic

acid-aspartic acid (SDD) motif present in the viral polymerases of all segmented negative-strand RNA viruses (Tordo et al., 1992). A mixture of TSWV virions and detergent did not abolish the RdRp activity, suggesting that the L protein is the viral polymerase and that it is bound to the RNPs (Adkins et al., 1995). Each TSWV virion has approximately 10 to 20 copies of the L protein associated to its RNPs (Adkins, 2000).

The Thysanoptera, *Frankliniella occidentalis* and other thrips vector species

The insect order Thysanoptera (from the Greek thysanos and pteron, which mean fringe and wing, respectively) is comprised exclusively of tiny, slender insects with fringed wings commonly known as thrips. Other common names include thunderflies, thunderbugs, thunderblights, stormflies, and plant lice. The order, which belongs to the Hemipteroid assemblage, is composed of two suborders, the Tubulifera with a single family and the Terebrantia with eight families (Moritz et al., 2001). Thripidae is the largest family within the suborder Terebrantia, from which about 50 species are insect pests that cause damage to agricultural crops and to which all thrips vectors of tospoviruses belong (Mound, 1996). Thysanopterans display a great diversity with respect to morphological structures, food preference, and behavioral characteristics. However, they share some common but unique characteristics such as a postembryonic remetaboly development, a process that is intermediate between hemimetaboly (partial metamorphosis) and holometaboly (complete metamorphosis) (Heming, 1973); a haplodiploid genetic system with unfertilized eggs becoming males (haploid) and fertilized eggs becoming females (diploid) (Crespi, 1992; Jacobson et al., 2013); and asymmetrical mouthparts comprised of a resorbed right mandible and a fully developed left mandible that forms a narrow stylet used to feed from cells (Heming, 1980). Phytophagous thrips are piercing-sucking insects that feed by emptying plant cells of their cytoplasmic and other cellular contents (Childers and Achor, 1991; Chisholm and Lewis, 1984; Hunter and Ullman, 1989; Hunter and Ullman, 1992). Using electron microscopy, intact plant cell organelles have been observed inside thrips guts (Ullman et al., 1992).

The western flower thrips, *Frankliniella occidentalis* Pergande (Fig. 1.3), is the most economically important pest among thysanopterans due to the direct and indirect damage it causes to cultivated crops by feeding and transmitting tospoviruses, respectively. *F. occidentalis*

is a remarkably polyphagous insect pest with a host range that includes 244 plant species from 62 different botanical families (OEPP/EPPO, 1998; OEPP/EPPO, 2002). Furthremore, *F. occidentalis* is a successful invader due to its high fecundity (~75 eggs per female) combined with short generation times (~12 days from egg to adult), a capacity to develop insecticide resistance, and its thigmotactic behavior, whereby it prefers concealed spaces on or in plant organs, protecting it from harsh conditions and insecticide applications. All the aforementioned reasons make effective control of *F. occidentalis* difficult to achieve using the current available strategies (Morse and Hoddle, 2006).

F. occidentalis feeding damage on plants can cause silvering (discoloration), scars, leaf and fruit malformation, growth malfunction, plant stunting, and brown bumps but injury is often cosmetic rather than economic (Navas et al., 1991). However, severe *F. occidentalis* feeding damage could be translated to growth, production and quality reduction of leaves, flowers and fruits, respectively (Maris et al., 2004). Most importantly, *F. occidentalis* is a competent vector of five tospoviruses including TSWV, which makes this thrips species able to successfully acquire, maintain, and transmit these plant viruses (Table 1.2). Several reports confirm the worldwide importance of *F. occidentalis* vectoring TSWV due to its polyphagous nature and widespread geographical distribution (Allen and Broadbent, 1986; Chamberlain et al., 1992; Sakimura, 1962; Stobbs et al., 1992). Epidemic outbreaks of TSWV in different countries have indeed been related to the introduction and spread of *F. occidentalis* (Aramburu et al., 1997; Avila et al., 2006; Wijkamp et al., 1995). After the worldwide expansion of *F. occidentalis* in 1990, TSWV has become a serious threat in field-grown and greenhouses crops (Chatzivassiliou et al., 2000). Moreover, several populations of *F. occidentalis* have developed insecticide resistance (Bielza, 2008; Cifuentes et al., 2012; López-Soler et al., 2008) which increases the difficulty of controlling this pervasive insect pest.

Most thrips vector species share several behavioral characteristics such as high locomotory activity, high fecundity, a short generation time, a predisposition to parthenogenesis, a female-biased sex ratio, preference for concealed spaces, a wide range of plant hosts, and the habit of puncturing epidermal and mesophyll plant cells (Moritz et al., 2001). Of the 7,400 described species within the insect order Thysanoptera only 14 are known vectors of tospoviruses (Table 1.2), highlighting the specificity of these virus-vector interactions. Currently, the known thrips vector species of tospoviruses belong to the five genera

Frankliniella, *Thrips*, *Ceratothripoides*, *Dyctiothrips*, and *Scirtothrips* and these species vary with respect to virus and host specificity. Thrips vectors differ in the number of virus species they transmit, for example, *T. tabaci* and *F. occidentalis* transmit several species while others, like *F. zucchini* transmits only one virus species. Moreover, thrips vectors differ in their feeding habits and geographical distribution. For example, *F. occidentalis*, a polyphagous and globally-distributed species, transmits Chrysanthemum stem necrosis virus (CSNV), *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), *Tomato chlorotic spot virus* (TCSV), and TSWV. *T. tabaci* which was the first thrips species implicated as the vector of TSWV in 1927 also transmits Iris yellow spot virus (IYSV), and Tomato fruit yellow ring virus (TFYRV). Among the highly competent vectors of multiple tospovirus species, most are highly polyphagous insects. These characteristics may have increased their proximity to several tospoviruses in nature, and their coexistence and co-evolution may explain the capacity of these vectors to transmit these viruses to numerous plant host species across extensive geographical ranges. In contrast, those thrips vectors that transmit one or few tospovirus species tend to have a more restricted plant host range; for example, *D. betae* transmits only Polygonum ring spot virus (PolRSV) and has a very limited host range. Thus, compatible virus-vector interactions require that the virus and the vector populations co-occur and thrive under the same environmental conditions and constraints. Despite the significance of these virus-vector interactions, knowledge of the molecular basis of the interactions that make a thrips species an efficient vector and host of a particular tospovirus species remain elusive.

***Tomato spotted wilt virus-Frankliniella occidentalis* interaction**

Tospoviruses have an unusual and very specific relationship with their thrips vectors, the only insect group that can transmit these plant-infecting viruses (Bautista et al., 1995; Mound, 2002; Riley et al., 2011). The TSWV-*F. occidentalis* interaction, as with many other insect-vector interactions, is characterized by a very intricate association and only few other thrips species are able to acquire and transmit the virus. The western flower thrips, *F. occidentalis*, is the most efficient vector of TSWV and it transmits the virus in a persistent propagative fashion (Ullman et al., 1993; Wijkamp et al., 1993). TSWV is the best-characterized tospovirus with respect to interactions with *F. occidentalis*. Virus transmission by adult thrips occurs exclusively

if TSWV acquisition takes place during the larval stages, with the first instar larvae being the most efficient stage at acquiring the virus (Fig. 1.4) (Lindord, 1932; van de Wetering et al., 1996). Early second instar larvae can also acquire TSWV but to a lower extent than first instar larvae (Chatzivassiliou et al., 2002). Adult thrips that feed on virus-infected plants can acquire TSWV as well, however, this does not result in a productive infection and transmission does not occur (de Assis Filho et al., 2004; Ullman et al., 1992). Peters and colleagues reported that the latent period (LP; time required since virus acquisition until an insect vector is capable to transmit such virus) of TSWV is completed during the larval stages before pupation begins (Peters et al., 1995). TSWV must persists through molting and pupation of its thrips vector, during which the replacement of internal tissues and organs of the prepupae and pupae occurs, resulting in the transstadial transmission (when a virus is transmitted through the different life stages of an insect vector) of the virus (Moritz, 1997). However, TSWV is not transovarially transmitted (when a virus is transmitted from the mother to the embryos of an insect), thus an infected female thrips does not passes the virus vertically to her offspring (Wijkamp et al., 1996). Finally, TSWV is transmitted by adult male and female thrips to a susceptible plant host and the transmission cycle must begin again (Fig. 1.4).

TSWV dynamics are the result of complex interactions between the triad of virus, vector and plant host which are all influenced by environmental conditions (Peters et al., 1995). At the maturation stage, TSWV particles are clustered inside large membrane sacks where they are accumulated and retained within plant cells and depend upon uptake by thrips vectors for transmission to other plant hosts (Kikkert et al., 1999). TSWV acquisition can occur in feeding periods as short as 5 minutes (Razvyazkina, 1953; Wijkamp et al., 1996), but continuous feeding and longer acquisition access periods (AAP) could be required for a thrips vector to ingest and acquire enough virus particles to become viruliferous (Peters et al., 1995). The AAP for thrips to acquire TSWV can vary depending on the plant host from which the virus is acquired and also due to the distribution and availability of the virus within the plant tissues (Peters et al., 1995). A LP is required before TSWV transmission by the thrips vectors can occur, which length decreases with increasing temperatures (Wijkamp and Peters, 1993). Finally, an inoculation access period (IAP) to deliver TSWV virions into plant cells of 5 to 133 minutes have been reported and might depend on the feeding behavior of individual thrips (Peters et al., 1995; Wijkamp et al., 1996). However, virus inoculation by thrips occurs more readily during brief

probes involving little or no ingestion (Sakimura, 1962; Sakimura, 1963), as viruses require intact cells to initiate the infection process. TSWV particles are delivered to plants through the saliva of thrips (Hunter and Ullman, 1992; Ullman et al., 1997). Salivation is a major component of brief probes, while ingestion is not extensive or even null and plant cells are maintained intact during these shallow probes. van de Wetering and colleagues showed that female thrips produced more feeding scars (a cluster of dead cells emptied of their contents) than male thrips, while males transmitted TSWV with a higher efficiency than females (van de Wetering et al., 1998). Using electrical penetration graph (EPG), it has been recently shown that TSWV modifies the feeding behavior of its thrips vector as virus-infected males fed more than uninfected males and they made almost three times more noningestion probes (probes in which they salivate but left the cells undamaged) than their uninfected counterparts (Stafford et al., 2011).

TSWV possesses a dual tropism, since it replicates in both animal (thrips vectors) and plant cells (Kikkert et al., 1997; Kikkert et al., 2001). A successful acquisition, multiplication, dissemination, and transmission of TSWV by thrips involve the digestive system, the salivary glands, and different physiological barriers within these tissues (Fig. 1.5) (German et al., 1992; Moritz, 1997; Nagata et al., 1999; Tsuda et al., 1996; Ullman et al., 1992). TSWV transmission is dependent on virus uptake in the thrips midgut (mg), which is divided in three sections designated mg1, mg2, and mg3. Ullman and colleagues were able to show that TSWV envelope glycoproteins bind to the brush border plasmalemma of thrips using immunolabeling of serial sections from larval midguts (Ullman et al., 1995). Several reports suggest that the thrips midgut epithelial cells are indeed the initial site of TSWV entry and multiplication during virus acquisition (Nagata et al., 1999; Ullman et al., 1993; Ullman et al., 1995; Wijkamp et al., 1993). A time-course of infection by TSWV of its main thrips vector, *F. occidentalis*, was studied in detail by de Assis Filho *et al.*, who determined that the virus infection began in and is initially restricted to the epithelial cells of the mg1 (de Assis Filho et al., 2002). A dose-dependent process seems to regulate the virus accumulation in the midgut, where virus particles subsequently become available upon TSWV replication to further spread the viral infection to other tissues and organs (Nagata et al., 2000a). Once TSWV uptake and replication in the midgut epithelia takes place, it is followed by infection of the visceral and longitudinal muscle cells surrounding the mg1 region of the alimentary canal (Wijkamp et al., 1993). Subsequently,

TSWV moves laterally only through the visceral and longitudinal muscle cells from the mg1 into the mg2 and mg3 regions during the late larval stage (de Assis Filho et al., 2002). The viral infection can also be found in the foregut of late larval stage (Montero Astúa, 2012). The time-course of infection by TSWV in *F. occidentalis* identified that the cardiac valve (the junction between the foregut and midgut) and the ligaments are also infected (de Assis Filho et al., 2002). Recently, the infection of the tubular salivary glands by TSWV has also been observed (Montero Astúa and Whitfield, personal communication). Moreover, de Assis Filho and colleagues reported that the hindgut, pyloric valve (the junction between the midgut and the hindgut), and Malpighian tubules were never infected by TSWV (de Assis Filho et al., 2002).

Nagata *et al.* found that TSWV infection of various tissues and the potential for a thrips to become a transmitter is regulated by different barriers and processes related to thrips metamorphosis (Nagata et al., 1999). Adult thrips that acquire TSWV from an infected plant do not become viruliferous due to a midgut escape barrier (de Assis Filho et al., 2004; Ohnishi et al., 2001; Ullman et al., 1992). Thus, the midgut escape barrier seems to become increasingly effective as larval development proceeds and is complete in adult thrips (Nagata et al., 1999). This fact is in accordance with the hypothesis established by Harris in 1979, which explains that decreased gut permeability of vectors occurs with increased age of an insect, thus, reducing virus acquisition as an insect vector develops (Harris, 1979). In the TSWV-*F. occidentalis* pathosystem the dissemination of ingested virus particles through the midgut barriers is a significant component of vector competence (Bandla et al., 1998). The moment at which TSWV escapes from the midgut should be before pupation (Nagata et al., 2000a), when a drastic renewal of the midgut epithelium occurs and when the virus would be otherwise completely eliminated from the thrips vector (Moritz, 1997) as it has been shown for other arboviruses within their respective insect vectors (Hardy et al., 1983; Kaslow and and Welburn, 1997). TSWV is transtadially transmitted, which implies that virion particles are retained within certain internal organs and cells that are not lost during molting and pupation (Sakimura, 1962). However, comprehensive information about the specific changes that occur in thrips gut during development and molting is lacking.

Successful transmission of TSWV by its vector only occurs after the virus particles reach and infect the thrips principal salivary glands (Cho et al., 1988; Nagata et al., 1999; Sherwood et al., 2001; Tsuda et al., 1996; Wijkamp et al., 1993). Nagata and colleagues have proposed that

principal salivary gland invasion by TSWV occurs through the ligaments that connect the mg1 to the principal salivary glands (Nagata et al., 1999). The infection of the principal salivary glands occurred at 72 hours post acquisition of TSWV and it was simultaneously observed in the ligaments and the principal salivary glands (Nagata et al., 1999). Moreover, Montero Astúa and Whitfield (personal communication) observed that the tubular salivary glands, which connect the mg3 to the principal salivary glands, are also infected by TSWV right before the virus reached the principal salivary glands. TSWV has been visualized with transmission electron microscopy (TEM) budding across the apical salivary glands membrane into the lumen of the salivary ducts of viruliferous *F. occidentalis* (Ullman et al., 1991; Ullman et al., 1992), which must occur right before TSWV can be transmitted to a new plant host (Wijkamp et al., 1993). Importantly, a partial infection of the principal salivary glands by the virus is not sufficient for TSWV transmission by thrips (Nagata et al., 1999).

Three putative models for TSWV movement in thrips have been proposed. In the first one, the virus traverses the thrips midgut epithelial and muscle cells and once it reaches the hemocoel it circulates within the hemocytes to finally infect the salivary glands (Ullman et al., 1991). The second model is based on thrips ontogeny. In the first instar larvae the principal salivary glands and the visceral muscle cells of the midgut are in direct contact and are all compressed into one area of the thorax, while spatial separation occurs thereafter as thrips development proceeds to adulthood (Moritz et al., 2004). TSWV could then move from the midgut to the salivary glands when the midgut and principal salivary glands are in direct contact only (at the first and early second larval stage) but not once the principal salivary glands have moved forward into the cephalic case and are spatially separated from the midgut (during late second larval stage through adulthood). Both the tubular accessory salivary glands and thin ligament-like structures connect the midgut with the principal salivary glands. In the third and last model, these two anatomical structures form a bridge that could lead to virus spread from the initial place of virus uptake with the final place, which infection is a prerequisite for transmission to occur (Nagata et al., 1999; Nagata et al., 1999); Montero Astúa and Whitfield, personal communication).

TSWV is transmitted in a persistent propagative manner by *F. occidentalis*, in which the virus not only persists throughout the entire life span of its vector once it is acquired but it also replicates within tissues and organs of the insect host (Ullman et al., 1993; Wijkamp et al.,

1993). Two rounds of TSWV replication have been identified during virus circulation within the insect vector; the first and the second taking place in the midgut and principal salivary glands, respectively (Tsuda et al., 1996; Ullman et al., 1992; Ullman et al., 1993; Ullman et al., 1995; Wijkamp et al., 1993). It has been shown that TSWV accumulation occurs in the midgut, foregut and salivary glands only (Nagata et al., 1999). Changes in accumulation of TSWV N protein during developmental stages of thrips has also been shown (Tsuda et al., 1996; van de Wetering et al., 1996). Inoue *et al.* found that titers of N protein and virus transmission were higher and more efficient in *Frankliniella* species than in *Thrips* species, making the former more efficient vectors of TSWV than the later (Inoue et al., 2002). Similarly, it has been found that adult viruliferous *F. occidentalis* are more likely to transmit TSWV multiple times if it harbors a higher virus titer (Rotenberg et al., 2009). Despite the extensive replication of TSWV in *F. occidentalis* there is little to no pathogenic effect in this thrips species (Wijkamp et al., 1996). However, the molecular basis of the *F. occidentalis* response during TSWV infection is still unknown.

Responses of insects to viral infections

Unlike mammals, insects solely possess an innate immune system to cope with infections caused by nematode, fungal, bacterial, viral, and protozoan pathogens. Studies using the fruit fly model insect, *Drosophila melanogaster*, have shown that the innate immunity is a multilayered system that involves the production of antimicrobial peptides and reactive oxygen species (Meister et al., 1997; Ryu et al., 2008; Tzou et al., 2000), clotting and melanization (Scherfer et al., 2006; Sugumaran, 2002), phagocytic response (Agaisse et al., 2003; Dijkers and O'Farrell, 2007; Elrod-Erickson et al., 2000; Kocks et al., 2005), and RNA interference (RNAi) (Galiana-Arnoux et al., 2006; Wang et al., 2006)). Several lines of evidence have shown that most fungi and Gram positive bacteria activate the Toll pathway, while many Gram negative bacteria activate the immune deficiency (Imd) pathway (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 2003; Silverman and Maniatis, 2001; Tzou et al., 2002). However, the exact mechanism/s that insects use to fight viral infections of entomopathogenic viruses or arboviruses is not well understood.

Although knowledge about antiviral immunity in insects still in its infancy, major breakthroughs have been made in the last few years. For example, a lipase purified from the digestive juice of the silkworm, *Bombyx mori*, was shown to have antiviral activity against *Bombyx mori nucleopolyhedrovirus* (BmNPV; *Baculoviridae*: *Nucleopolyhedrovirus*) (Ponnuvel et al., 2003). This lipase was expressed in the midgut, specifically the anterior and middle portions, but not in other tissues such as the fat body, hemocytes, Malpighian tubules, silk gland, or trachea (Ponnuvel et al., 2003). Similarly, a serine protease from the digestive juice of the silkworm has antiviral activity against BmNPV (Nakazawa et al., 2004). Moreover, larval hemolymph from *Heliothis virescens* has been shown to possess antiviral activity against *Helicoverpa zea single nucleopolyhedrovirus* (HzSNPV; *Baculoviridae*: *Nucleopolyhedrovirus*) *in vitro* (Popham et al., 2004). This virucidal activity is specifically caused by hemolymph phenoloxidases as shown by complete blockage of its antiviral properties when treated with phenoloxidase inhibitors (Shelby and Popham, 2006). Sim and colleagues found that the heat shock protein cognate 70B (HSC70B) was up-regulated during *O'nyong-nyong virus* (ONNV; *Togaviridae*: *Alphavirus*) infection of the African malaria mosquito, *Anopheles gambiae* (Sim et al., 2005). Suppresion of HSC70B using RNAi showed that this protein suppresses ONNV replication in the mosquito vector (Sim et al., 2007).

Baculovirus replication in insect cells initiates apoptosis (Clem et al., 1991) while viral proteins such as P49 is able to prevent the apoptotic response by inhibiting an initiator caspase (Zoog et al., 2002). It has also been reported that cells of *Helicoverpa zea* larvae infected by *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV; *Baculoviridae*: *Nucleopolyhedrovirus*) are encapsulated by hemocytes and finally cleared (Washburn et al., 1996). Autophagy, a nonapoptotic form of programmed cell death that involves the bulk degradation of cellular cytoplasmic contents has been shown to play an antiviral role against mammalian and plant viruses (Seay et al., 2005). Later it was determined that autophagy plays a direct antiviral role against *Vesicular stomatitis virus* (VSV; *Rhabdoviridae*: *Vesiculovirus*) in *Drosophila melanogaster*, which decreased viral replication most probably triggered by the recognition of the VSV viral glycoprotein (Shelley et al., 2009).

Functional analysis of H4 and N5 genes from *Microplitis demolitor virus* (MdBV; *Polydnaviridae*: *Bracovirus*), which is a polydnavirus symbiotically associated with the parasitoid wasp *Microplitis demolitor*, showed a role of these proteins as inhibitors of NF-κB

that suppress the Toll and Imd pathways in the parasitized larval moth (Thoetkiattikul et al., 2005). Furthermore, MdBV encodes a protein, Egf1.0, which inhibits the phenoloxidase (PO) cascade resulting in a disabled melanization response (Beck and Strand, 2007). It has been shown that the serine protease inhibitor Egf1.0 suppresses the PO cascade by two different mechanisms, inhibiting the activity of prophenoloxidase activating proteinase 1 and 3 and by preventing processing of serine proteinase homologue 1 and 2 (Lu et al., 2008). Moreover, the protein tyrosine phosphatase 2 (PTP-2) from MdBV causes the granulocytes of the fall armyworm, *Spodoptera frugiperda*, to apoptose (Suderman et al., 2008). Thus, MdBV seems to have several and efficient mechanisms to suppress the antiviral defense responses of its insect host to ensure not only its own survival but also the survival of the larvae of the parasitoid wasp that it is symbiotically associated with.

Using a DNA microarray analysis to compare the midgut global gene expression profiles between mosquitoes fed on *Sindbis virus* (SINV; *Togaviridae*: *Alphavirus*) infected blood and mosquitoes fed on non-infected blood revealed that the most dramatic changes involved 3 genes that had a 25- to 40-fold change increase in challenged mosquitoes; these genes were synaptic vesicle protein-2, potassium-dependent sodium calcium exchanger, and a homologue of *Caenorhabditis elegans* Unc-93, a putative component of a two-pore potassium channel, suggesting that SINV infection of the yellow fever mosquito, *Aedes aegypti*, alters the transport processes in the insect vector (Sanders et al., 2005). Furthermore, changes in transcript expression were observed in infected mosquitoes that also suggested an involvement of the Toll and Janus kinase (JNK) pathways as a complex response to viral infection (Sanders et al., 2005). In a similar study, transcripts of a putative RNA editase (CsRED1), a protein tyrosine phosphatase (CsLAR), a homologue to the *wingless* receptor in the fruit fly (CsFZ2), and putative translation machinery components (CseIF3, CseIF5A, and CsRPS6) were found to be more abundant in midguts from *Epizootic hemorrhagic fever orbivirus*-fed biting midges (*Culicoides sonorensis*) than in their non-exposed counterparts (Campbell and Wilson, 2002). Moreover, subtractive cDNA libraries and macroarrays identified transcripts involved in pathogen recognition (lectins) and those encoding antimicrobial peptides (defensin and cecropin), receptors that activate the innate immune response (Toll-3), and members of the signal transduction pathways (JNK kinase) to be up-regulated in the western flower thrips larvae, *F. occidentalis*, infected by TSWV (Medeiros et al., 2004). The transcriptional up-regulation of

these genes after viral infection was confirmed by Northern blot analysis (Medeiros et al., 2004). Transcripts involved in the RNAi pathway were not detected by Medeiros *et al.* despite the fact that they are known to be expressed in *F. occidentalis* larvae (Rotenberg and Whitfield, 2010).

Infection of *Aedes albopictus*-derived mosquito cells by *Semliki Forest virus* (SFV; *Togaviridae*: *Alphavirus*) significantly reduced cellular gene expression (Fragkoudis et al., 2008). Furthermore, activation of Imd and STAT pathways prior to SFV infection reduced virus gene expression and RNA levels, but SFV did not activate either of these two pathways (Fragkoudis et al., 2008). In contrast, *Drosophila C virus* (DCV; *Dicistroviridae*: *Cripavirus*) activates the Jak/STAT signaling pathway in *Drosophila* (Dostert et al., 2005). However, the activation of this pathway is required but not sufficient to trigger a complete antiviral response in the fruit fly (Dostert et al., 2005). The Vago gene from *Culex quinquefasciatus* is up-regulated during West Nile virus (WNV; *Flaviviridae*: *Flavivirus*) infection and restricts the viral infection via activation of the Jak/STAT pathway (Paradkar et al., 2012). The Toll pathway was also found to play a role as an antiviral response in *D. melanogaster* against *Drosophila X virus* (DXV: *Birnaviridae: Entomobirnavirus*) (Zambon et al., 2005) and in *Aedes aegypti* against Dengue virus (DENV; *Flaviviridae*: *Flavivirus*) (Xi et al., 2008).

RNA interference (RNAi) was found to be an antiviral mechanism used by *Drosophila* and *Aedes* to impair DXV (Zambon et al., 2006) and DENV (Franz et al., 2006) infection, respectively. Viral titers of ONNV were significantly reduced when *Anopheles gambiae* mosquitoes were injected with double-stranded RNA (dsRNA) specific to the viral nonstructural protein P3 (Keene et al., 2004). Silencing of argonaute 2 (AGO2), which is involved in the RNAi pathway, results in higher viral titers and faster spread of ONNV in *A. gambiae* than in mosquitoes injected with a beta-galactosidase dsRNA control (Keene et al., 2004). Interestingly, *Flock house virus* (FHV; *Nodaviridae*: *Alphanodavirus*) is able to induce and suppress RNAi in *Drosophila* S2 cells and the viral protein B2 acts specifically as a silencing suppressor in transgenic plants expressing GFP (Li et al., 2002). Expression of the full-length premembrane coding region of DENV-2 from double-stranded SINV in *A. albopictus* cells conferred complete resistance to DENV-2 challenge (Gaines et al., 1996), showing that pathogen-derived resistance in insects can be used as a strategy to control arbovirus transmission. Later *A. aegypti* injected intrathoracically with double-stranded SINV containing DENV sequences from the four serotypes were highly resistant to challenge with DENV from which the effector sequences were

derived (Adelman et al., 2001), suggesting that pathogen-derived resistance is also functional in whole insects. Moreover, RNAi confers an antiviral defense against the negative-sense RNA VSV in *Drosophila* as shown by the high susceptibility of the RNAi-defective mutant flies to the virus (Mueller et al., 2010). Initially it was thought that RNAi in the model insect, *Drosophila melanogaster*, mediates antiviral immunity only locally (Roignant et al., 2003). However, subsequent studies have shown that following a local response, an RNAi signal spreads systemically through the insect's body to confer antiviral immunity in distal tissues within the fruit fly (Saleh et al., 2009). These authors proposed a model to explain the systemic spread of an RNAi signal in *Drosophila* in which viral infection causing cell death or lysis will free dsRNA into the hemocoel that can be taken up and processed by distal uninfected cells to protect them from viral infection. How the RNAi signal spread systemically in an insect during a viral infection that does not cause cell death and lysis remains an open question.

More than 75% of the plant viruses recognized by the International Committee on Taxonomy of Viruses (ICTV) are transmitted by insect vectors (Hogenhout et al., 2008). However, only a handful of studies have started to work towards elucidating the molecular mechanisms of these intricate and complex virus-vector interactions. For example, a massive parallel pyrosequencing-based transcriptome analysis of the small brown planthopper, *Laodelphax striatellus*, revealed that a number of transcripts were up-regulated in insects infected with *Rice stripe virus* (RSV; *Tenuivirus*) compared to naïve ones (Zhang et al., 2010). However, there was no difference in transcripts abundance of RNAi components between RSV infected and non-infected planthoppers (Zhang et al., 2010). A transcriptomic analysis between non-infected and infected whiteflies showed that *Tomato yellow leaf curl China virus* (TYLCCV; *Geminiviridae*: *Begomovirus*) circulation and persistence in its insect vector caused changes in the cell cycle and primary metabolism (Luan et al., 2011). TYLCCV infection of the whitefly vector also activated immune responses such as autophagy and antimicrobial peptide production but suppressed the expression of genes involved in the Toll signaling pathway and the mitogen-activated protein kinase (MAPK) pathway (Luan et al., 2011). Similarly, a global analysis of the transcriptional response of the white-backed planthopper, *Sogatella furcifera*, revealed that the *Southern rice black-streaked dwarf virus* (SRBSDV; *Reoviridae*: *Fijivirus*) persistent and propagative infection of the insect vector perturbs cellular processes, primary metabolism, the ubiquitin-proteasome pathway, and cell cytoskeleton organization while

activates genes involved in autophagy, antimicrobial peptide production, and RNAi (Xu et al., 2012). Interestingly, 58% of the differentially expressed transcripts were up-regulated by SRBSDV infection (Xu et al., 2012). The up- and down-regulation of specific sets of genes in different virus-vector combinations clearly suggests the relationship of coevolved adaptations that ensure the survival of the insect vector and the transmission of the plant virus in specific interactions. A study using a proteomics approach comparing vector and non-vector aphids with F2 populations found that luciferase and cyclophilin might be potentially involved in luteovirus transcytosis through different membranes as they were only identified in aphid groups that transmit luteoviruses but not in those that are unable to transmit them (Yang et al., 2008b). Moreover, a similar study identified a serine protease inhibitor (SERPIN-4) to be up-regulated in transmission competent aphids while a putative proteasome subunit beta 7 was down-regulated in these same group (Cilia et al., 2011). Thus, transcriptomics and/or proteomics approaches can be used to further study and understand the interactions and responses of insects to plant viruses that replicate in their vectors.

In my doctoral dissertation I have used proteomic approaches coupled with computational searches to advance our understanding of the TSWV-*F. occidentalis* interaction. Identification of thrips molecules that respond to the virus infection of the insect vector as well as those that directly interact with TSWV provides new insights on the molecular basis of this interaction and new targets to design control strategies to manage this agriculturally important pathosystem. Furthermore, the RNA interference tools developed here to study the function of vATPase-B in adult female thrips represent the first proof of concept that this cellular mechanism is operational in *F. occidentalis* and show that it has the potential to be translated into field and greenhouse tools on the war against thrips and tospoviruses.

Figures and Tables

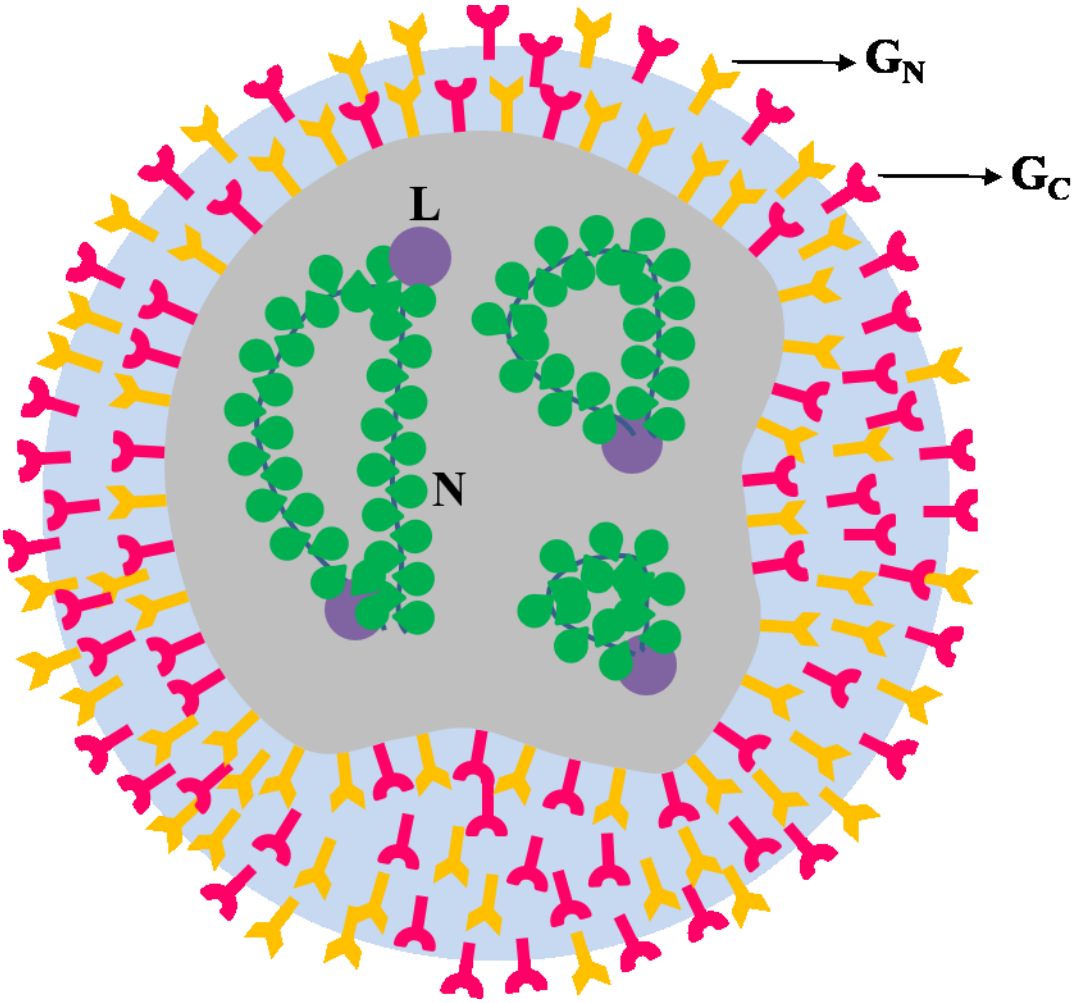

Figure 1.1 TSWV virion.

The TSWV virion has a host Golgi-derived membrane (blue) in which two viral glycoproteins are inserted. The glycoproteins G_N (yellow) and G_C (fuchsia), the nucleocapsid (green), and the RNA-dependent RNA-polymerase (purple) are the four structural proteins that make up the virus particle. Three RNA molecules designated small (S), medium (M), and large (L) are contained in the interior of the virion. The RNAs are coated by nucleocapsid (N) protein, which forms the ribonucleoprotein (RNP) complexes. A few copies (10-20) of the RNA-dependent RNA-polymerase (L) protein, which is the viral polymerase, are also present in each particle.

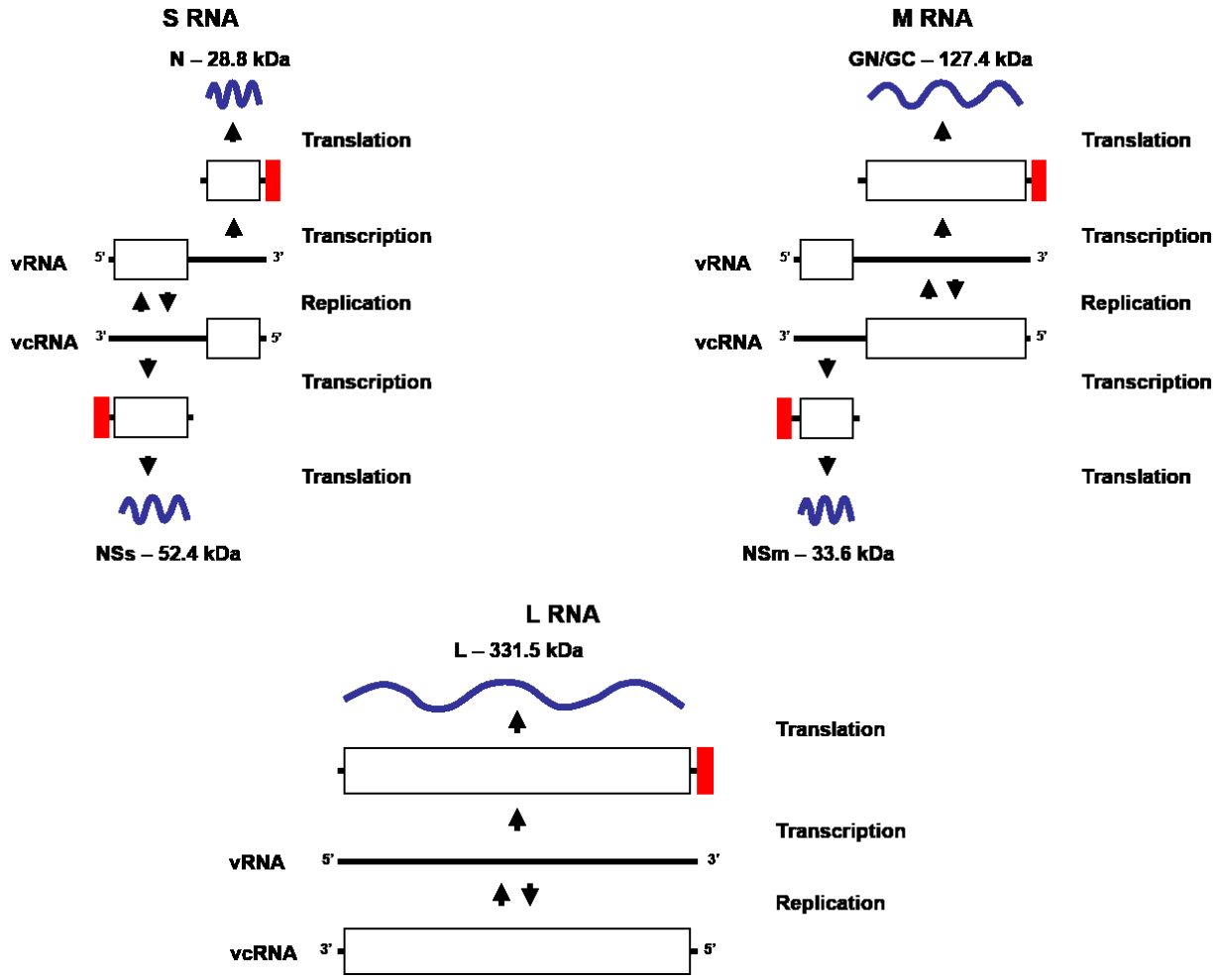

Figure 1.2 TSWV genomic organization and coding strategy.

The S and M RNA segments possess an ambisense coding strategy to express two viral genes from each segment. The S RNA encodes a nonstructural small (NSs) protein from the viral complementary (vc) sense and the nucleocapsid (N) protein from the viral (v) sense as subgenomic RNAs (sgRNAs). The glycoprotein (G_N/G_C) precursor is encoded from the v sense, while a nonstructural medium (NSm) protein is encoded from the vc sense from the M RNA using sgRNAs as well. The L RNA is of complete negative polarity. The RNA-dependent RNA-polymerase (L) protein is the only protein encoded from messenger RNA (mRNA) from the L RNA. All TSWV nonstructural and structural proteins are encoded in the v and vc sense, respectively. The red rectangles represent host-derived 5' caps, the open boxes indicate the open reading frames (ORFs), and the blue flexuous lines represent the expressed viral proteins.

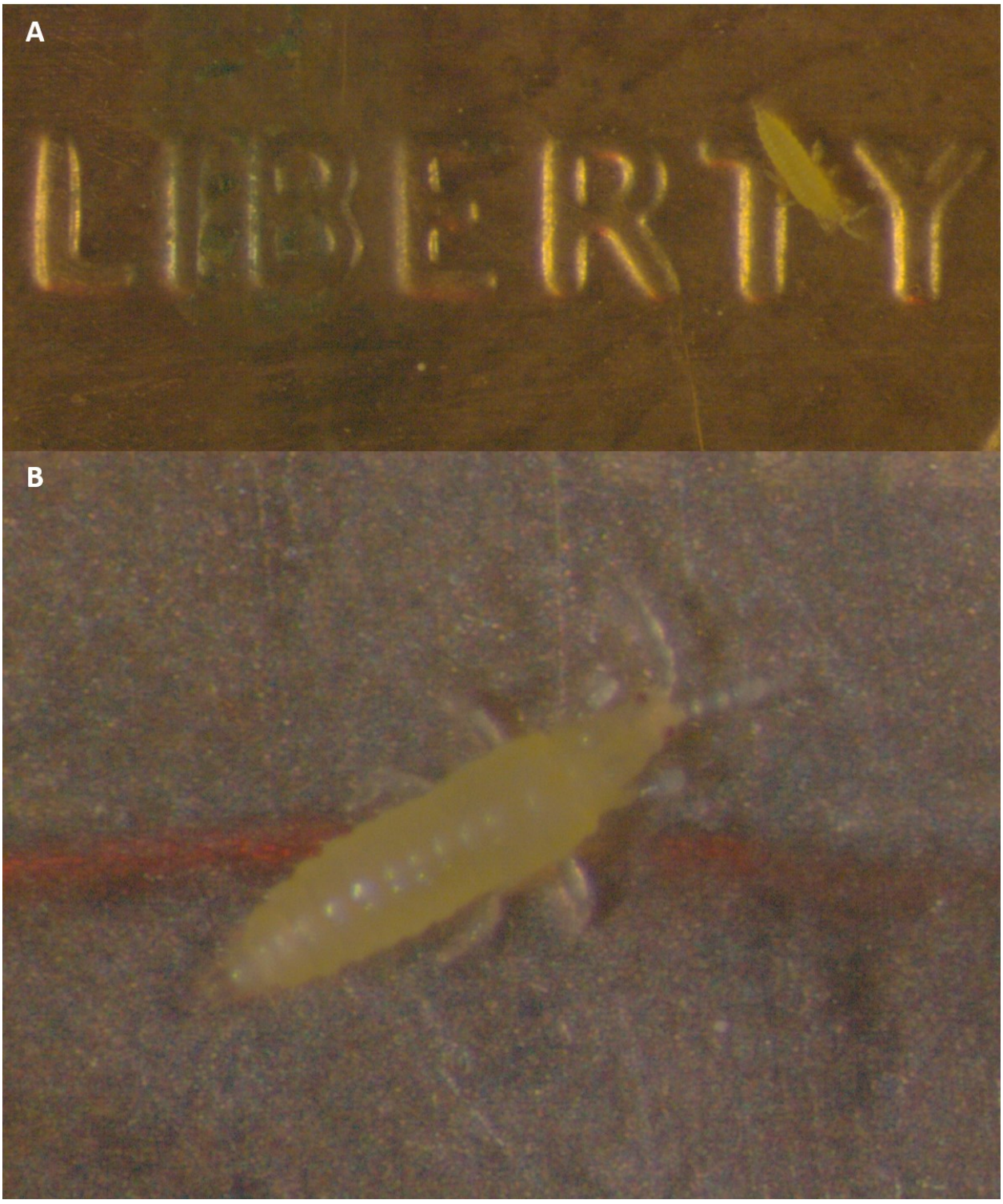

Figure 1.3 *Frankliniella occidentalis* first instar larvae.

First instar larvae of *Frankliniella occidentalis* on the word "LIBERTY" of a penny for size reference (A) and a close up of the insect (B).

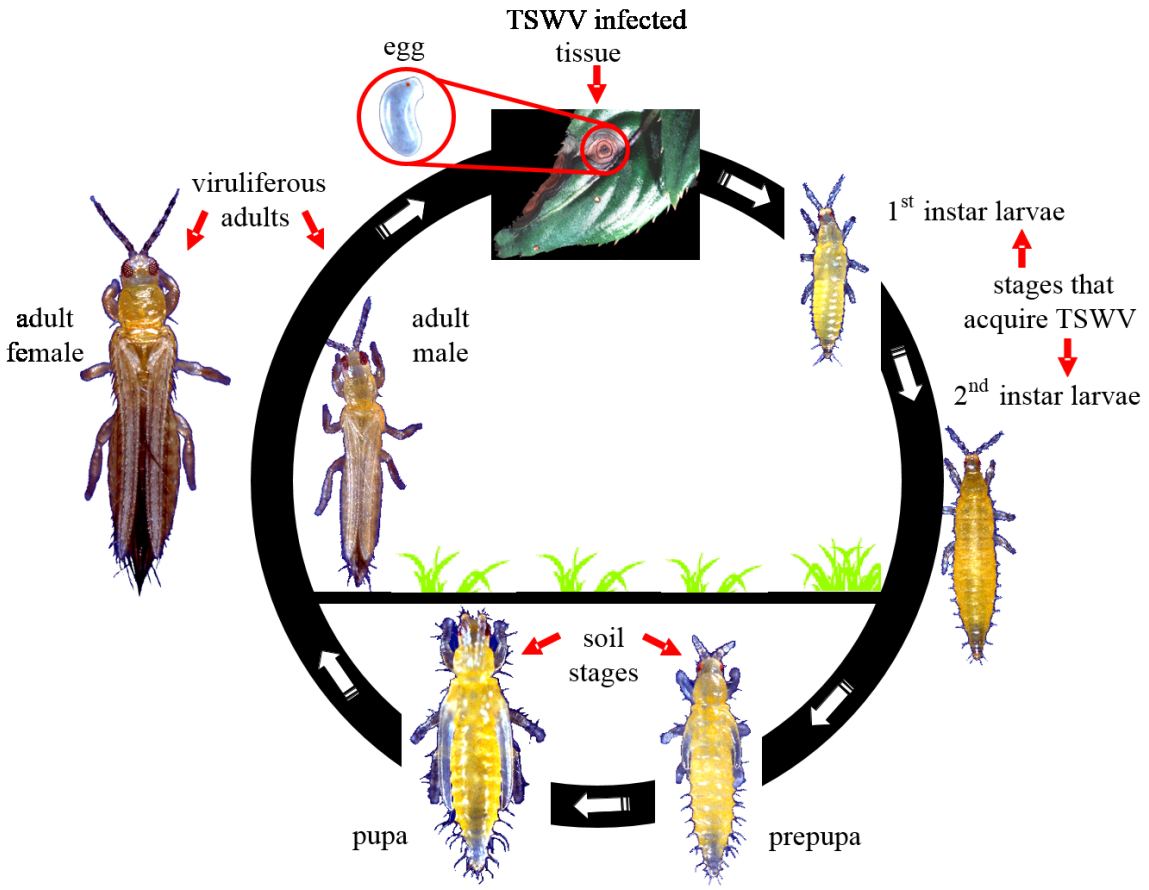

Figure 1.4 TSWV transmission cycle by *Frankliniella occidentalis*.

Adult females lay their eggs in TSWV-infected plant tissue. In a few days the 1st instar larvae hatch from the eggs, which is also the most efficient stage to acquire the virus. The 2nd instar larvae can also acquire the virus but at a lower frequency. The pupal stages (prepupa and pupa), which usually occurs in the soil, are non-feeding and sedentary. When the adult male and female thrips emerge from the pupal stages, they feed and transmit the virus efficiently during brief probes.

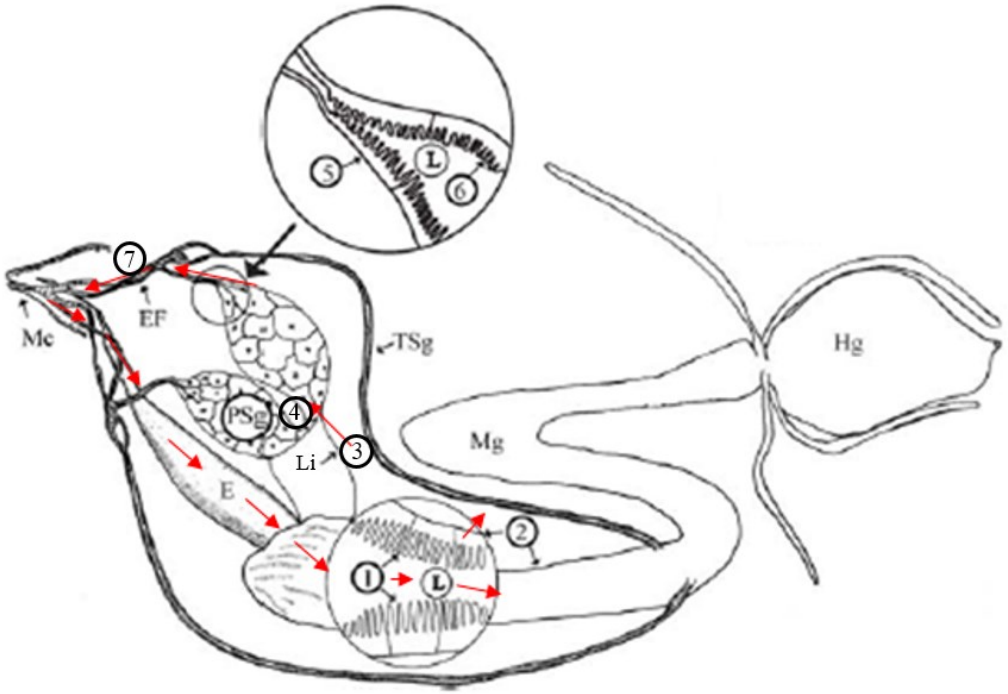

Figure 1.5 Internal morphology of thrips organs and putative route of TSWV dissemination within *Frankliniella occidentalis*. The virus particles enter the body of the thrips vector through the mouthcone (Mc) during feeding and travel to the esophagus (E) until they reach the lumen (L) of the midgut (Mg). TSWV enters the Mg epithelia through the apical membrane of the brush border (1). Virus replication takes place in Mg epithelial cells and then moves into the visceral and longitudinal muscle cells of the Mg where it continues replicating (2). The virus crosses the basal lamina by an unknown mechanism and must exit those cells in order to infect the ligaments (Li) and tubular salivary glands (TSg) (3) and finally the principal salivary glands (PSg) (4). After a second round of replication in the PSg (5), the virus must exit through the apical membrane into the lumen of the PSg (6) and mixed with salivary secretions within the efferent salivary ducts (EF) (7), which is produced and released during thrips feeding on plants. Modified from Whitfield et al., 2005a.

Table 1.1 Tospovirus species (approved and tentative).

Approved *Tospovirus* species	Acronym	Reference
Groundnut bud necrosis virus	GBNV	(Reddy et al., 1968)
Groundnut ringspot virus	GRSV	(DEAVILA et al., 1993)
Groundnut yellow spot virus	GYSV	(Satyanarayana et al., 1996)
Impatiens necrotic spot virus	INSV	(DEAVILA et al., 1992)
Tomato chlorotic spot virus	TCSV	(DEAVILA et al., 1990)
Tomato spotted wilt virus	TSWV	(Samuel et al., 1930)
Watermelon silver mottle virus	WSMV	(Yeh and Chang, 1995)
Zucchini lethal chlorosis virus	ZLCV	(Rezende et al., 1997)

Tentative *Tospovirus* species	Acronym	Reference
Alstroemeria necrotic streak virus	ANSV	(Hassani-Mehraban et al., 2010)
Bean necrotic mosaic virus	BNMV	(de Oliveira et al., 2011)
Calla lily chlorotic spot virus	CCSV	(Chen et al., 2005)
Capsicum chlorosis virus	CaCV	(McMichael et al., 2002)
Chrysanthemum stem necrosis virus	CSNV	(Duarte et al., 1995)
Hippeastrum chlorotic ringspot virus	HCRV	(Dong et al., 2013)
Groundnut chlorotic fan-spot virus	GCFV	(Chu et al., 2001)
Groundnut ringspot virus/Tomato chlorotic spot virus reassortant	GRSV/TCSV	(Webster et al., 2011)
Iris yellow spot virus	IYSV	(Cortes et al., 1998)
Melon severe mosaic virus	MeSMV	(Ciuffo et al., 2009)

Melon yellow spot virus	MYSV	(Kato et al., 2000)
Mulberry vein banding virus	MuVBV	(Meng et al., 2013)
Pepper necrotic spot virus	PNSV	(Torres et al., 2012)
Physalis severe mottle virus	PhySMV	(Cortez et al., 2001)
Polygonum ringspot virus	PolRSV	(Ciuffo et al., 2008)
Soybean vein necrosis virus	SVNV	(Zhou et al., 2011)
Tomato necrosis virus	TNeV	(reported in Plyusnin et al., 2012)
Tomato necrotic ringspot virus	TNRV	(Seepiban et al., 2011)
Tomato fruit yellow ring virus	TYRV	(Winter et al., 2006)
Tomato zonate spot virus	TZSV	(Dong et al., 2008)
Watermelon bud necrosis virus	WBNV	(Jain et al., 1998)

Table 1.2 Thrips vectors of tospoviruses.

Thrips vectors	Common name	*Tospovirus* species	Reference
Ceratothripoides claratis	Oriental tomato thrips	Capsicum chlorosis virus*	(Premachandra et al., 2005)
Dictyothrips betae		Polygonum ring spot virus*	(Ciuffo et al., 2010)
Frankliniella bispinosa	Florida flower thrips±	*Tomato spotted wilt virus*	(Avila et al., 2006)
Frankliniella cephalica	Florida flower thrips±	*Tomato spotted wilt virus*	(Ohnishi et al., 2006)
Frankliniella fusca	tobacco thrips	*Impatiens necrotic spot virus*	(Naidu et al., 2001)
		Tomato spotted wilt virus	(Sakimura, 1963)
Frankliniella gemina		Chrysanthemum stem necrosis virus*	(reviewed in Ullman et al., 1997)
		Groundnut ringspot virus	(de Borbon et al., 1999)
		Tomato spotted wilt virus	(de Borbon et al., 1999)
Frankliniella intonsa	European flower thrips	*Groundnut ringspot virus*	(Wijkamp et al., 1995)
		Impatiens necrotic spot virus	(Sakurai et al., 2004)
		Tomato chlorotic spot virus	(Wijkamp et al., 1995)
		Tomato spotted wilt virus	(Wijkamp et al., 1995)
Frankliniella occidentalis	western flower thrips	Chrysanthemum stem necrosis virus*	(Nagata and de Avila, 2000)
		Groundnut ringspot virus	(Wijkamp et al., 1995)
		Impatiens necrotic spot virus	(DeAngelis et al., 1993)
		Tomato chlorotic spot virus	(Nagata et al., 2004)
		Tomato spotted wilt virus	(Wijkamp et al., 1995)
Frankliniella schultzei	common blossom thrips±, tomato thrips±	Chrysanthemum stem necrosis virus*	(Nagata and de Avila, 2000)
		Groundnut bud necrosis virus	(Meena et al., 2005)

		Groundnut ringspot virus	(Wijkamp et al., 1995)
		Impatiens necrotic spot virus	(reviewed in Ullman et al., 1997)
		Tomato chlorotic spot virus	(Wijkamp et al., 1995)
		Tomato spotted wilt virus	(Sakimura, 1969)
Frankliniella zucchini		*Zucchini lethal chlorosis virus*	(Nakahara and Monteiro, 1999)
Scirtothrips dorsalis	chilli thrips[±], yellow tea thrips[±]	*Groundnut bud necrosis virus*	(Meena et al., 2005)
		Groundnut chlorotic fan-spot virus*	(Chen et al., 1996)
		Groundnut yellow spot virus	(Gopal et al., 2010)
Thrips palmi	melon thrips	Calla lily chlorotic spot virus*	(Chen et al., 2005)
		Capsicum chlorosis virus	(reviewed in Ullman et al., 1997)
		Groundnut bud necrosis virus	(Reddy et al., 1992)
		Melon yellow spot virus*	(Kato et al., 2000)
		Watermelon bud necrosis virus*	(reviewed in Ullman et al., 1997)
		Watermelon silver mottle virus	(Iwaki et al., 1984)
Thrips setosus	light-brown soybean thrips[±], Japanese flower thrips[±]	*Tomato spotted wilt virus*	(Tsuda et al., 1996)
Thrips tabaci	onion thrips	Iris yellow spot virus*	(Cortes et al., 1998)
		Tomato spotted wilt virus	(Wijkamp et al., 1995)
		Tomato fruit yellow ring virus*	(Golnaraghi et al., 2007)

Virus names in italics indicate *Tospovirus* species approved by the International Committee for the Taxonomy of Viruses.
* = tentative *Tospovirus* species
[±] = thrips common name is not recognized by the Entomological Society of America

Chapter 2 - Proteomic analysis of *Frankliniella occidentalis* and differentially expressed proteins in response to *Tomato spotted wilt virus* infection

Abstract

Tomato spotted wilt virus (TSWV) is transmitted by *Frankliniella occidentalis* in a persistent propagative manner. Despite the extensive replication of TSWV in thrips' midgut and salivary glands, there is little to no pathogenic effect on *F. occidentalis*. I hypothesize that the first instar larva (L1) of *F. occidentalis* mounts a response to TSWV that protects it from pathogenic effects caused by virus infection and replication in various insect tissues. Using a partial thrips transcriptome previously obtained by Sanger sequencing and 454-Titanium sequencing of cDNA generated from *F. occidentalis* non-exposed and exposed to TSWV, respectively, the L1 thrips proteome that resolved on a two-dimensional (2-D) gel was characterized. Forty seven percent of the resolved protein spots were identified using the thrips transcriptome, while only 23% were identified using the Metazoan non-redundant (nr) protein database from NCBI. I then compared the proteomes of TSWV-infected and non-infected L1 to identify proteins that display differential abundances in response to virus. Using four biological replicates, 26 spots containing 37 proteins were significantly altered in response to TSWV. Gene ontology (GO) assignments for 32 of these proteins revealed biological roles associated with the infection cycle of other plant- and animal-infecting viruses and antiviral defense responses. These findings support the hypothesis that the *F. occidentalis* L1 display a complex reaction to TSWV infection and provide new insights towards unraveling the molecular basis of this interaction.

Introduction

Insects and other arthropods, such as ticks and mites, are the primary vectors of several animal- and plant-infecting viruses. These vector-borne viruses rely on their invertebrate vector

to be disseminated and to infect their primary animal or plant host. Both animal- and plant-infecting viruses have evolved different transmission strategies in that some attach to the cuticle lining of the mouthparts of the arthropod while others traverse different tissues of the vector's body, in which case virus replication within the invertebrate host may or may not occur. Usually, these arthropod-virus interactions are characterized by some degree of specificity. The cellular mechanisms that determine vector specificity and virus transmission are governed by the interactions between viral components and unknown molecules from the arthropod vector. In contrast to the extensive knowledge of the functions of viral genes and the biological aspects of these interactions, very little is known about the molecular responses that arthropod vectors deploy during viral replication in different tissues within their body.

Viruses in the genus *Tospovirus*, the only plant-infecting members of the virus family *Bunyaviridae*, are transmitted exclusively by insects in the order Thysanoptera. Insects within this order (from the Greek thysanos and pteron that mean fringe and wing, respectively) are extremely small, slender insects with fringed wings, commonly known as thrips. The order, which belongs to the Hemipteroid assemblage, is composed of two suborders, the Tubulifera, with a single family, and the Terebrantia with eight families (Moritz et al., 2001). Thripidae is the largest family within the suborder Terebrantia, from which about 50 species are insect pests that cause damage to agricultural crops and to which all vectors of tospoviruses belong (Mound, 1996). Thysanopterans display a great diversity with respect to morphological structures, food preference, and behavioral characteristics. However, they share some common but unique characteristics, such as a postembryonic remetaboly development (Heming, 1973), a hapodiploid genetic system (reviewed in Crespi, 1992), and asymmetrical mouthparts that form a narrow stylet (Heming, 1980). Moreover, most vector species share several behavioral characteristics, such as high locomotory activity, high fecundity combined with a short generation time, a strongly female-biased sex ratio, a preference for concealed spaces, a wide range of host plants, and the habit of piercing and sucking from epidermal and mesophyll plant cells (Moritz et al., 2004). Interestingly, of the 5,500 described species of Thysanoptera (Mound, 2002) only 14 are known vectors of tospoviruses, indicative of the specificity of these virus-vector interactions.

The western flower thrips, *Frankliniella occidentalis* Pergande, is the most economically important insect pest among thysanopterans due to its extremely wide host range, broad geographical distribution, and competence to transmit 5 of the 17 recognized *Tospovirus* species.

The polyphagous nature of *F. occidentalis* combined with its short reproductive cycle (~12 days, egg to adult) and high fecundity (~75 eggs per female) have contributed to the success of this insect pest as an invasive species. For decades, the use of insecticides has been the primary strategy for controlling this insect pest. However, several *F. occidentalis* populations from different geographical areas have developed insecticide resistance (Bielza, 2008; López-Soler et al., 2008; Robb et al., 1995). Additionally, *F. occidentalis* displays thigmotactic behavior, whereby the insect prefers concealed spaces on or in plant organs, protecting it from harsh conditions. For all the aforementioned reasons, effective control of *F. occidentalis* is difficult to achieve using the current available strategies (Morse and Hoddle, 2006).

Tospoviruses are segmented, single-stranded, ambisense RNA viruses enclosed in a host-derived membrane. *Tomato spotted wilt virus* (TSWV) is the best-characterized tospovirus with respect to interactions with its most efficient vector, *F. occidentalis*. TSWV is transmitted in a persistent propagative fashion in which it journeys through the thrips' body and replicates in various organs of the insect vector. Acquisition of TSWV by thrips is restricted to the first and early second larval stages, and acquisition efficiency decreases as development proceeds. The virus persists through the prepupal and pupal life stages and adulthood. Once a virion is acquired from an infected plant by a larval thrips, the virus enters into and replicates in the midgut epithelial cells and then moves to the muscle cells of the gut. Subsequently, the virus infects and replicates in the salivary glands. Both adult males and females will transmit the virus to a permissive plant host during nondestructive, brief probes.

During the early steps of virus infection of larval thrips, the virus particles most likely interact with proteins in the midgut lumen of the thrips vector to initiate the infection process. The virus replicates in the midgut epithelial cells, where it likely uses host cellular components to complete its replication cycle. Escape from the midgut involves traversing the basal lamina to spread to other organs of the insect. Although TSWV replicates in its thrips vector, pathological effects have not been observed in *F. occidentalis* (Wijkamp et al., 1996), resulting in efficient transmission of TSWV by this thrips species. One possible explanation for the apparent lack of pathology of the insect vector is that the controlled expression of immune-related proteins by thrips successfully modulates the virus titer, as suggested or demonstrated for arthropod vectors of animal-infecting viruses (Myles et al., 2008; Sánchez-Vargas et al., 2009). The tight regulation of proteins involved in other biological processes, such as metabolism, development,

growth and reproduction, membrane and protein trafficking, reduction/oxidation (redox), and homeostasis may also counterbalance some of the negative effects that virus infection might otherwise have in the vector.

Unlike mammals, insects solely possess an innate immune system to cope with infections caused by nematode, fungal, bacterial, viral, and protozoan pathogens. It has been shown in *Drosophila melanogaster* and other insects that the innate immunity is a multilayered system that involves production of antimicrobial peptides and reactive oxygen species (Ha et al., 2009; Meister et al., 1997; Tzou et al., 2000)), clotting and melanization (Agaisse et al., 2003; Scherfer et al., 2006), encapsulation and phagocytosis (Kocks et al., 2005; Washburn et al., 1996), apoptosis and autophagy (Clem et al., 1991; Seay et al., 2005; Shelly et al., 2009), and RNA interference (RNAi) (Galiana-Arnoux et al., 2006; Wang et al., 2006). A better understanding of the molecular mechanisms that insects use to defend themselves against entomopathogenic and vector-borne viruses will enable the design of novel strategies to control these agriculturally and clinically important viruses and their vectors.

I hypothesize that the first instar larva (L1) of *F. occidentalis* mounts a response during TSWV infection of various tissues and organs of the vector that protects the insect from pathogenic effects while allowing viral replication and spread to take place through the thrips' body for efficient virus transmission to occur. To begin to address the mechanisms underlying vector competency and lack of pathology in the TSWV-thrips interaction, I used the recently generated transcriptomic resources for *F. occidentalis* to identify proteins in healthy and TSWV-infected larval thrips. Two-dimensional (2-D) gel electrophoresis and matrix-assisted laser desorption ionization – tandem time of flight (MALDI-TOF/TOF) mass spectrometry coupled with computational analysis allowed me to identify 52% of the resolved proteins from healthy thrips, from which a higher proportion (47%) matched the *F. occidentalis* transcriptome collection. Fifteen proteins from naïve thrips were functionally annotated as being part of the cell killing or immune system processes. The proteomic tools generated in this study were then used to study *F. occidentalis* larva and its interaction with TSWV. I conducted an analysis of differentially expressed proteins between L1s of *F. occidentalis* that were exposed or not exposed to TSWV-infected leaves and harvested 24 hr following a 3-hr acquisition access period (AAP). Overall, I found that the newly-generated *F. occidentalis* transcript sequence collection (*Fo* Seq) greatly improved the identification of thrips proteins over the existing arthropod

sequences available and that TSWV infection resulted in differential expression of proteins involved in primary metabolic and cellular processes, as well as defense-related proteins of significance to a persistent propagative plant- and animal-infecting virus.

Results

Characterization of the larval thrips proteome

Using 400 µg of total protein extracted from 600 healthy L1s of *F. occidentalis*, I clearly resolved 194 protein spots in a 2-D gel with a pH range of 3 to 10 (Fig. 2.1). MALDI-TOF/TOF mass spectrometry coupled with computational analysis using the *Fo* Seq and the Metazoan nr protein database from NCBI allowed me to identify 52% of the 194 resolved protein spots (Table 2.1). The distribution of identified protein spots reflected that a higher proportion of peptides (47%) matched the *Fo* Seq with a considerably lower proportion of peptides (23%) matching sequences from the Metazoan database (Fig. 2.2). Out of the 92 and 45 proteins spots identified with the *Fo* Seq and the Metazoan database, respectively, 36 protein spots (19%) were identified with both databases. Fifty six proteins (29%) were exclusively identified with the *Fo* Seq, while nine (5%) were identified with the Metazoan database only. It is worth mentioning that from the nine protein spots that were identified with the Metazoan database, two corresponded to cysteine proteases from *F. occidentalis* that were not represented in the subject database. Furthermore, there were 9 and 13 protein spots comprised of two different proteins as determined by comparison to the *Fo* Seq and the Metazoan database sequences, respectively (Table 2.1). Eighty protein spots (41%) had identities but no significant matches and thirteen spots (7%) were not identified with either of the databases searched (Fig. 2.2). Searches using the Prokaryote nr protein database from NCBI yielded 27 protein spots (14%) with identities, however, these particular matches had less significant Mascot scores than those obtained with the *Fo* Seq and Metazoan sequences. As such, we consider these proteins to be of insect origin and/or highly conserved across kingdoms.

The subset of *Fo* Seq and Metazoan sequences that matched thrips larval proteins were subjected to BLASTp analysis to identify matches to protein sequences available at NCBI (Fig. 2.3). The majority of these *Fo* Seq and Metazoan sequences matched proteins of other insect species. Protein sequences of the red flour beetle, *Tribolium castaneum*, scored the highest and

produced the greatest number of matches to the larval thrips proteins identified in this study. Seven and three proteins from *F. occidentalis* had sequence similarities to proteins of the body louse (*Pediculus humanus corporis*) and the pea aphid (*Acyrthosiphon pisum*), respectively, representing two other orders in the Hemipteroid assemblage. Moreover, 57% of our identified protein spots had provisional functional annotations obtained with Blast2GO analysis (Fig. 2.4). In relation to host defense response, 15 protein spots were functionally annotated as proteins associated with cell killing or immune system processes. These spots comprised six different proteins, which are tubulin alpha-1 chain, beta tubulin, glutaredoxin 5, heat shock protein, cysteine protease, and lethal giant larvae homologue (Table 2.2).

Identification of differentially expressed proteins in response to virus infection

To identify *F. occidentalis* proteins that are differentially expressed in response to TSWV infection, a merged composite image of the 2-D gels for the virus-exposed and non-exposed treatments (eight in total) was generated by the software Ludesi REDFIN 3. There were 488 protein spots present in the merged image. I identified 26 protein spots that varied in density between the virus-exposed and non-exposed treatments, of which 11 and 15 were found to be significantly ($P < 0.05$) and marginally ($P = 0.051$ - 0.1) different by at least 1.1-fold, respectively, between treatments as determined by ANOVA (Fig. 2.5, Table 2.3). Among these 26 protein spots, 62% were down-regulated in the TSWV-exposed treatment. Electrospray ionization (ESI) mass spectrometry coupled with Mascot search using the *Fo* Seq and the Metazoan nr protein database resulted in the identification of 37 proteins within the 26 spots. Moreover, one protein spot (spot 310) was not identified with either of the databases searched, and none of the differentially expressed spots contained peptides that matched TSWV proteins. The majority of the identified proteins had sequence similarities to proteins from other insects.

Eighty six percent of the identified proteins were provisionally annotated using Blast2GO (Fig. 2.6). Special attention was given to GO terms with particular relevance to biological processes and molecular functions associated with the life cycle of the insect vector and to a persistent propagative vector-borne virus. Among the 32 provisionally annotated proteins, 2 are proteins involved in reproduction and growth, 8 in development, 21 in metabolic process, 9 in localization and transport, 14 in response to stimuli, 8 in reduction/oxidation (redox) and

homeostasis, 22 in binding, and 21 in catalytic activity. The provisional annotations of these differentially expressed thrips proteins indicate the multifunctionality of these proteins in several aspects of the insect life cycle and most likely during virus infection. Among the 14 proteins in the category of response to stimuli, 9 proteins were clearly associated with innate immune defenses (Skp1, 40S ribosomal protein S3, mitochondrial ATP synthase α subunit, actin, lysozyme C, thioredoxin-dependent peroxidase, pyruvate dehydrogenase kinase 3, stress-induced phosphoprotein 1, and heat shock cognate 71 protein).

Discussion

Despite the importance of *F. occidentalis* as a worldwide insect pest and vector of tospoviruses, the thrips response to virus infection at the molecular level is just beginning to be studied. In 2010, the first EST sequencing project for *F. occidentalis* L1 was reported (Rotenberg and Whitfield, 2010). Here I demonstrate its utility for exploring the thrips proteome. Additionally, I have conducted a proteomic analysis of the young L1 stage of *F. occidentalis* and determined the number and identity of differentially expressed proteins between TSWV-exposed and non-exposed L1s using the *F. occidentalis* transcriptome collection as the primary database. The *Fo* Seq therefore provides a unique sequence resource not only for studying *F. occidentalis* and other thrips species but also for better understanding the evolutionary biology of the overlooked insect order Thysanoptera with regard to other insects and arthropods in general.

The proteomic analysis of the L1 stage allowed the identification of more than half of the resolved protein spots, with a high proportion of peptides matching the *Fo* Seq compared to those peptides identified with the Metazoan nr protein database from NCBI. Moreover, 48% of the resolved proteins were not identified with any of the databases searched because peptide identification relies on *in silico* digestion of protein sequences present in public or private databases (Wolschin and Amdam, 2007). The inability to identify proteins from 2-D gels is a common phenomenon for organisms without a sequenced genome or those having a partial transcriptome only. Interestingly, 29% of the proteins with significant matches were exclusively identified with the *Fo* Seq, indicating that these might be proteins unique to the insect order Thysanoptera or species-specific proteins.

Several proteins resolved and annotated in this study may have potential relevance to virus entry and host response to virus infection. An integrin, a known receptor for some viruses in the family *Bunyaviridae,* was identified from naïve L1s (spot 10). Integrins are a large family of heterodimeric transmembrane glycoproteins that mediate cell adhesion of and binding to the extracellular matrix (Hynes, 1992). It has been shown that several diverse families of viruses use integrins as receptors to bind to and enter into their host cells. Several hantaviruses (family *Bunyaviridae*) have been found to use integrins as their cellular receptors to enter into and infect their host cells (Gavrilovskaya et al., 1998; Gavrilovskaya et al., 1999). Further characterization of thrips integrins and possible interactions with TSWV glycoproteins is warranted.

With regards to insect host defense response to infectious agents, six types of putative immune-related proteins were identified from naïve L1s. Both alpha- and beta- tubulin were identified in the L1 proteome, and various tubulin proteins have been shown to play a role in phagocytosis (Gotthardt et al., 2006; Lim et al., 2007) and in regulation of apoptosis (Lin et al., 2009). A protein spot that matched lethal giant larvae homologue was found, and one possible function for this protein is the induction of salivary gland cells autophagic cell death. Autophagy is required for degradation of the cytoplasmic contents of salivary gland cells during development, and it is an essential component of antiviral defenses against *Vesicular stomatitis virus* (VSV) in *D. melanogaster* (Berry and Baehrecke, 2007; Shelly et al., 2009). Two protein spots were identified as cysteine proteases, which also play a role in apoptosis and the ubiquitin cycle (Balakirev et al., 2003; Sun and Chen, 2004). Glutaredoxin 5, which is involved in cell redox homeostasis to maintain cell integrity and functionality (Belli et al., 2004), was also classified as an immune-related protein in healthy *F. occidentalis* larva. I also identified several protein spots as heat shock proteins, which are expressed in response to biotic and abiotic stresses (De Maio, 1999). It is well known that many of these proteins play roles other than those involved in defense mechanisms against foreign organisms. Thus, it is plausible that their expression in naïve larval thrips reflects their multifaceted roles in different aspects of the thrips life cycle or that there is a basal level of expression before a pathogen invasion takes place.

The high vector competency of *F. occidentalis* for TSWV transmission combined with the lack of measurable pathological effects on the insect vector despite virus replication in different insect tissues (Wijkamp et al., 1996), led me to study the response of the *F. occidentalis* L1 during the early stages of TSWV infection. An analysis of virus titer in L1s at 2 and 21 hr

post-virus access has indicated that the relative abundance of virus significantly increased during this time frame (Schneweis and Rotenberg, personal communication). The average 14-fold increase in relative abundance of TSWV N RNA suggests that the viral polymerase is active within 24 hr of thrips exposure to virus and transcription and/or replication may be occurring at the time point sampled for the proteomic analysis. Findings from the analysis of titer by real-time quantitative RT-PCR is similar to increases in virus titer observed using serological techniques (Tsuda et al., 1996). I have also detected TSWV N protein in thrips 24 hr after virus exposure (Fig. 2.7). In all, I found that 26 spots containing 37 proteins were up- or down-regulated in response to TSWV infection; however, in the majority of the cases, TSWV had a negative effect on thrips gene expression at the protein level (Table 2.3). As observed in other proteome studies using 2-D gels (Yang et al., 2008b), I found several single spots that contained two different proteins. At this time, it is not known if one or both of these proteins were differentially regulated by TSWV infection. Using a finer scale pH gradient to separate these comigrating proteins in future studies may aid in a better resolution of these proteins.

The statistical analysis of protein expression over four biological replications of the experiment revealed that only 5.3% of the protein spots (26/488) resolved from the pools of 600 insects were differentially regulated by virus infection. This relatively low but significant number of responsive proteins may be explained by i) inclusion of non-infected thrips in the cohorts of thrips (10% have no detectable virus); ii) inclusion of non-infected tissues as is the case with whole insect bodies (only the midgut epithelial and muscle cells are infected during the early stages of the TSWV replication cycle in larval thrips (Wijkamp et al., 1993)), and/or iii) inclusion of viruliferous insects harboring low titers of virus that may result in little to no effect on protein expression. Using cut-off significance *P* values of 0.05 to 0.1 in addition to a *P* value < 0.05 as our primary criteria for differential expression allowed the detection of protein changes that otherwise might be missed due to the aforementioned reasons. Moreover, the use of four biological replications (i.e., independent experiments with inherent natural and technical variation) to identify differentially expressed proteins provides confidence in the effects due to virus infection.

Among the twenty one proteins potentially involved in metabolism, spot 201 was identified as a triosephosphate isomerase, an enzyme which plays an important role in glycolysis and is essential for efficient energy production (Jung et al., 2002). Interestingly, triosephosphate

isomerase deficiency, a unique glycolytic enzymopathy known to affect humans and other mammals, is characterized by susceptibility to pathogen infections (Schneider, 2000), which contrasts with the up-regulation of this protein in TSWV-infected thrips. The up-regulated spot 137 matched a mitochondrial ATP synthase alpha subunit that is a membrane-bound enzyme involved in ATP synthesis and/or hydrolysis and transport of protons across membranes (Devenish et al., 2000). A mitochondrial ATP synthase alpha subunit of the brown planthopper, *Laodelphax striatellus*, was found to be up-regulated at the transcript level during infection with *Rice stripe virus* (RSV, Tenuivirus) (Zhang et al., 2010). Furthermore, an enolase, which is a metalloenzyme, was identified in spot 161 as being up-regulated by TSWV infection. Consistent with our findings, a putative enolase transcript was also identified to be up-regulated in RSV-infected *L. striatellus* compared to their naïve counterparts (Zhang et al., 2010). Matrix metalloproteases have recently been documented to play a role in baculovirus escape from gut tissue through activation of effector caspases (Means and Passarelli, 2010). The resulting remodeling of the basal lamina lining of tracheal cells associated with the intestine allows baculoviruses to escape from the midgut epithelial cells and establish systemic infections in their lepidopteran hosts (Means and Passarelli, 2010). Larval acquisition of TSWV is required for transmission of virus; however, when adult thrips feed on virions, they enter the midgut and replicate but do not escape the gut tissues (Ullman et al., 1992). Thus, a midgut escape barrier is hypothesized to govern vector specificity and competency. Remodeling of the midgut basal lamina in larval thrips during TSWV infection, similar to the described mechanism of escape for baculoviruses, could be one explanation for the differences in virus dissemination that have been observed in larval versus adult thrips.

Virus infection of insects by entomopathogenic and vector-borne viruses can cause both positive and negative effects on the development of the insect host (Burand and Park, 1992; Reese et al., 2009). I found vitellogenin and actin (both within spot 374) to be up-regulated in the TSWV-exposed thrips. Vitellogenin is the precursor of the lipoproteins and phosphoproteins that make up most of the protein content of yolk. Interestingly, spot 378 which also contains actin was down-regulated during TSWV infection (in contrast with actin being up-regulated in spot 374). These contradictory results reflect the complex response that *F. occidentalis* displays during TSWV infection. It is well known that members of the actin gene family are widely dispersed in the genome of metazoans and plants and that they can be differentially regulated

under varying conditions (Fyrberg et al., 1981; Meagher, 1991). A 60S ribosomal protein L9 (spot 72), which is involved in translation elongation and termination, was found to be up-regulated in the TSWV-exposed cohorts. Overall, these findings suggest that TSWV might have a direct positive effect at least to some extent on the development and metabolism of its most efficient insect vector, *F. occidentalis*. For example, it was reported that larval thrips exposed to TSWV reached larger body sizes than their non-exposed counterparts, which ultimately resulted in a shorter period of vulnerability to two species of predatory mites (Belliure et al., 2008). The findings of five proteins involved in thrips development being up-regulated during virus infection combined with results obtained by Belliure and colleagues (Belliure et al., 2008) suggest that TSWV infection of its thrips vector is responsible at least in part for the positive effects observed in this virus-vector interaction, such as a faster larval growth and no detrimental effect during virus infection.

I have also identified nine proteins putatively involved in localization and membrane and protein transport as being differentially expressed. These proteins are potentially important for the viral infection cycle steps of entry and exit from insect vector cells. For example, vacuolar proton ATP synthase (v-ATPase) subunit E, identified within spot 134, was up-regulated by virus infection. v-ATPases are membrane-bound enzymes that catalyze ATP hydrolysis to transport solutes across membranes and lower the pH in organelles (Nelson et al., 2000). Up-regulation of v-ATPase might have implications in TSWV entry to the midgut epithelial cells of its thrips vector, since this protein is involved in clathrin-coated vesicle trafficking (Forgac, 1999), the known mechanism of entry for other bunyaviruses (Jin et al., 2002; Simon et al., 2009). An apolipoprotein, a protein that binds to and transports lipids through the hemolymph of insects and the circulatory system of mammals, was identified in spot 481 as being down-regulated by TSWV infection. A glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was identified in spot 182 and within spot 196 as being down-regulated by TSWV infection in both spots. In contrast, this protein was consistently found up-regulated in three different vector-competent genotypes of the greenbug (Cilia et al., 2011), and G3PDH has been found to be potentially involved in the transcytosis of *Beet western yellows virus* in the green peach aphid (*Myzus persicae*) (Seddas et al., 2004). G3PDH has been found to be important in the infection process of other plant- and animal-infecting viruses. For example, G3PDH negatively regulates the replication of *Bamboo mosaic virus* and its associated satellite RNA *in vitro* (Prasanth et al.,

2011), and G3PDH mutants directly inhibit the accumulation and plus-strand synthesis of *Tomato bushy stunt virus* in yeast and plants (Huang and Nagy, 2011). It is also known that G3PDH binds to membranes and regulates endocytosis (Sirover, 1997) and exocytosis (Glaser and Gross, 1995). Additional research of TSWV entry and exit in thrips will aid in understanding the relevance of these proteins in the virus infection process.

Insects rely solely on the innate immune responses to defend themselves against invading microbes. I found 14 proteins that were ascribed a functional annotation associated to response to stimuli, which includes responses to biotic and abiotic stresses. For example, spot 88 was identified as an UV excision repair protein, designated rad23, which is involved in a multiprotein and proteosome complex that targets nucleotide excision repair on the genome and ubiquitinated proteins for degradation, respectively, during abiotic stresses (Bertolaet et al., 2001a; Bertolaet et al., 2001b; Zhao et al., 2006). Among the 14 proteins within the category of response to stimuli, nine are potentially involved in the defense response of *F. occidentalis* to TSWV infection. Interestingly, 5 out of these 9 proteins were down-regulated in the TSWV-exposed cohorts. A thioredoxin-dependent peroxidase (also peroxiredoxin) that among several functions acts as an inhibitor of apoptosis (Zhang et al., 1997) was identified in spot 319, which was down-regulated by TSWV infection. A protein showing similarity to a stress-induced phosphoprotein 1, which plays a role in signaling in the Jak-STAT pathway (reviewed in Haura et al., 2005), was identified in spot 340. The Jak-STAT pathway is a major component of the innate immune response of mammals and insects against viruses (Dostert et al., 2005; Dupuis et al., 2003; Souza-Neto et al., 2009). Lysozyme C, which was identified in spot 794 as being down-regulated by TSWV infection, has been well documented to play a role in killing bacteria (Fleming, 1922). Moreover, the down-regulated spot 803 was identified as heat shock cognate 71 protein, which has been shown to be modulated during influenza virus infection (Melville et al., 1999). In contrast, spots 836 and 137 were both up-regulated during virus infection. Spot 836 was identified as 40S ribosomal protein S3, which plays a role in apoptosis, and is involved in the negative regulation of NFκB transcription factor activity that includes the well-studied Toll and Imd pathways. These two signaling pathways have been shown to play an important antiviral role in insects (reviewed in Sabin et al., 2010). The virus up-regulated spot 137 was identified as a mitochondrial ATP synthase alpha subunit, which GO term suggests a possible role in engulfment during phagocytosis to clear pathogen infections. Characterizing the

components of the innate immune system that recognize and respond to TSWV may be important for understanding vector competency and designing novel control strategies.

I found that 5 of the 37 identified proteins did not have a Blast2GO annotation, but their identity (based on protein sequence similarities found within the NCBI nr protein database) suggests possible roles for these proteins. Spot 196, which was down-regulated in the virus-exposed group, was identified as cyclophilin. Cyclophilins have been shown to play a role in virus infection and have been implicated as a determinant of insect vector competency. For example, incorporation of cyclophilin A into the newly synthesized human immunodeficiency virus 1 (HIV-1) particles is required for virion attachment to host cells (Saphire et al., 1999), while its recruitment by the GAG polyprotein during HIV-1 infection (Braaten et al., 1997) promotes HIV-1 replication (Braaten and Luban, 2001). Cyclophilins were identified in greenbugs (*Schizaphis graminum*) that are vectors of *Cereal yellow dwarf virus* (Family *Luteoviridae*) but not in non-vectors of the same aphid species (Yang et al., 2008b). Two different proteins co-resolved within spot 283, a protein yellow-like and a 26S protease regulatory subunit 7, which have been associated with melanization and behavior of *D. melanogaster* (Drapeau et al., 2003; Wittkopp et al., 2002) and the ATP-dependent degradation of ubiquitinated proteins (Chen et al., 1997), respectively. The identification of several proteins with roles in ubiquitin-mediated protein degradation suggests that this pathway may be differentially activated in *F. occidentalis* during TSWV infection.

Despite detection of TSWV in 90% of the thrips subsampled from 4 biological replicates, I did not detect viral proteins in the analysis of differentially expressed proteins between TSWV-exposed and non-exposed larval thrips. A possible explanation for the inability to identify virus proteins in the current analysis may be (1) the early time point used to collect larval thrips, (2) the detection limit of a 2-D gel stained with Coomassie Brilliant Blue G-250, or (3) the comigration of viral proteins with other proteins. However, I detected TSWV N in Western blots of 2-D gels from virus-exposed larvae (600 individuals with 90% infection rate) and purified virus (positive control) but not in the noexposed cohort (Fig. 2.7). This indicates that viral proteins are expressed in the virus-exposed cohorts and that changes in their protein profile are indeed attributed to TSWV infection. Moreover, I did not identify most of the immune-related proteins that were identified by Medeiros *et al.* (Medeiros et al., 2004) during thrips infection by TSWV, with the exception of lectin C. In this previous study, the authors used a virus isolate

and thrips population different from those used in this study. Furthermore, they conducted a macroarray analysis of subtractive cDNA libraries of TSWV-infected second instar larvae (96 hr post AAP), which may have been the cause of the different findings obtained in the current study using proteomic tools to identify not only immune-related proteins but all proteins responsive to viral infection.

The proteomic analysis of the response of *F. occidentalis* to TSWV infection has identified a suite of candidate proteins that respond to virus infection. Further analysis of transcriptome responses will enable a meta-analysis and comparison of changes at the mRNA and protein level that occur in virus-infected thrips. At this time, functional genomic assays using RNA interference (RNAi) have not been developed for thrips. Thus, the development of RNAi technology for thrips will enable studies to describe the functional roles of the differentially expressed proteins in TSWV-*F. occidentalis* interactions.

Materials and Methods

Frankliniella occidentalis cultures

Our virus-free colony of *F. occidentalis* originated from an isolate collected from the Kamilo Iki Valley on the island of Oahu, Hawaii in the early 1980's (Bautista et al., 1995). Insects were reared and maintained on green bean pods (*Phaseolus vulgaris*) in 16-oz clear plastic deli cups with lids fitted with thrips-proof screen at 22°C (± 2°C) under laboratory conditions as previously described (Bautista et al., 1995; Ullman et al., 1992). To generate first instar larvae for our experiments, beans were incubated with adult thrips for 3 days to allow adult females to oviposit. Adult thrips were then removed and the beans were incubated at 23°C for 24 hr to collect emergent larval thrips (0 to 24 hr old).

Collection and protein extraction from healthy larval thrips for proteomic analysis

Six hundred emergent larval thrips (0 to 24 hr old) were manually collected using a fine paintbrush (000), pooled into a 1.7 mL nuclease- and protease-free microcentrifuge tube, flash-frozen in liquid nitrogen, and stored in -80°C until further use. Total proteins were extracted in 200 µL of Rehydration/Sample Buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl)-

dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM 1,4-dithiothreitol (DTT), 0.2% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue (Bio-Rad, Hercules, CA, USA) using a sterile Kontes pestle (Fisher Scientific). After centrifugation at 12,000 x *g* for 5 min, the protein supernatant was quantified for 2-D gel electrophoresis using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Wilmington, DE, USA) following the manufacturer's instructions.

Two-dimensional gel electrophoresis and MALDI-TOF/TOF mass spectrometry

Following total protein extraction and quantification, 400 µg of protein supernatant were applied to an 11 cm IPG strip with a pH 3 to 10 gradient (Bio-Rad), for overnight passive rehydration at 25°C. For the first separation of proteins, the IPG strip was subjected to isoelectric focusing (IEF) using a Protean IEF Cell (Bio-Rad). The IEF was carried out using the following voltage step-gradient program: 250 V for 2,000 Vh; 8,000 V for 18,000 Vh; and 8,000 V for 20,000 Vh at 20°C and a maximum current setting of 70 µA/strip. After IEF, the IPG strip was equilibrated for 15 min in Equilibration Buffer I (6 M urea, 2% SDS, 0.375 Tris-HCl [pH 8.8], 20% glycerol, and 2% [w/v] DTT) (Bio-Rad), followed by another 15 min in Equilibration Buffer II (same as Equilibration Buffer I but containing 2.5% iodoacetamide (IAA) instead of DTT) (Bio-Rad) to reduce and carbamidomethylate the focused proteins, respectively. For the second separation of proteins, the IPG strip was placed across a 10-20% Criterion Tris-HCl Precast Gel (Bio-Rad) and overlaid with agarose. Electrophoresis was run in Tris-glycine buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3) (Bio-Rad) using an electrophoresis Criterion unit (Bio-Rad) at a constant voltage of 150 V until the yellow dye front completely migrated out of the bottom of the gel. Using a clinical rotator at low rotation speed, the 2-D gel was rinsed with double-distilled (dd) water for 15 min and then stained overnight with Bio-Safe Coomassie Brilliant Blue G-250 (Bio-Rad). After staining, the 2-D gel was washed with dd water until the blue background was no longer visible. The 2-D gel was kept at 4°C until further use.

The 2-D gel was scanned with a Bruker Daltonics Proteineer spII imaging system (Bruker Daltonics, Billerica, MA, USA). All the clearly defined protein spots were manually excised from the 2-D gel using a cork borer and deposited individually in microcentrifuge tubes.

One hundred microliters of 25 mM ammonium bicarbonate were added to each tube, which then were incubated for 30 min at room temperature to destain the protein spots. Proteins were destained with 50 µL of acetonitrile (ACN) (biotech grade solvent >99.93%) (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature. The gel pieces were dried by speed vacuum for 15 min at 4°C. The samples were subjected to tryptic digestion using 100 ng of proteomics-grade trypsin (Trypsin Gold, Promega, Madison, WI) in 20 µL of dd water at 37°C overnight (~ 16 hr). Trypsinized peptides were then extracted from the gel pieces with 5% trifluoroacetic acid (TFA) in 50% [v/v] ACN/water at 37°C for 30 min. The resulting peptide solutions were dried by speed vacuum for 4 hr and then reconstituted in 10 µL of 20 mg/mL 2, 5-dihydroxylbenzonic acid in 33% ACN/0.1% TFA. Peptide solutions were then mixed (1:1) with matrix α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich). For each sample, 2 µL of peptide/matrix solution was dispensed onto a Bruker stainless steel matrix-assisted laser desorption ionization (MALDI) target plate (Bruker Daltonics). Mass spectra (MS and MS/MS) were obtained using a Bruker Daltonics Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics), and positively charged ions were acquired in the reflector mode over a *m/z* range of 500 to 1,500 using delayed extraction. The spectra were processed by the FlexAnalysis 3.0 and Biotools 3.0 software programs (Bruker Daltonics) without further smoothing or spectrum processing. Monoisotopic masses were obtained using the SNAP algorithm with at least 5 for the signal/noise ratio of peak intensity. Measurements were externally calibrated with 8 different peptides ranging from 757.39 to 3147.47 (Peptide Calibration Standard II, Bruker Daltonics). The generated peak lists were transferred to ProteinScape 1.3 software program, which uses the MASCOT 2.2 (www.matrixscience.com) (Matrix Science, Boston, MA, USA) search engine for protein identification.

Protein identification

Peptide mass lists were compared to the *Fo* Seq (*F. occidentalis* transcriptome collection) and the Metazoan nr protein database of the National Center of Biotechnology Information (NCBI) for protein identification. Furthermore, I conducted a sequence search against the Prokaryotic nr protein database of NCBI to identify proteins from bacterial origin within the *F. occidentalis* resolved proteome. The NCBI database searches were conducted on August 23,

2010. For protein identification, we used an error tolerance search algorithm and the six possible translation open reading frames. Moreover, we used the following search parameters for protein identification: carbamidomethyl-cysteine as a fixed modification and methionine oxidation as a variable modification, with one missed tryptic cleavage allowed. The MS and MS/MS data collected were used to conduct a combined search. MASCOT 2.2 software was used to compare databases of peptide sequences and their theoretical fragmentation patterns with a given MS and MS/MS spectrum. Protein spots that had significant matches ($P \leq 0.05$) were considered to be identified. Additionally, we considered to be identified protein spots that had marginally significant matches ($P = 0.051$ to 0.1) in order to provide a less stringent but conservative identification. Complete sequences from the *Fo* Seq that matched protein spots under the described criteria were subjected to a protein database search using BLASTp from NCBI to determine their homology to known proteins. Furthermore, I conducted a functional annotation of the sequences that matched the thrips proteins analyzed in this proteomic work using Blast2GO against NCBI nr sequence database.

TSWV maintenance

TSWV (isolate TSWV-MT2) was periodically maintained by thrips transmission on *Emilia sonchifolia* as previously described (Ullman et al., 1993). Virus was mechanically inoculated onto young *E. sonchifolia* plants using thrips-inoculated *Emilia* leaf tissue. Systemically infected tissue from the mechanically inoculated plants was used as the source of virus acquisition by emergent larval thrips 12 days post inoculation (dpi).

Acquisition efficiency of TSWV-exposed L1 thrips

Acquisition efficiency, *i.e.*, proportion of TSWV-positive insects, was determined after a 24 hr clearing period on healthy green bean pods following a 3 hr acquisition access period (AAP) on virus-infected *E. sonchifolia* tissue for each biological replicate. Ten individual insects were subsampled, frozen, and stored for endpoint RT-PCR amplification of TSWV N RNA. Total RNA was extracted from individual insects using a Chelex 100 (Biorad) method developed by Boonham *et al.* (Boonham et al., 2002). Ten microliters of the RNA sample served as the template for cDNA synthesis using the Verso cDNA Synthesis Kit (Thermo Scientific,

Wilmington, DE, USA) with a 3:1 ratio of random hexamers to anchored olido (dT) primers and Verso RT Enhancer to remove contaminating DNA, following the manufacturer's protocol. Negative and positive template controls were included in the cDNA and PCR steps. PCR was performed in 25 μL reaction mixtures using the PCR Go Taq System I (Promega, Madison, WI, USA) at 95°C for 2 min followed by 40 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. The TSWV N primers used for endpoint RT-PCR (200 nM each in final reaction) to detect virus infection in individual larvae were described by Rotenberg and colleagues (Rotenberg et al., 2009). *F. occidentalis* actin endpoint RT-PCR primers previously tested (Boonham et al., 2002) were used for detection of insect cDNA to insure that total RNA template was present in TSWV-negative insects. Amplified products were visualized in 1.5% agarose gels using ethidium bromide and a UV transilluminator (Bio-Rad).

Sample preparation for proteomic analysis of virus-exposed and non-exposed L1 thrips

A virus acquisition experiment was conducted to determine the protein-level response of L1 thrips to TSWV infection. The experiment consisted of two treatments, virus-exposed and non-exposed L1s, and each experiment was conducted five independent times, *i.e.*, biological replicates. Four replicates were used for differential protein analysis and one replicate was used for Western blot analysis using the methods from Whitfield *et al.* (Whitfield et al., 2004).

Cohorts of emergent larval thrips (0 to 18 hr old) were collected, starved for 30 min, and then exposed to bouquets of virus-infected *E. sonchifolia* leaves as described above (exposed group), or bouquets of healthy, non-infected *E. sonchifolia* (non-exposed group) for a 3 hr AAP. The bouquets were subsequently replaced by virus-free green bean pods, and the cohorts were allowed a 24 hr clearing period. From each treatment, groups of 600 L1s (27 to 45 hr old) were then collected manually and stored at -80°C as described above, and 10 insects were subsampled from each treatment to assess acquisition efficiency of the exposed group, or virus contamination in the non-exposed group. We determined that acquisition efficiency of the L1 cohorts ranged from 80% to 90% and that non-exposed thrips were virus-free. We chose cohorts with 90% infection as our four biological replicates for the proteome analysis and one replicate for the Western blot analysis.

Analysis of differentially expressed proteins

To identify proteins that are differentially expressed in the L1 of *F. occidentalis* during TSWV infection, we subjected proteins extracted from virus-exposed and non-exposed L1 thrips by the trichloroacetic acid-acetone (TCA-A) extraction method used by Cilia *et al.* (Cilia et al., 2009; Cilia et al., 2011) to 2-D electrophoresis. To standardize the relative abundance of proteins across treatments and biological replications, we used 375 µg of protein extract per sample. Images of the 2-D gels were generated using a Bruker Daltonics Proteineer spII imaging system (Bruker Daltonics). The images were uploaded into the software program Ludesi REDFIN 3 (http://www.ludesi.com/redfin/) for analysis of the differentially expressed proteins. The 2-D gels were manually warped and then automatically aligned to each other, and protein spots delineation was edited when necessary. Protein spots were selected as being differentially expressed between the two treatments if they showed an ANOVA p-value of < 0.1 (with a p-value < 0.05 being significant and a p-value $= 0.051$ to 0.1 being marginally significant) and a $>$ 1.1-fold change in spot density.

ESI-MS/MS and identification of differentially expressed proteins

Protein spots that were identified as differentially expressed between TSWV-exposed and non-exposed thrips were excised from one pair of 2-D gels and processed as previously described here. The gel-extracted peptides were reconstituted in a solution of 0.1% formic acid and 2% ACN and processed in a Bruker Daltonics HCT Ultra Ion Trap mass spectrometer (Bruker Daltonics) coupled with a microcolumn switching device (Switchos; LC Packings), an autosampler (Famos: LC Packings), and a nanogradient generator (UltiMate Nano HPLC; LC Packings) for electrospray ionization tandem (ESI-MS/MS) mass spectrometry to generate peptide sequences of extensive coverage. Samples were loaded on a C_{18} reversed-phase capillary column (75 µm inside diameter [i.d.] by 15 cm, PepMap, Dionex) in conjunction with an Acclaim C_{18} PepMap trapping column (300 µm i.d. by 10 mm, PepMap, Dionex). Peptides were separated by a nanoflow linear ACN gradient using buffer A (0.1% formic acid, 2% ACN) and buffer B (0.1% formic acid, 80% ACN) starting from 5% buffer B to 60% over 45 min at a flow rate of 200 nL/min. The column was washed with 95% buffer B for 5 min. The system control

software, Hystar 3.2, was used to control the entire process. The eluted peptides were then injected into an HCT Ultra Ion Trap mass spectrometer (Bruker Daltonics). The mass spectrometer was set up in the data-dependent MS/MS mode to alternatively acquire full scans (*m/z* acquisition range from 300 to 1,500 Da). The four more intense peaks in any full scan were selected as precursor ions and fragmented by collision energy. MS/MS spectra were interpreted, and peak lists were generated by DataAnalysis 3.4 and Biotools 3.0 software programs (Bruker Daltonics), respectively. Generated MS/MS spectra were compared to *in silico* digested sequences from the *Fo* Seq and the Metazoan nr protein database of NCBI. Furthermore, we used the genome sequence of TSWV (accession numbers: NC_002051 for the S RNA, NC_002050 for the M RNA, and NC_002052 for the L RNA) to identified protein spots that correspond to viral proteins in TSWV exposed thrips. Other parameters used were as previously described here. Protein spots were considered as identified when they had Mascot scores that represented a p-value < 0.05. The sequences that matched proteins that were differentially expressed under the described parameters were also subjected to BLASTp from NCBI to obtain their homology to known proteins and to Blast2GO to assign them a provisional functional annotation.

Figures and Tables

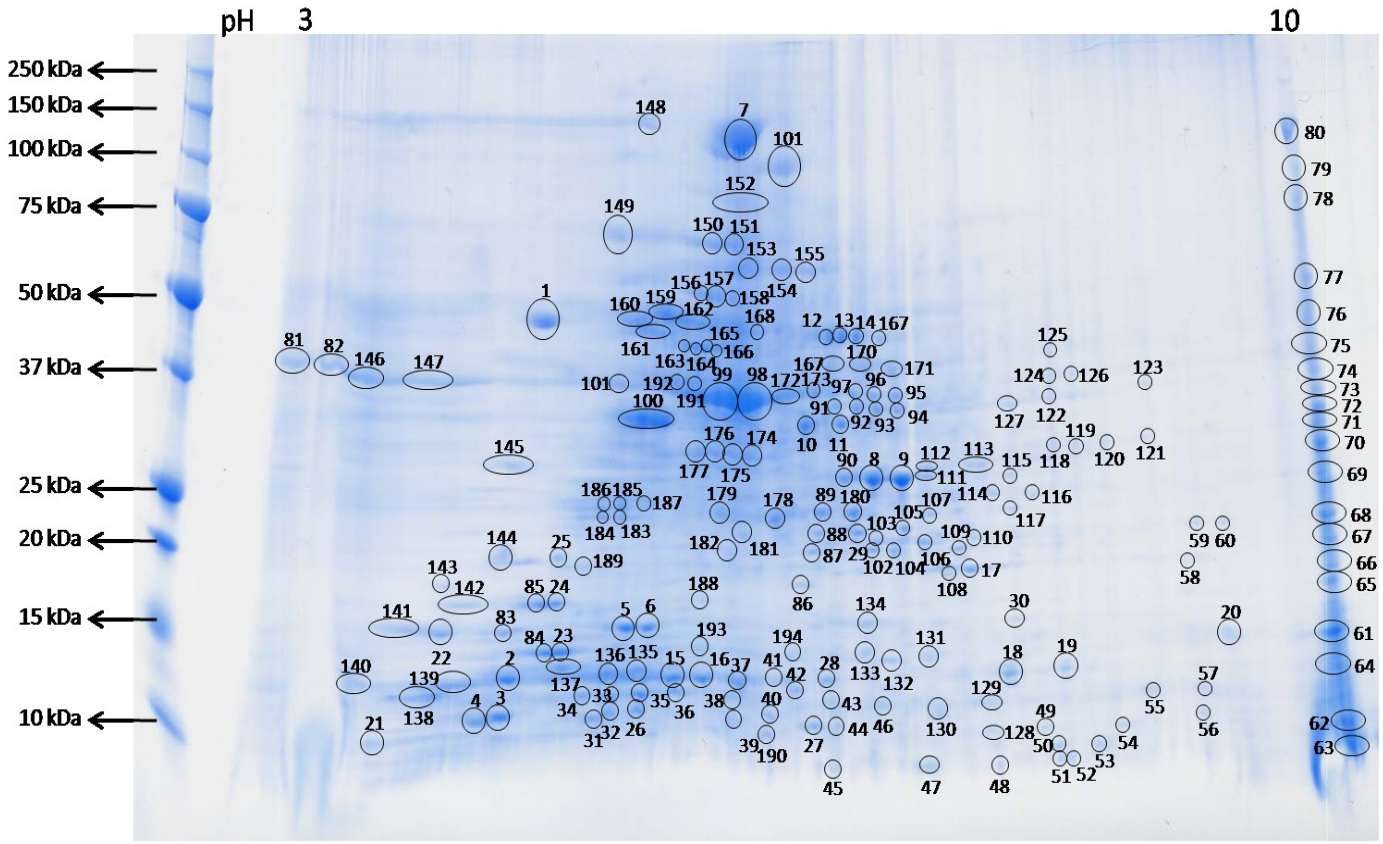

Figure 2.1 Two-dimensional (2-D) gel of the resolved proteome from healthy *Frankliniella occidentalis* first instar larvae. Four hundred micrograms of total protein extracted from 600 pooled first instar larvae of *F. occidentalis* were resolved in a 2-D gel using an 11 cm IPG strip with a pH 3 to 10 gradient, followed by standard SDS-polyacrylamide gel electrophoresis (10-20% polyacrylamide). The 2-D gel was stained by Coomassie Brilliant Blue G-250 for protein visualization. One hundred and ninety four protein spots were clearly resolved, excised, further processed, and subjected to MALDI-TOF/TOF mass spectrometry for protein identification. Molecular mass (in kilodaltons) is shown on the *y* axis and pI (as pH range) is shown on the *x* axis.

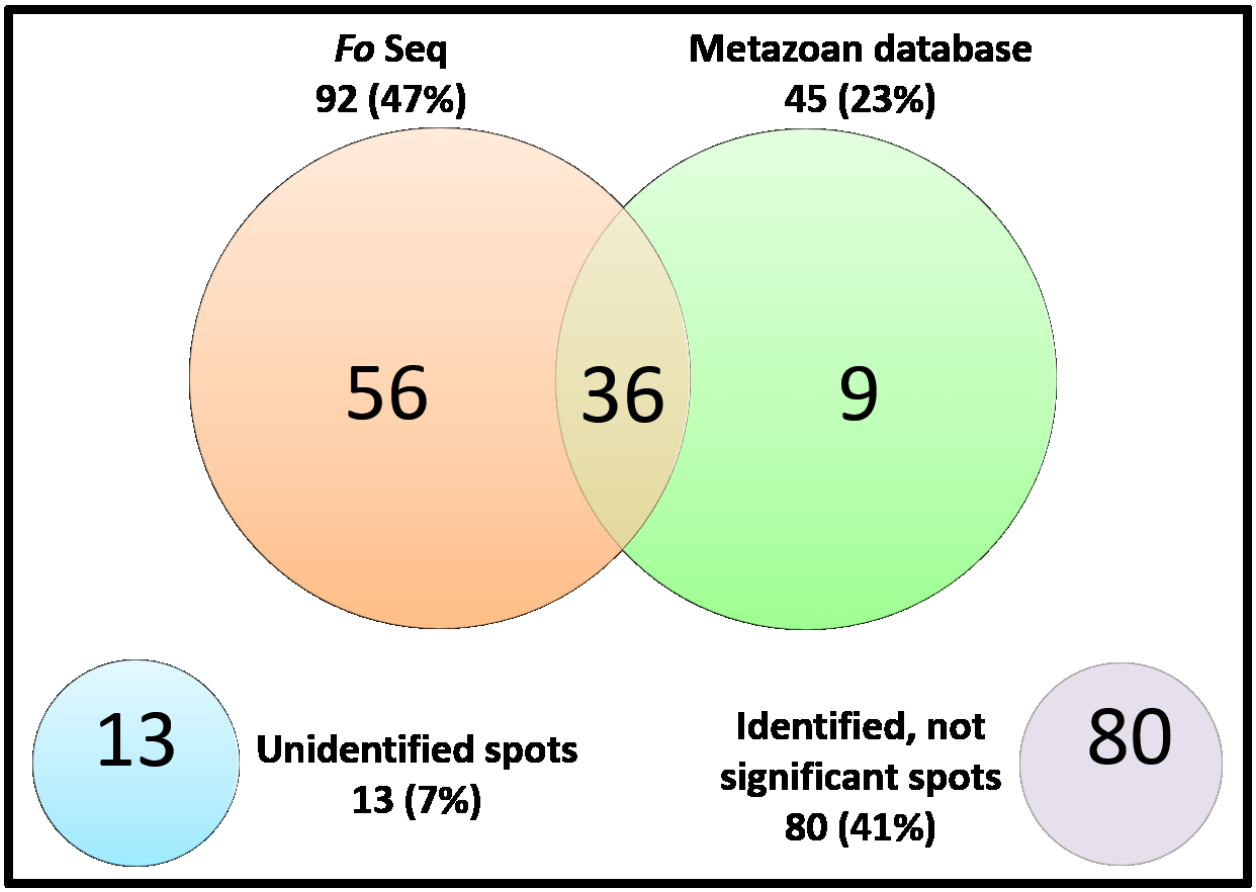

Figure 2.2 Venn diagram of the identified proteins from healthy *Frankliniella occidentalis* first instar larvae.

The large circles display the number and proportion (in parenthesis) of proteins that were identified using the *Fo* Seq (orange) and the Metazoan nr protein database from NCBI (green)**.** The overlap indicates the number of proteins that were identified with both databases, while the numbers inside each circle represent the proteins that were unique to either database. The small circles display the number and proportion (in parenthesis) of proteins that were unidentified (blue) or identified without having significant matches (purple).

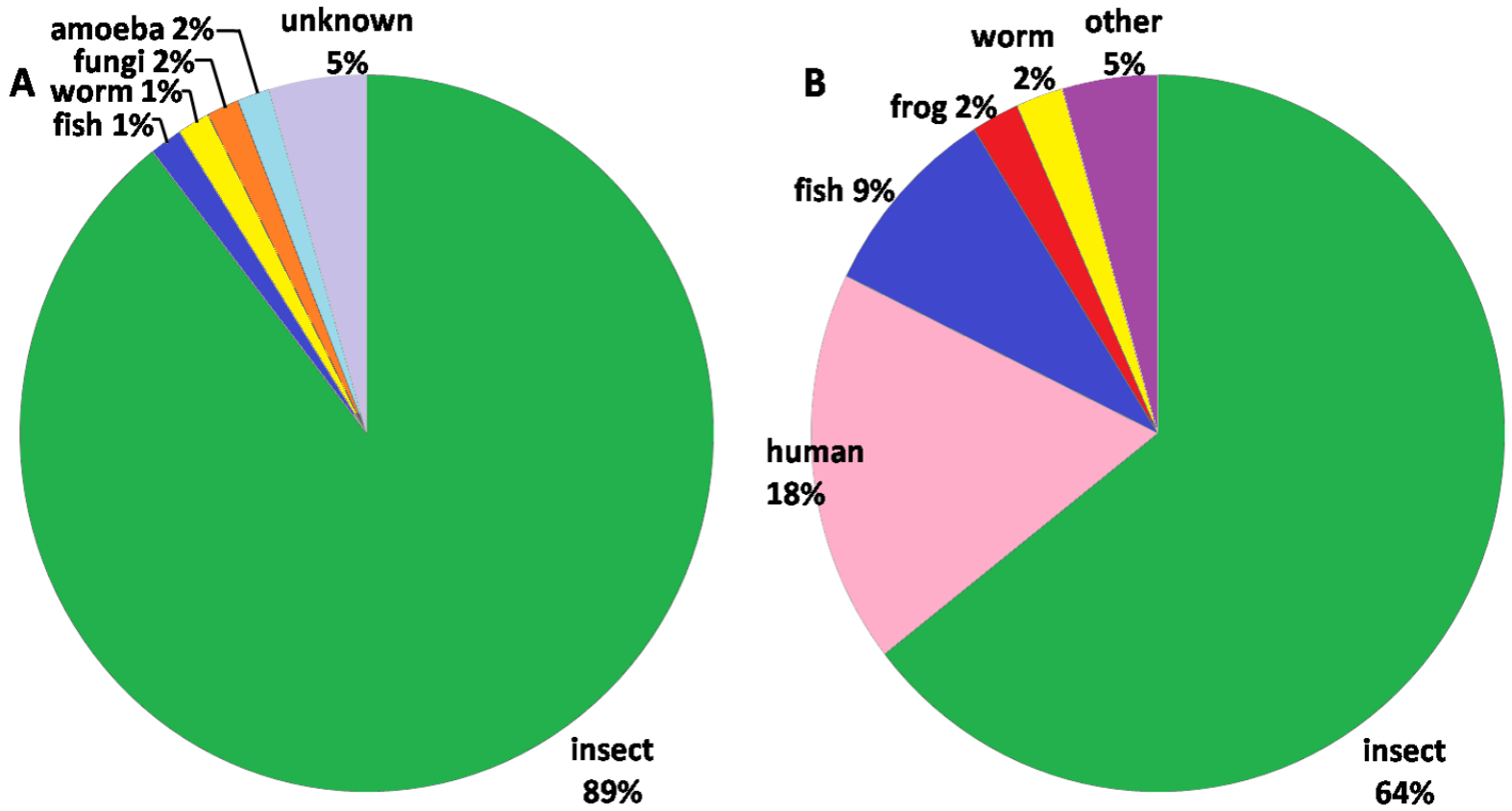

Figure 2.3 Distribution of classes of organisms with protein sequences similar to those of *Frankliniella occidentalis* proteins. These pie charts display the classes of organisms that had protein sequences similar to the sequences of the *Fo* Seq (A) and the Metazoan nr protein database from NCBI (B) used to identify proteins from healthy larval thrips.

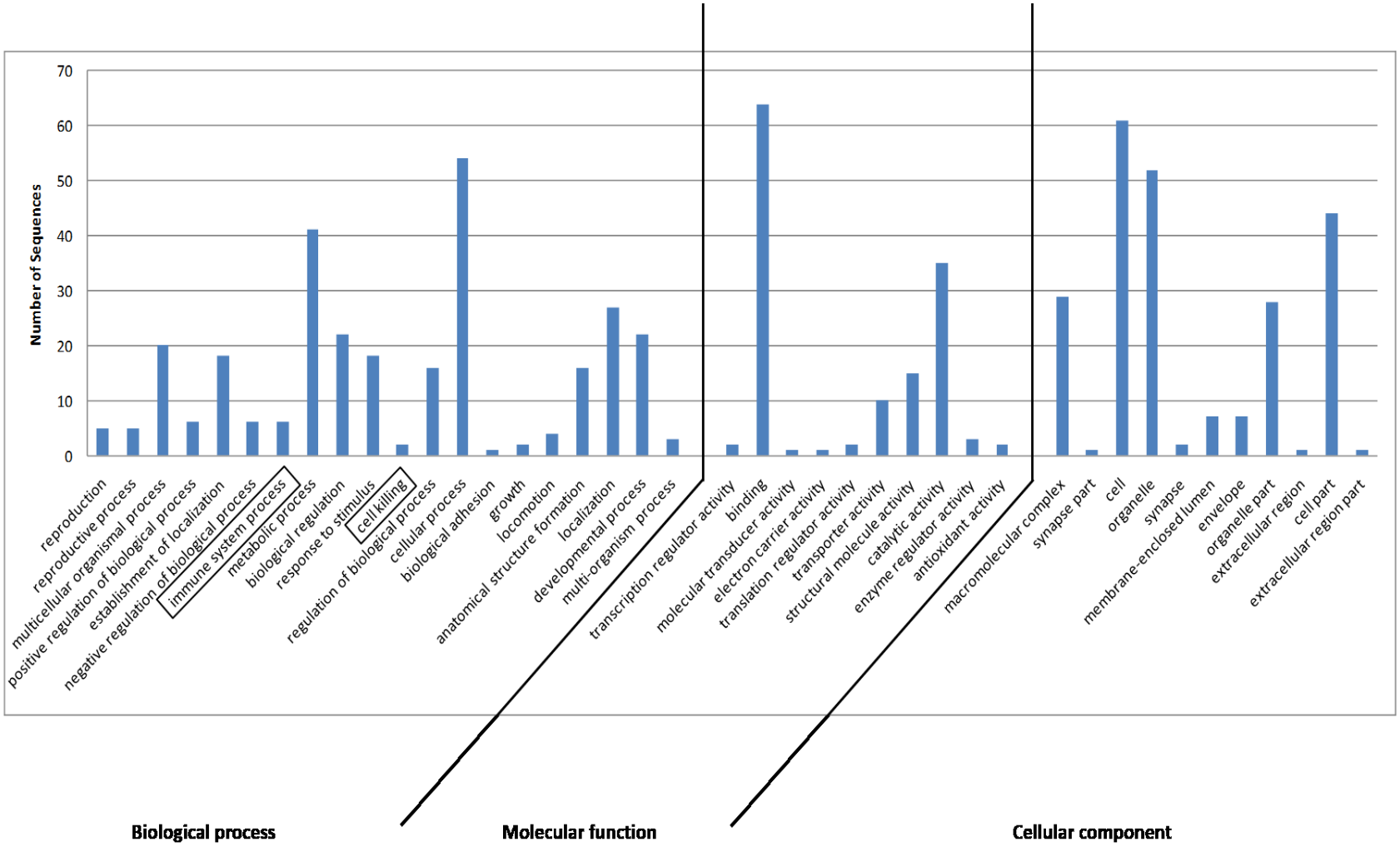

Figure 2.4 Distribution of *Frankliniella occidentalis* identified proteins by provisional biological processes, molecular functions, and cellular component categories.

Each category represents a gene ontology (GO) term assigned by Blast2GO analysis, while the height of the bars indicates the number of proteins that were classified in each category.

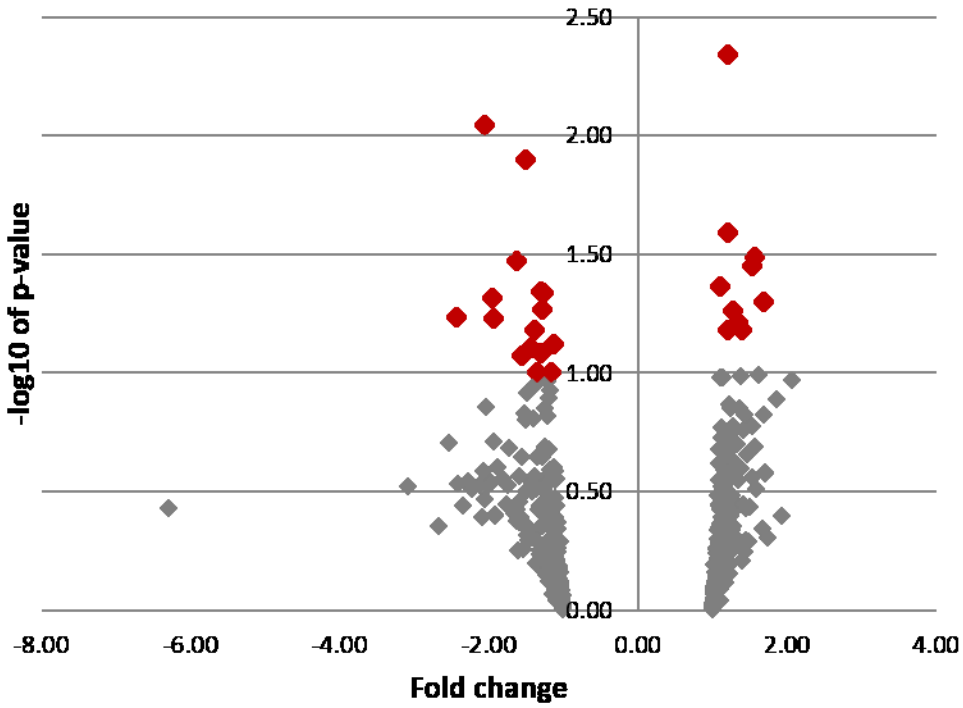

Figure 2.5 Volcano plot of protein spots from the analysis of differentially expressed proteins between TSWV-exposed and non-exposed *Frankliniella occidentalis* first instar larvae.

The relative abundance of the differentially expressed protein spots from the 4 biological replicates performed are presented in the volcano plot as red diamonds. The direction of the change (up- or down-regulation) of protein spots that were differentially expressed between treatments is shown as fold change on the *x* axis, while the magnitude of the change as $-\log^{10} P$ value is shown on the *y* axis.

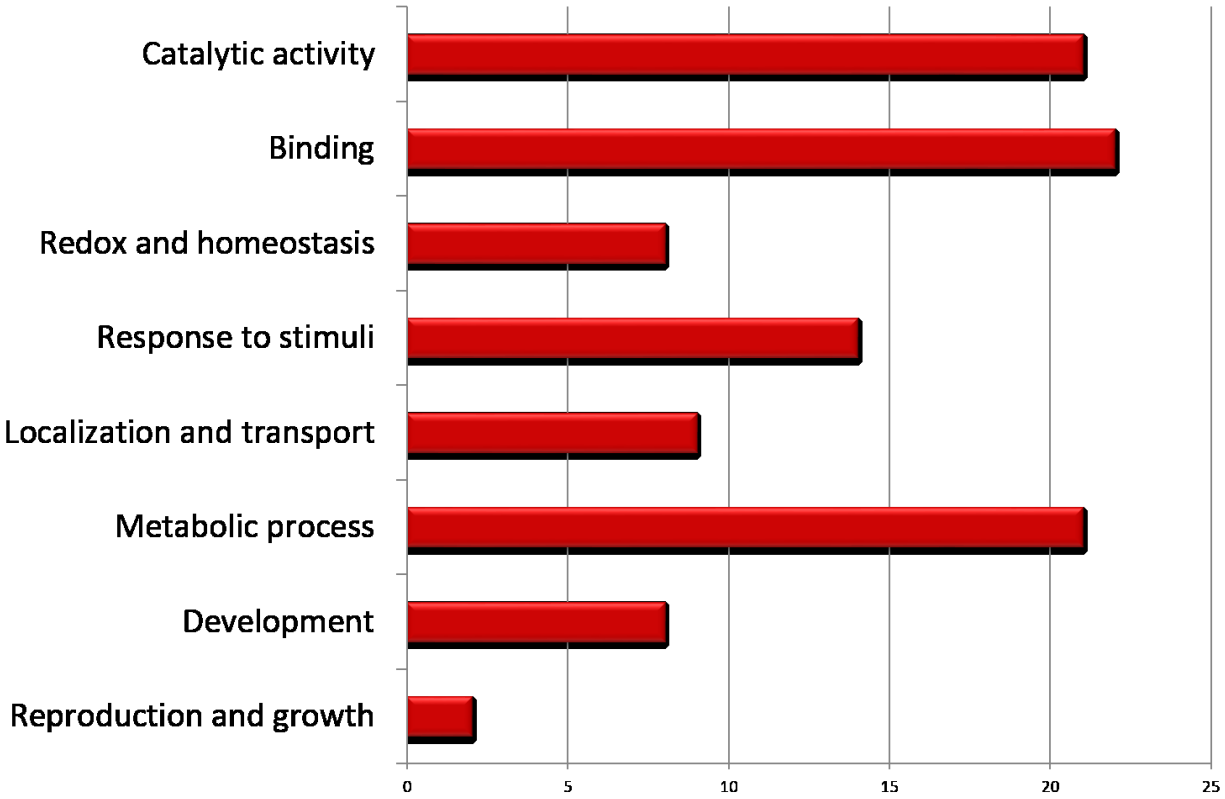

Figure 2.6 Distribution of *Frankliniella occidentalis* proteins that were differentially expressed between TSWV-exposed and non-exposed first instar larvae by provisional biological processes and molecular function.

Special attention was given to gene ontology (GO) terms with relevance to biological processes and molecular functions associated with the life cycle of *F. occidentalis* and to a persistent propagative vector-borne virus. Each category represents a GO term assigned by Blast2GO analysis, while the length of the bars indicates the number of proteins that were classified in each category.

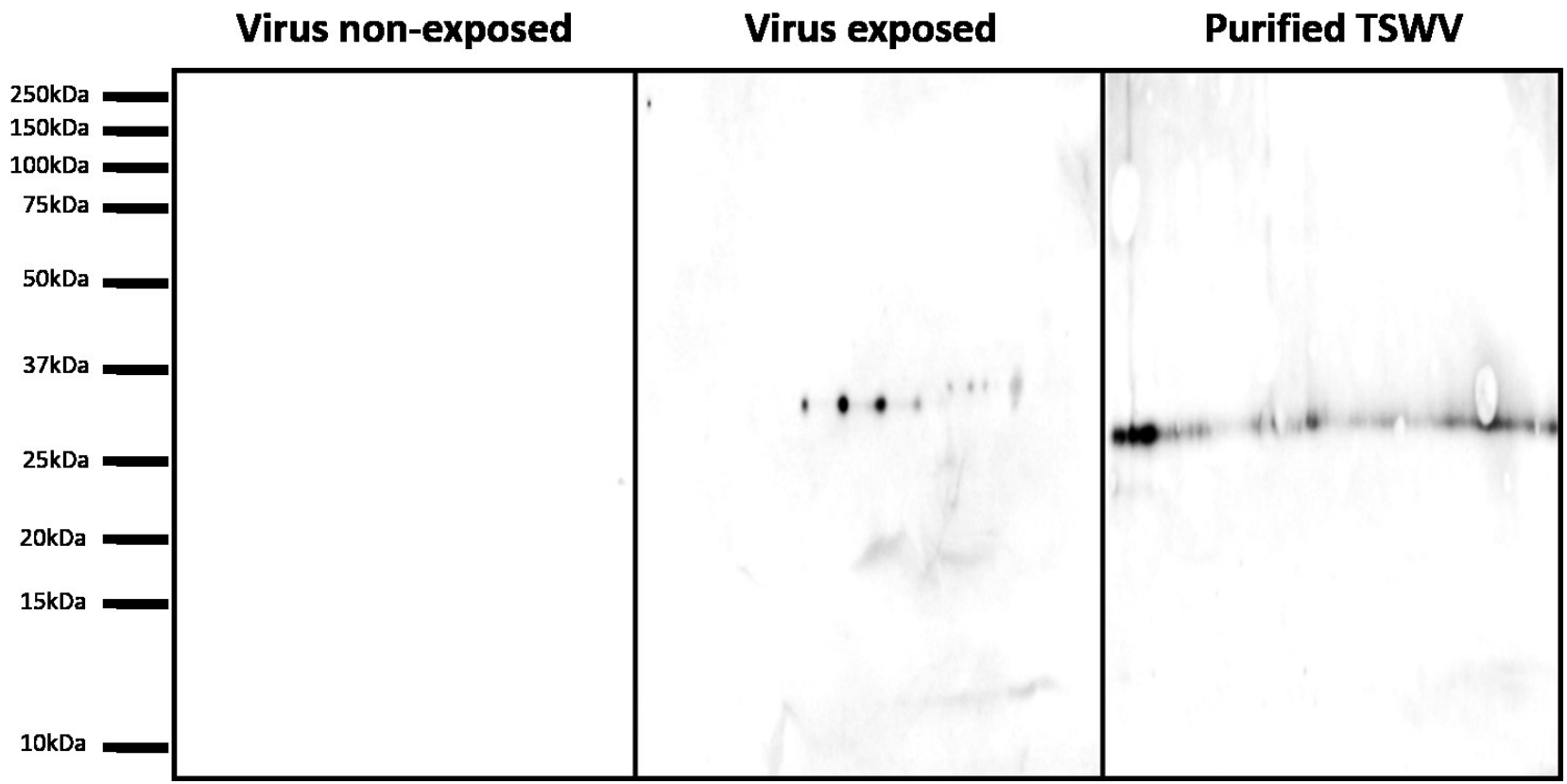

Figure 2.7 Western blots of two-dimensional (2-D) gels from TSWV-exposed and non-exposed *Frankliniella occidentalis* first instar larvae and purified virus.

Three hundred and seventy five micrograms of total proteins extracted from 600 pooled first instar larvae of *F. occidentalis* that were exposed (90% infection rate) or non-exposed to TSWV were resolved in 2-D gels using an 11 cm IPG strip with a pH range of 3 to 10, followed by standard SDS-polyacrylamide gel electrophoresis (10-20% polyacrylamide). Additionally, proteins from TSWV purified from *Emilia sonchifolia* were resolved in a 2-D gel and used as positive control. The proteins from these 2-D gels were transferred to a nitrocellulose membrane that were tested with a polyclonal antibody against TSWV N protein (~29 kDa).

Table 2.1 Identified proteins from healthy *Frankliniella occidentalis* first instar larvae.

Spot #	Database used for identification[a]	*F. occidentalis* transcript identifier	Protein ID[d]	Organism with sequence similarity	Accession number	Mascot score		# of peptides matched	
						MS	MS/MS	MS	MS/MS
1	*Fo*	CL2461Contig1_S454	similar to calreticulin isoform 1	*Apis mellifera*	XP_392689.2	100	524	21	6
1	M		calreticulin	*Drosophila melanogaster*	gi|6063416		51		1
3	*Fo*	CL1605Contig1_S454	cuticular protein RR-1 family member 16	*Nasonia vitripennis*	NP_001161311.1		362		3
3	M		hCG1650121, isoform CRA_a	*Homo sapiens*	gi|119567926		43		1
4	*Fo*	contig04259	cuticular protein RR-1 motif 43	*Bombyx mori*	NP_001166711.1		148		1
5	*Fo*	CL5080Contig1_S454	conserved hypothetical protein	*Tribolium castaneum*	XP_970222.2	90	370	9	4
6	*Fo*	CL5080Contig1_S454	conserved hypothetical protein	*Tribolium castaneum*	XP_970222.2	80	250	6	4
6	M		similar to ENSANGP00000015016	*Nasonia vitripennis*	gi|156548106		53		1
7	*Fo*	contig26596	myosin heavy chain, isoform N	*Drosophila melanogaster*	NP_001162990.1	166	87	23	1
7[c]	M		AGAP010147-PA	*Anopheles gambiae* str. PEST	gi|158299190	94		26	
7[c]	M		myosin heavy chain, nonmuscle or smooth muscle	*Aedes aegypti*	gi|157110721		154		2
8	*Fo*	CL4382Contig1_S454	cuticular protein RR-1 family member 39	*Nasonia vitripennis*	NP_001161274.1	121	286	10	4
9	*Fo*	CL4382Contig1_S454	cuticular protein RR-1 family member 39	*Nasonia vitripennis*	NP_001161274.1	84	72	9	1
10	*Fo*	contig17167	cuticular protein tweedle motif 1	*Bombyx mori*	NP_001166628.1		54		1
10	M		integrin alpha 7A subunit	*Homo sapiens*	gi|2654173		44		1
11[b]	*Fo*	FOAA-aab46e12.g1	---	---	---	67		7	
11[b]	*Fo*	CL3474Contig1_S454	conserved hypothetical protein	*Culex quinquefasciatus*	XP_001867362.1		238		2
12	*Fo*	contig14713	cuticular protein 111, RR-3 family	*Acyrthosiphon pisum*	XP_001950838.1	85	174	15	4
13	*Fo*	contig14713	cuticular protein 111, RR-3 family	*Acyrthosiphon pisum*	XP_001950838.1	97	193	15	5
14	*Fo*	contig14713	cuticular protein 111, RR-3 family	*Acyrthosiphon pisum*	XP_001950838.1	74	198	13	4
15	*Fo*	CL3385Contig1_S454	cuticular protein 47Ef CG13214-PA	*Tribolium castaneum*	XP_968350.1	80	236	7	3
16	*Fo*	CL3385Contig1_S454	cuticular protein 47Ef CG13214-PA	*Tribolium castaneum*	XP_968350.1		338		3
16	M		CG14676	*Drosophila melanogaster*	gi|24644452		50		1
18	*Fo*	CL208Contig1_S454	monocarboxylate transporter	*Pediculus humanus corporis*	XP_002426810.1		108		1
19	*Fo*	CL208Contig1_S454	monocarboxylate transporter	*Pediculus humanus corporis*	XP_002426810.1		65		1
20	*Fo*	CL4854Contig1_S454	peptidyl-prolyl cis-trans isomerase 2	*Nasonia vitripennis*	XP_001607048.1		47		1
22	*Fo*	CL169Contig1_S454	calmodulin	*Culex quinquefasciatus*	XP_001849785.1		51		1

Spot #	Database used for identification[a]	*F. occidentalis* transcript identifier	Protein ID[d]	Organism with sequence similarity	Accession number	Mascot score		# of peptides matched	
						MS	MS/MS	MS	MS/MS
22[c]	M		KRT9 protein	*Homo sapiens*	gi|113197968	207		19	
22[c]	M		cytokeratin 9	*Homo sapiens*	gi|435476		66		1
23	*Fo*	CL54Contig1_S454	myosin 1 light chain	*Apis mellifera*	XP_393544.2		136		2
23	M		myosin 1 light chain-like protein	*Maconellicoccus hirsutus*	gi|121543987		110		1
24	*Fo*	CL2263Contig1_S454	myosin regulatory light chain 2	*Bombyx mori*	NP_001091813.1		71		1
25	*Fo*	contig05467	cuticular protein 78, RR-1 family	*Anopheles gambiae* str. PEST	XP_318996.4		35		1
26	*Fo*	CL4310Contig1_S454	ATP synthase alpha subunit	*Aedes aegypti*	XP_001655906.1	82	214	13	3
26	M		putative mitochondrial ATP synthase alpha subunit precursor	*Toxoptera citricida*	gi|52630965		57		1
27	M		keratin 10	*Homo sapiens*	gi|186629	99		14	
28	*Fo*	CL1680Contig1_S454	endocuticle structural glycoprotein SgAbd-2	*Pediculus humanus corporis*	XP_002428599.1		464		4
29	*Fo*	contig11827	cuticule protein	*Pediculus humanus corporis*	XP_002423073.1		225		3
30	*Fo*	CL4382Contig1_S454	cuticular protein RR-1 family member 39	*Nasonia vitripennis*	NP_001161274.1	83	328	9	5
30	M		heat shock 70kDa protein 8	*Danio rerio*	gi|94732277	71		8	
31[b]	*Fo*	contig07826	BRCA1-associated ring domain protein	*Danaus plexippus*	EHJ70219.1	63		7	
31[b]	*Fo*	CL4704Contig1_S454	cAMP-dependent protein kinase inhibitor beta	*Pediculus humanus corporis*	XP_002430533.1		40		1
31	M		unknown	*Homo sapiens*	gi|11692692		62		2
32	*Fo*	CL4310Contig1_S454	ATP synthase alpha subunit	*Aedes aegypti*	XP_001655906.1		55		2
33	*Fo*	CL1081Contig1_S454	sortilin-related receptor L	*Nasonia vitripennis*	NP_001123523.1		75		2
34[b]	*Fo*	contig27831	---	---	---	67		9	
34[b]	*Fo*	contig00544	nicotinic acetylcholine receptor alpha 6 subunit 4	*Tribolium castaneum*	NP_001153541.1		41		1
34	M		predicted protein	*Nematostella vectensis*	gi|156384837	77		10	
35	M		ribosomal protein P2 isoform A	*Lysiphlebus testaceipes*	gi|62083357		65		1
38	M		chain B, complex between nucleosome core particle (H3,H4,H2a,H2b) and 146 bp long DNA Fragment	*Xenopus laevis*	gi|3745759	74		7	
42	*Fo*	CL3413Contig1_S454	cellular FABP-like protein 2	*Tribolium castaneum*	NP_001164131.1	117	77	10	2
43	*Fo*	CL798Contig1_S454	elongation factor 2 isoform 1	*Apis mellifera*	XP_392691.2		95		1

Spot #	Database used for identification[a]	*F. occidentalis* transcript identifier	Protein ID[d]	Organism with sequence similarity	Accession number	Mascot score		# of peptides matched	
						MS	MS/MS	MS	MS/MS
43	M		elongation factor	*Caenorhabditis elegans*	gi|156279		95		1
44	*Fo*	FOAA-aaa81c05.g1	vacuolar ATP synthase subunit G	*Acyrthosiphon pisum*	NP_001119628.1		67		1
44	M		vacuolar ATPase G subunit	*Maconellicoccus hirsutus*	gi|121543630		67		1
49	*Fo*	contig26395	---	---	---		134		3
50	*Fo*	contig11827	cuticule protein	*Pediculus humanus corporis*	XP_002423073.1		147		2
51[b]	*Fo*	CL3568Contig1_S454	ubiquitin	*Culicoides sonorensis*	AAV84265.1	72		7	
51[b]	*Fo*	FOAA-aab28b04.g1	hypothetical protein UM04588.1	*Ustilago maydis* 521	XP_760735.1		123		2
51[c]	M		ubiquitin	*Littorina littorea*	gi|164510076	91		7	
51[c]	M		ubiquitin	*Drosophila melanogaster*	gi|158759		123		2
52[b]	*Fo*	CL3568Contig1_S454	ubiquitin	*Culicoides sonorensis*	AAV84265.1	68		6	
52[b]	*Fo*	FOAA-aab28b04.g1	hypothetical protein UM04588.1	*Ustilago maydis* 521	XP_760735.1		131		2
52[c]	M		ubiquitin	*Littorina littorea*	gi|164510076	84		6	
52[c]	M		ubiquitin	*Drosophila melanogaster*	gi|158759		131		2
53	*Fo*	contig11827	cuticule protein	*Pediculus humanus corporis*	XP_002423073.1		54		1
56	*Fo*	contig03553	similar to ENSANGP00000011747	*Nasonia vitripennis*	XP_001599992.1	74	56	8	1
58	*Fo*	contig25916	similar to CG2852-PA	*Nasonia vitripennis*	XP_001604234.1	64		10	
59	*Fo*	contig15005	hypothetical protein	*Tribolium castaneum*	XP_976233.2		77		1
61	*Fo*	CL4854Contig1_S454	peptidyl-prolyl cis-trans isomerase 2	*Nasonia vitripennis*	XP_001607048.1	63	95	14	2
62	*Fo*	FOAA-aaa17c02.g1	similar to H2A histone	*Tribolium castaneum*	XP_967411.1		36		1
63	*Fo*	contig23633	---	---	---		51		1
65[b]	*Fo*	CL66Contig1_S454	muslce protein 20-like protein	*Anoplophora glabripennis*	AAY68367.1	92		14	
65[b]	*Fo*	contig17431	similar to sugar transporter	*Nasonia vitripennis*	XP_001604576.1		70		2
66	*Fo*	CL198Contig1_S454	agrin	*Schistosoma mansoni*	XP_002577398.1		42		1
80	*Fo*	contig00662	similar to CG13124-PA	*Apis mellifera*	XP_393579.2		34		1
81	M		actin	*Drosophila melanogaster*	gi|156763		253		3
90	*Fo*	CL4382Contig1_S454	cuticular protein RR-1 family member 39	*Nasonia vitripennis*	NP_001161274.1	75	48	9	1
92	*Fo*	contig05881	hypothetical protein	*Danio rerio*	XP_001333755.3	69		7	
93	M		melanoma-associated antigen E2	*Homo sapiens*	gi|20162570	71		11	
98	M		skeletal alpha-actin	*Sparus aurata*	gi|6653228	84		13	

Spot #	Database used for identification[a]	*F. occidentalis* transcript identifier	Protein ID[d]	Organism with sequence similarity	Accession number	Mascot score		# of peptides matched	
						MS	MS/MS	MS	MS/MS
102	*Fo*	CL1656Contig1_S454	glutathione S-transferase	*Culex quinquefasciatus*	XP_001847604.1	98	85	14	1
104	*Fo*	contig26349	endocuticle structural glycoprotein SgAbd-2	*Pediculus humanus corporis*	XP_002428599.1	63	126	6	3
105	*Fo*	CL29Contig1_S454	similar to Cbp20	*Nasonia vitripennis*	XP_001608109.1		104		1
105	M		similar to CG15006-PA	*Apis mellifera*	gi|110764439		47		1
109	*Fo*	FOAA-aab00f06.g1	GK13576	*Drosophila willistoni*	XP_002072646.1		54		2
111	*Fo*	CL4382Contig1_S454	cuticular protein RR-1 family member 39	*Nasonia vitripennis*	NP_001161274.1	88	113	8	2
113	*Fo*	CL3105Contig1_S454	glyceraldehyde-3-phosphate dehydrogenase	*Bombyx mori*	NP_001037386.1	84		7	
114	*Fo*	contig25068	hypothetical protein	*Entamoeba dispar* SAW760	XP_001737751.1	66		6	
116	*Fo*	CL18Contig1_S454	heat shot protein cognate 4	*Apis mellifera*	NP_001153522.1	118	35	10	1
116	M		heat shock 70kDa protein 8	*Danio rerio*	gi|94732277	103		7	
132	*Fo*	contig19365	superoxide dismutase	*Culex quinquefasciatus*	XP_001866335.1	70		6	
134	*Fo*	CL862Contig1_S454	phospholipid hydroperoxide glutathione peroxidase	*Pediculus humanus corporis*	XP_002429001.1		37		2
135	*Fo*	CL3385Contig1_S454	cuticular protein 47Ef CG13214-PA	*Tribolium castaneum*	XP_968350.1	77	34	7	1
141	*Fo*	contig16281	---	---	---		36		1
141	M		AGAP007997-PA	*Anopheles gambiae* str. PEST	gi|118789514		48		1
145	*Fo*	CL1184Contig1_S454	nascent polypeptide associated complex protein alpha subunit	*Apis mellifera*	XP_623555.1		101		1
145	M		nascent polypeptide associated complex protein alpha subunit	*Drosophila melanogaster*	gi|1632784		69		1
146[b]	*Fo*	contig27610	actin	*Hypochilus thorelli*	ABZ91668.1	86		9	
146[b]	*Fo*	contig01320	actin related protein 1	*Nasonia vitripennis*	NP_001157191.1		293		4
146[c]	M		skeletal muscle alpha-actin	*Cyprinus carpio*	gi|37813312	145		14	
146[c]	M		unnamed protein product	*Mus musculus*	gi|74195718		309		5
147	*Fo*	contig01320	actin related protein 1	*Nasonia vitripennis*	NP_001157191.1	98	84	11	2
147[c]	M		AGAP011516-PA	*Anopheles gambiae* str. PEST	gi|158293921	144		15	
147[c]	M		actin A3	*Bombyx mori*	gi|5751		78		1
150	*Fo*	contig00252	similar to ENSANGP00000012893	*Nasonia vitripennis*	XP_001606463.1	63		7	
151	*Fo*	CL18Contig1_S454	heat shot protein cognate 4	*Apis mellifera*	NP_001153522.1	66		13	
153	*Fo*	contig16955	similar to vATPase subunit A	*Nasonia vitripennis*	XP_001604685.1	120	171	22	4

Spot #	Database used for identification[a]	*F. occidentalis* transcript identifier	Protein ID[d]	Organism with sequence similarity	Accession number	Mascot score		# of peptides matched	
						MS	MS/MS	MS	MS/MS
153	M		ATPase	*Homo sapiens*	gi|291866	94	130	15	3
154	*Fo*	contig17167	cuticular protein tweedle motif 1	*Bombyx mori*	NP_001166628.1	64		6	
157	*Fo*	CL2000Contig1_S454	similar to ENSANGP00000014839	*Nasonia vitripennis*	XP_001600045.1	125	102	14	2
157[c]	M		similar to 60 kDa heat shock protein, mitochondrial precursor	*Apis mellifera*	gi|66547450	83		10	
157[c]	M		60kDa heat shock protein	*Drosophila melanogaster*	gi|1653979		71		1
158	*Fo*	CL2000Contig1_S454	similar to ENSANGP00000014839	*Nasonia vitripennis*	XP_001600045.1	84	48	10	1
158	M		similar to 60 kDa heat shock protein, mitochondrial precursor	*Drosophila melanogaster*	gi|1653979		48		1
159	*Fo*	contig04030	troponin t	*Culex quinquefasciatus*	XP_001851541.1		78		1
159	M		RNA binding motif protein 25, isoform CRA_a	*Homo sapiens*	gi|119601492		47		1
160	*Fo*	contig15776	similar to AGAP012407-PA	*Tribolium castaneum*	XP_975184.2		37		1
161[b]	*Fo*	CL618Contig1_S454	beta-tubulin 1	*Monochamus alternatus*	ABY66392.1	103		17	
161[b]	*Fo*	CL1556Contig1_S454	beta-tubulin	*Bombyx mori*	NP_001036964.1		70		4
161	M		beta-tubulin	*Theromyzon tessulatum*	gi|127906328	126		14	
162	*Fo*	CL788Contig1_S454	tubulin alpha-1 chain	*Pediculus humanus corporis*	XP_002429121.1	95	164	12	4
162[c]	M		alpha-tubulin	*Cryptocercus punctulatus*	gi|119117127	103		12	
162[c]	M		alpha-tubulin at 84B	*Drosophila melanogaster*	gi|17136564		156		3
163	*Fo*	CL321Contig1_S454	ATP synthase beta subunit	*Tribolium castaneum*	NP_001164361.1		96		1
163[c]	M		putative ATP synthase beta subunit	*Maconellicoccus hirsutus*	gi|124487966	109		11	
163[c]	M		ATP synthase beta subunit	*Drosophila melanogaster*	gi|287945		270		3
164	*Fo*	CL321Contig1_S454	ATP synthase beta subunit	*Tribolium castaneum*	NP_001164361.1		83		1
164[c]	M		ATP synthase beta subunit	*Aedes aegypti*	gi|157132308	128		13	
164[c]	M		beta-subunit	*Bos taurus*	gi|104		178	2	
165	*Fo*	CL321Contig1_S454	ATP synthase beta subunit	*Tribolium castaneum*	NP_001164361.1	69	54	7	1
165[c]	M		putative ATP synthase beta subunit	*Maconellicoccus hirsutus*	gi|124487966	149		14	
165[c]	M		ATP synthase subunit family member (atp-2)	*Caenorhabditis elegans*	gi|25144756		160		2
166	*Fo*	CL321Contig1_S454	ATP synthase beta subunit	*Tribolium castaneum*	NP_001164361.1		81		1
166[c]	M		ATP synthase beta subunit	*Aedes aegypti*	gi|157132308	103		11	
166[c]	M		beta-subunit	*Bos taurus*	gi|104		211		3

Spot #	Database used for identification[a]	*F. occidentalis* transcript identifier	Protein ID[d]	Organism with sequence similarity	Accession number	Mascot score		# of peptides matched	
						MS	MS/MS	MS	MS/MS
167	*Fo*	CL1451Contig1_S454	GI17663	*Drosophila mojavensis*	XP_002002963.1	90		15	
168[b]	*Fo*	contig02378	alpha-tubulin 1	*Monochamus alternatus*	ABU24274.1	66		9	
168[b]	*Fo*	CL1922Contig1_S454	vATPase 55kD subunit B	*Drosophila melanogaster*	NP_476908.1		70		3
170	*Fo*	CL4706Contig1_S454	similar to AGAP007827-PA	*Tribolium castaneum*	XP_967559.1	72		8	
171	*Fo*	CL4706Contig1_S454	similar to AGAP007827-PA	*Tribolium castaneum*	XP_967559.1	68		8	
172	*Fo*	contig01320	actin related protein 1	*Nasonia vitripennis*	NP_001157191.1	98	256	13	3
172[c]	M		similar to actin-87E isoform 1	*Apis mellifera*	gi|66509780	154		18	
172[c]	M		RecName: Full=Actin, muscle-type	*Molgula oculata*	gi|3121741		303		4
173	*Fo*	CL299Contig1_S454	similar to RE12057p isoform 2	*Acyrthosiphon pisum*	XP_001943291.1		59		1
173	M		actin A3	*Bombyx mori*	gi|5751		59		1
174	M		similar to tubulin, beta, 2	*Apis mellifera*	gi|48095547	73		10	
176	*Fo*	contig01307	similar to pupal cuticle protein 78E	*Tribolium castaneum*	XP_969263.1	64		8	
177	*Fo*	contig01307	similar to pupal cuticle protein 78E	*Tribolium castaneum*	XP_969263.1	65	80	9	2
178	*Fo*	contig17167	cuticular protein tweedle motif 1	*Bombyx mori*	NP_001166628.1	65	186	6	3
179	*Fo*	contig17167	cuticular protein tweedle motif 1	*Bombyx mori*	NP_001166628.1		134		3
181	*Fo*	CL357Contig1_S454	eukaryotic initiator factor 4a	*Tribolium castaneum*	NP_001177648.1		107		2
181	M		similar to eukaryotic initiation factor 4A (ATP-dependent RNA helicase eIF4A) (eIF-4A) isoform 2	*Apis mellifera*	gi|66551115		100		2
184	M		cysteine protease CP7 precursor	*Frankliniella occidentalis*	gi|15593246		70		1
185	M		cysteine protease CP7 precursor	*Frankliniella occidentalis*	gi|15593246		104		2
187	*Fo*	contig22086	similar to cuticular protein 47Ef CG13214-PA	*Tribolium castaneum*	XP_968350.1		53		1
192	*Fo*	CL4870Contig1_S454	mitochondrial peptidase beta subunit	*Culex quinquefasciatus*	XP_001863592.1	90		14	
193	*Fo*	contig01320	actin related protein 1	*Nasonia vitripennis*	NP_001157191.1		71		1
193	M		beta-actin	*Culex pipiens pipiens*	gi|90811719		95		2

[a] Protein identification was obtained using the *Fo* Seq (*Fo*) and the Metazoan nr protein database from NCBI (M).

[b] Different *F. occidentalis* transcripts matched peptide sequences from a single protein spot within the 2-D gel of healthy larval thrips.

[c] Different sequences from the Metazoan nr protein database matched the same peptide stretches from a single protein spot within the 2-D gel of healthy larval thrips.

[d] ID, identification.

Table 2.2 *Frankliniella occidentalis* proteins from naïve first instar larvae with putative roles in insect innate immunity.

Spot #	*F. occidentalis* transcript identifier or NCBI accession number	Protein ID	Putative role in defense
28, 162	CL1680Contig1_S454, CL788Contig1_S454/ gi|17136564	tubulin alpha-1 chain	phagocytosis
161, 174	CL618Contig1_S454/ CL1556Contig1_S454/ gi|127906328, gi|48095547	beta tubulin	regulation of apoptosis
134	CL862Contig1_S454	glutaredoxin 5	cell redox homeostasis
19, 25, 26, 30, 116, 157, 158	CL208Contig1_S454, contig05467, CL4310Contig1_S454, gi|94732277, CL18Contig1_S454/gi|94732277, CL2000Contig1_S454/gi|66547450/gi|1653979, CL2000Contig1_S454//gi|1653979	heat shock protein	response to biotic/abiotic stress
184, 185	gi|15593246, gi|15593246	cysteine protease	ubiquitin cycle
181	CL357Contig1_S454	lethal giant larvae homologue	induction of salivary gland cells autophagic cell death

Table 2.3 *Frankliniella occidentalis* proteins differentially expressed between *Tomato spotted wilt virus*-exposed and non-exposed first instar larvae.

Spot #	*p*-value from ANOVA	Fold change	Database searched (Sequence matched)	Mascot score	Protein ID	Identified motifs	*E* - value
Up-regulated in response to TSWV infection							
201	0.0046	1.22	*Fo*Seq (CL4589Contig1_S454)	625	triosephosphate isomerase	TIM_phosphate binding	5e-110
			NCBI (gi|259016078)	151	triosephosphate isomerase	TIM_phosphate binding	4e-41
47[a]	0.0257	1.22	*Fo*Seq (CL2263Contig1_S454)	422	myosin 3 light chain	Ca^{+2} binding site, EFh	2e-15
			NCBI (gi|312371061)	334	hypothetical protein AND_22684 (similar to Skp1)	Skp1	4e-80
836[a]	0.0326	1.57	*Fo*Seq (CL63Contig1_S454)	488	similar to voltage-dependent anion-selective channel 2	Porin3, Porin3_Tom40	6e-118
			NCBI (RS3_AMBME)	124	40S ribosomal protein S3	40S_S3_KH, KH-II, Ribosomal_S3_C	1e-160
137	0.0355	1.54	*Fo*Seq (CL4310Contig1_S454)	264	mitochondrial ATP synthase α subunit	ATPase_alpha	0.0
			NCBI (gi|52630965)	261	mitochondrial ATP synthase α subunit	ATPase_alpha	0.0
271[a]	0.0433	1.12	*Fo*Seq (CL4116Contig1_S454)	499	cxpwmw03 (similar to electron transfer flavoprotein subunit alpha)	ETF_alpha, AANH-like	3e-153
			NCBI (gi|328786330)	129	saccharopine dehydrogenase-like isoform 1	GTP_cyclohydrol, TFold, NADB-Rossmann	0.0
374[a]	0.0508	1.69	*Fo*Seq (contig16594)	284	vitellogenin	lipoprotein N-terminal	5e-113
			NCBI (gi|156763)	155	actin	actin	0.0

Spot #	*p*-value from ANOVA	Fold change	Database searched (Sequence matched)	Mascot score	Protein ID	Identified motifs	*E* - value
277	0.0551	1.28	*Fo*Seq (CL3352Contig1_S454)	215	alpha-tocopherol transfer protein-like isoform 1	SEC14	1e-164
			NCBI (no match)	---------	---------	---------	---------
134	0.0616	1.36	*Fo*Seq (CL2397Contig1_S454)	557	vacuolar proton ATP synthase subunit E	V-ATP_synt-E	4e-71
			NCBI (gi|46561760)	300	vacuolar proton ATP synthase subunit E	V-ATP_synt-E	7e-110
161	0.0662	1.40	*Fo*Seq (CL4706Contig1_S454)	782	AGAP007827-PA isoform 1 (similar to enolase)	enolase-like	0.0
			NCBI (gi|157121051)	434	enolase	enolase-like	0.0
72[a]	0.0668	1.21	*Fo*Seq (CL1656Contig1_S454)	956	glutathione S-transferase	thioredoxin-like, GST_Sigma	4e-52
			NCBI (gi|66565444)	149	60S ribosomal protein L9	ribosomal_L6	2e-101
Down-regulated in response to TSWV infection							
244	0.0090	2.05	*Fo*Seq (contig19201)	389	cytochrome b-c1 complex subunit 2	peptidase_M16, peptidase M16_C	2e-103
			NCBI (no match)	---------	---------	---------	---------
481[a]	0.0128	1.50	*Fo*Seq (contig15458)	323	apolipoprotein D	lipocalin_2	4e-84
			NCBI (K1C10_HUMAN)	534	keratin type I cytoskeletal 10	Filament	0.0
310	0.0338	1.62	*Fo*Seq (no match)	---------	---------	---------	---------
			NCBI (no match)	---------	---------	---------	---------

Spot #	*p*-value from ANOVA	Fold change	Database searched (Sequence matched)	Mascot score	Protein ID	Identified motifs	*E* - value
88	0.0466	1.26	*Fo*Seq (CL423Contig1_S454)	401	predicted hypothetical protein (similar to rad23)	RAD_23, UBA, XPC binding	1e-77
			NCBI (gi|242023622)	169	UV excision repair protein rad23	RAD_23, UBA, XPC binding	3e-164
794	0.0460	1.29	*Fo*Seq (contig30771)	214	---------	---------	---------
			NCBI (LYSC_CHICK)	263	lysozyme C	LYZ1, Lys	6e-110
378[a]	0.0486	1.95	*Fo*Seq (CL1213Contig1_S454)	495	AGAP009685-PA (similar to aspartate aminotransferase)	AAT_I	0.0
			NCBI (gi|156763)	215	actin	actin	0.0
319	0.0548	1.28	*Fo*Seq (contig17796)	232	peroxiredoxin 1-like	thioredoxin-like	5e-83
			NCBI (gi|58377838)	88	thioredoxin-dependent peroxidase	thioredoxin-like	2e-111
819[a]	0.0589	2.42	*Fo*Seq (CL827Contig1_S454)	360	zinc finger protein 1	ZPR1_znf, zf-ZPR1, ZPR1	0.0
			NCBI (PDK3_HUMAN)	264	pyruvate dehydrogenase kinase 3	HATPase_C, BCDHK_Adom3, Bae5, Vick	0.0
340[a]	0.0597	1.92	*Fo*Seq (CL2403Contig1_S454)	523	hypothetical protein SINV_09553 (similar to stress-induced phosphoprotein 1)	Tad D, TPR	0.0
			NCBI (gi|170027766)	137	electron transfer flavoprotein – ubiquinone oxidoreductase	Pyr_Redox	0.0
430[a]	0.0667	1.38	*Fo*Seq (CL862Contig1_S454)	425	phospholipid hydroperoxide glutathione peroxidase	thioredoxin-like, GSH_peroxidase	2e-67
			NCBI (gi|242008321)	243	conserved hypothetical protein (similar to actin-depolimerization factor 1)	ADF	1e-81

Spot #	*p*-value from ANOVA	Fold change	Database searched (Sequence matched)	Mascot score	Protein ID	Identified motifs	*E* - value
196[a]	0.0763	1.12	*Fo*Seq (CL4854Contig1_S454)	512	cyclophilin	cyclophilin_ABH, CLD	6e-83
			NCBI (gi|307643755)	228	glyceraldehyde-3-phosphate dehydrogenase	Gp_dh_C	3e-109
803	0.0787	1.43	*Fo*Seq (CL18Contig1_S454)	362	heat shock protein 70 cognate 4	HSP70, Dnak, HscA	0.0
			NCBI (HSP7C_BOVIN)	218	heat shock cognate 71 protein	HSP70, Dnak, HscA	0.0
283[a]	0.0833	1.29	*Fo*Seq (contig16705)	625	protein yellow-like	MRJP	2e-138
			NCBI (gi|157134067)	248	26S protease regulatory subunit 7	P-loop NTPase	3e-162
296	0.0851	1.55	*Fo*Seq (contig14504)	240	arginine kinase	phosphagen_ kinase	2e-129
			NCBI (gi|161088212)	227	arginine kinase	phosphagen_ kinase	4e-130
206	0.0997	1.15	*Fo*Seq (contig14504)	640	arginine kinase	phosphatase_kinase	1e-103
			NCBI (gi|161088180)	496	arginine kinase	phosphatase_kinase	1e-104
182	0.0998	1.35	*Fo*Seq (CL3105Contig1_S454)	422	glyceraldehyde-3-phosphate dehydrogenase	Gp_dh_C/N	3e-101
			NCBI (gi|156547538)	435	glyceraldehyde-3-phosphate dehydrogenase	Gp_dh_C/N	2e-173

[a] Different proteins were identified within a single spot using the *Fo* Seq and the Metazoan nr protein database from NCBI.

Chapter 3 - Identification of *Frankliniella occidentalis* proteins that directly interact with *Tomato spotted wilt virus*

Abstract

Tomato spotted wilt virus (TSWV), the type species of the *Tospovirus* genus, is transmitted from plant to plant by only few species of thrips in a persistent propagative fashion. The western flower thrips, *Frankliniella occidentalis*, is the most efficient thrips vector of TSWV. Acquisition of the virus that results in successful transmission of TSWV is restricted to the larval stages of the insect vector, with acquisition efficiencies decreasing as larval development proceeds. Once acquired, TSWV binds to and enters into the midgut, replicates in the midgut epithelial cells and surrounding muscle cells, then journeys to and replicates in the salivary gland tissues for transmission to occur. Although the biological aspects of this virus-vector interaction are well characterized, the thrips molecules that directly interact with TSWV virions during the infection process of the insect vector remain poorly understood. To begin to identify the thrips proteins that directly interact with TSWV, overlay assays of *F. occidentalis* larval proteins resolved by two-dimensional (2-D) gel electrophoresis were conducted. Overlay assays using purified TSWV virions or a TSWV glycoprotein (G_N), shown previously to play a role in viral attachment to thrips guts, enabled the detection of eight and eleven protein spots, respectively. Electrospray ionization (ESI) mass spectrometry coupled with Mascot searches identified that four unique proteins (cyclophilin, enolase, cuticular protein, and endocuticle structural glycoprotein) interacted with purified TSWV virions, while two proteins (mitochondrial ATP synthase α subunit and endocuticle structural glycoprotein) interacted with recombinant G_N. Homologous proteins to the thrips proteins identified here as putative interactors have been also identified in other virus-vector interactions, suggesting that these *F. occidentalis* proteins are promising candidates that warrant further examination. Identification of thrips proteins putatively involved in the viral infection of the insect vector provides new insights into the molecular basis of this interaction and novel targets to design control strategies to manage this agronomically important pathosystem.

Introduction

Vector-borne diseases caused by animal- and plant-infecting viruses are among the most important medical, veterinary, and agricultural problems worldwide (Gubler, 1998; Gubler, 2002). The virus family *Bunyaviridae* is composed of four arthropod-borne genera (*Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*) and one rodent-borne genus (*Hantavirus*). Viruses within this family can cause severe morbidity, mortality, and agricultural crop losses wherever they and their vectors can thrive and persist (de Oliveira et al., 2012; Guler et al., 2012; Herder et al., 2012; McMullan et al., 2012; Walter and Barr, 2011). The genus *Tospovirus* is the only genus in this family whose members infect plants. Eight approved species have been reported by the International Committee on the Taxonomy of Viruses while twenty one tentative species have been proposed to be members of this genus (http://ictvonline.org/virusTaxonomy.asp?msl_id=28). However, the type species of the genus, *Tomato spotted wilt virus* (TSWV), is the best characterized tospovirus with respect to genome organization, coding strategy, protein function, and the biological interaction with its insect vector (reviewed in Whitfield et al., 2005a).

TSWV has a tripartite, single-stranded, ambisense RNA genome enclosed in a host-derived lipid membrane that forms pleomorphic virions of about 80 to 120 nanometers in diameter (Gonzalez-Scarano and Nathanson, 1996; Shope, 1985). It encodes six viral proteins from five open reading frames (ORFs) using a negative (L RNA segment) or an ambisense (M and S RNA segments) coding strategy (de Haan et al., 1990; de Haan et al., 1991; Kikkert et al., 1997; Kormelink et al., 1992). The L protein, encoded from the L RNA segment, is the RNA-dependent RNA-polymerase and around 10 to 20 copies are packaged within a single virion (Adkins et al., 1995; Adkins, 2000). The nonstructural medium (NSm) protein and the glycoproteins are both encoded from the M RNA segment (Kormelink et al., 1992). The NSm protein is involved in cell-to-cell and systemic movement of the virus in plants (Kormelink et al., 1994; Lewandowski and Adkins, 2005; Li et al., 2009; Storms et al., 1998; Storms et al., 1995). The viral glycoproteins are encoded as a polyprotein that is cleaved to generate the two mature glycoproteins and are designated G_N and G_C based on their location at the N- and C-terminus in the polyprotein (Adkins et al., 1996). The viral glycoproteins are involved in attachment and

fusion during the entry of the virus into the midgut epithelial cells of the insect vector (Whitfield et al., 2004; Whitfield et al., 2008; Whitfield et al., 2005b). The S RNA segment encodes the nonstructural small protein (NSs), which is a functional silencing suppressor, and the nucleocapsid (N) protein that encapsidates the genomic RNA to form the ribonucleocapsids that are packaged into the virions (Bucher et al., 2003; de Haan et al., 1990; Richmond et al., 1998; Schnettler et al., 2010; Takeda et al., 2002).

Viruses in the genus *Tospovirus* are exclusively transmitted by thrips in a persistent propagative fashion (Ullman et al., 1993; Wijkamp et al., 1993). For acquisition to occur, larval thrips have to feed on virus-infected plants and obtain virus particles that will travel through their digestive tract and attach to a yet unidentified receptor to enter the midgut epithelial cells (Lindord, 1932; Ullman et al., 1995; van de Wetering et al., 1996). It is in the midgut and surrounding muscle tissues where the virus undergoes the first round of replication within its insect vector (Tsuda et al., 1996; Ullman et al., 1992; Ullman et al., 1995; Wijkamp et al., 1993). By an unknown mechanism, the virus must escape the midgut and most likely using the ligament-like structures and the accessory salivary glands it reaches the principal salivary glands where a second round of replication takes place (Ullman et al., 1995; Wijkamp et al., 1993). Finally, adult thrips are able to transmit the virus during short, sampling probes where a mix of saliva and virus particles are inoculated into intact plant cells (Sakimura, 1962; Sakimura, 1963). Tospoviruses are not transovarially transmitted from virus-infected adults to their offspring (Wijkamp et al., 1996). Despite the knowledge acquired during the last three decades about the TSWV genome, coding strategies, proteins, and biological interaction with its insect vector, the thrips molecules that play an intrinsic role in vector competency during TSWV entry, escape, and spread through the insect's body are just beginning to be described.

The western flower thrips, *Frankliniella occidentalis* Pergande, is one of the most efficient vectors of TSWV and other tospoviruses (Riley et al., 2011). It is the most economically important insect pest among thysanopterans due to its extremely wide host range, broad geographical distribution, and competence to transmit five species of tospoviruses. In the last years, new genomic resources have been developed for this thrips species with the generation of a partial transcriptome (Badillo-Vargas et al., 2012; Rotenberg and Whitfield, 2010). In 2014, the *F. occidentalis* genome has been sequenced by the i5K initiative and it reveals thus far that it encodes for over 17,000 proteins (Rotenberg and Whitfield, personal

communication). Using two-dimensional (2-D) gel electrophoresis coupled with mass spectrometry, an initial effort to begin to unravel the *F. occidentalis* response to TSWV infection has also been reported (Badillo-Vargas et al., 2012). However, *F. occidentalis* proteins that directly interact with TSWV or its viral attachment protein (G_N) remain elusive. Two previous studies attempted to identify thrips proteins that directly interact with TSWV or the viral glycoproteins (G_N and G_C). A 94-kDa protein from all the developmental stages of the two major vector species of TSWV, *F. occidentalis* and *Thrips tabaci*, exhibited specific binding to virus particles (Kikkert et al., 1998). The authors suggested that the 94-kDa protein may represent a receptor involved in circulation of TSWV through the insect vector because it was present throughout the thrips body. Moreover, a 50-kDa protein from larval and adult *F. occidentalis* was detected with monoclonal antibodies against TSWV glycoproteins in virus overlay assays (Bandla et al., 1998). The plasmalemma of larval thrips midguts was specifically labeled when using anti-idiotype antibodies to monoclonal antibodies against TSWV glycoproteins (Bandla et al., 1998). In both cases, either the 94- and 50-kDa proteins from thrips were not identified nor further characterized as cellular receptors for TSWV infection of the insect vector.

The cellular mechanisms that determine virus transmission are governed by the intrinsic interactions between viral components and proteins from the insect vectors. Virus overlay assays, also known as Far-Western blot, have been routinely used to further understand viruses infecting vertebrates by using mammalian cells to determine the interaction of viral attachment proteins with their corresponding cellular receptors (Boyle et al., 1987; Compton et al., 1992; Crane et al., 1991; Dalziel et al., 1991; Dveksler et al., 1993; Gershoni et al., 1986; Jin et al., 1994; Karger and Mettenleiter, 1996; Mizukami et al., 1996; Tayyari et al., 2011). Among plant viruses, the approach has been limited to investigate a few relationships between viruses and their insect vectors. For example, a screening of proteins from the green peach aphid, *Myzus persicae*, using *Potato leafroll virus* (PLRV; *Luteoviridae*: *Polerovirus*) and virus overlay assays identified that the plant virus directly interacts with symbionin, a protein that is abundantly synthesized by the primary bacterial endosymbiont *Buchnera aphidicola* (Vandenheuvel et al., 1994). Similarly, *Tomato yellow leaf curl virus* (TYLCV; *Geminiviridae*: *Begomovirus*) was found to interact with symbionin of the bacterial endosymbiont *Hamiltonella* from the whitefly *Bemisia tabaci* (Morin et al., 1999; Gottlieb et al., 2010). Moreover, TYLCV specifically bound

to *B. tabaci* heat shock protein 70 in virus overlay assays (Goetz et al., 2012). Recent technical advances in proteomic approaches and mass spectrometry have further impacted the use of overlay assays by better resolving insect proteins in two dimensions using 2-D gel electrophoresis. The helper-component protease (HC-Pro) of *Tobacco etch virus* (TEV; *Potyviridae*: *Potyvirus*) was found to bind specifically to a ribosomal protein (homologous to the laminin receptor precursor) of *M. persicae* in protein blots from 2-D gels (Fernandez-Calvino et al., 2010). Here, I have used virus overlay assays of 2-D gels to identify *F. occidentalis* larval proteins that directly interact with purified TSWV virions. Furthermore, I have conducted overlay assays using a recombinant G_N protein produced in bacterial cells to identify *F. occidentalis* larval proteins that specifically interact with the TSWV viral attachment protein. The overlay assays revealed that eight unique protein spots containing four different proteins specifically interacted with purified TSWV virions, while eleven protein spots containing two different proteins interacted with recombinant G_N. Sequence analysis of these putative *F. occidentalis* interacting larval proteins revealed their homology to proteins associated with the infection cycle of other vector-borne or entomopathogenic viruses. Validation of these protein-protein interactions using a heterologous insect expression system for additional virus overlay assays and custom-made antibodies for immunolocalization of these proteins within *F. occidentalis* larvae are currently underway. Ultimately, identification of these candidate proteins from *F. occidentalis* that directly interact with TSWV or its attachment protein provides new insights into the thrips molecules that might be involved in TSWV attachment and entry (cuticular protein and endocuticle structural glycoprotein), endocytosis/exocytosis (cyclophilin and mitochondrial ATP synthase α subunit), and basal lamina remodeling for escape from different tissues within the insect vector.

Results

Identification of larval thrips proteins that bound to purified *Tomato spotted wilt virus* in overlay assays of two-dimensional gels

To identify *F. occidentalis* proteins that directly interact with TSWV, larval thrips proteins extracted from whole insect bodies were separated by two-dimensional (2-D) gel electrophoresis and virus overlay assays using purified TSWV virions were carried out. Three

(spots 19, 21, and 25) and five (spots 7, 8, 20, 22, and 24) protein spots were detected in 4 and 3 replicates of the virus overlay assay, respectively (Figure 3.1). Using electrospray ionization (ESI) mass spectrometry, four types of proteins were consistently identified from three independent picking gels within these 8 protein spots (Table 3.1). A thrips protein similar to cuticular protein was identified in spots 19 and 21. Processing of the third protein spot that was also identified in the four replicates of the virus overlay assay resulted in the identification of a cuticular protein and an unknown protein with significant similarity to enolase both in spot 25. Among the protein spots detected in three replicates, an endocuticle structural glycoprotein, a cuticular protein, and a cyclophilin were identified in spots 8, 20, and 22, respectively. Two different proteins were identified in spots 7 (endocuticle structural glycoprotein and cyclophilin) and 24 (enolase and cuticular protein). All the thrips proteins identified within the eight spots that bound to purified TSWV had sequence similarities to proteins from other insect species (e.g. *Pediculus humanus corporis*, *Tribolium castaneum*, *Acyrthosiphon pisum*, etc.). Protein spots observed only in one or two replicates of the virus overlay assay were not further analyzed using ESI mass spectrometry. Moreover, no thrips proteins, different to the non-specific binding seen in the negative control, were observed to bind in an overlay assay using a healthy plant extract obtained from a sucrose gradient centrifugation process (also used to purified TSWV virions) of virus-free *Datura stramonium* plants (Figure 3.2).

Identification of larval thrips proteins that bound to a recombinant G_N glycoprotein in overlay assays of two-dimensional gels

Recombinant G_N expressed in bacterial cells was used to perform overlay assays of larval thrips proteins resolved in 2-D gels to identify a candidate TSWV receptor within *F. occidentalis* as the G_N glycoprotein from TSWV has been hypothesized to be the attachment viral protein (the ligand) that binds to a receptor in the insect vector. Eleven spots were identified in 2 biological replicates of the G_N overlay assay (Figure 3.3). Only two types of proteins were consistently identified from two independent picking gels within these 11 protein spots that bound to the recombinant G_N (Table 3.2). Spot 1 and 2 were both identified as a mitochondrial ATP synthase α subunit, while spots 3 through 11 were all identified as an endocuticle structural glycoprotein. It is worth to mention that an endocuticle structural glycoprotein was also identified in two spots

(7 and 8) detected in the virus overlay assay. Despite the fact that these two endocuticle structural glycoproteins are different in length and in sequence identity at the nucleotide and protein level, they both possess the Chitin_bind_4 motif. These eleven thrips proteins also were similar to proteins from other insect species (e.g. *Aedes aegypti* and *Harpegnathos saltator*).

Sequence analysis of thrips proteins that interacted with TSWV or recombinant G_N glycoprotein in overlay assays of two-dimensional gels

Thrips proteins that interacted with purified TSWV virions or recombinant G_N in overlay assays of 2-D gels were identified using the *Fo* Seq database reported previously (Badillo-Vargas et al., 2012). Sequence analysis was conducted to identify the complete open reading frame (ORF) and the predicted translated protein of the sequences from the *Fo* Seq database that matched *F. occidentalis* proteins identified in the overlay assays. Features of the predicted ORFs of cyclophilin (CL4854Contig1_S454), enolase (CL4706Contig1_S454), cuticular protein (CL4900Contig1_S454), endocuticle structural glycoprotein (contig01248), and mitochondrial ATP synthase α subunit (CL4310Contig1_S454) from *F. occidentalis* and their predicted translated proteins selected for further characterization are shown in Table 3.3. Moreover, Blastp analysis allowed the identification of specific motifs in these 5 thrips proteins.

The Cyclophilin_ABH motif (from residues 53 to 217) was identified in the *F. occidentalis* cyclophilin, which has a predicted ORF of 618 nucleotides to encode a protein of 206 amino acids. The *F. occidentalis* cyclophilin shares 88% identity with that of the jewel wasp, *Nasonia vitripennis* (GeneBank accession No: XP_001607048.1) (Table 3.4). The Cyclophilin_ABH motif identified in the *F. occidentalis* cyclophilin suggests that this interacting protein that bound to purified TSWV is either cyclophilin A, B or H. Sequence alignment of the *F. occidentalis* cyclophilin amino acid sequence with those from other organisms showed that it is most similar to a cyclophilin-like from the pea aphid, *Acyrthosiphon pisum* (GeneBank accession No: BAH70637), with an 84% identity. This *A. pisum* cyclophilin-like is predicted to be cyclophilin A. The cyclophilin protein from *F. occidentalis* has 37 and 46% identity with cyclophilin B (GeneBank accession No: NP_001156707) and cyclophilin H (GeneBank accession No: XP_001943358) from *A. pisum*, respectively. Moreover, it shares 62% identity with a cyclophilin from the human body louse, *Pediculus humanus corporis* (GeneBank

accession No: XP_002425817), and 73% identity to the cyclophilin A from human, *Homo sapiens* (GeneBank accession No: NP_066953). Thus, this *F. occidentalis* cyclophilin has been designated as cyclophilin A. This *F. occidentalis* cyclophilin is predicted to have 8 phosphorilation sites, 5 N-myristoylation sites, and 2 N-glycosylation sites. The shift observed in isoelectric point between the *F. occidentalis* cyclophilin protein identified in spots 7 and 22 (Fig. 3.1) might be due to N-myristoylation of this protein as this posttranslational modification changes the electrostatic charges of proteins.

The *F. occidentalis* enolase has a predicted ORF and protein of 1,302 nucleotides and 434 amino acids, respectively, which has a Metal_binding motif in it (from residues 361 to 374). This protein was most similar to an unknown protein from the mountain pine beetle, *Dendroctonus ponderosae*, sharing 87% identity (GeneBank accession No: AEE62848.1) (Table 3.4). Blastp analysis showed that this unknown protein from *D. ponderosae* is most similar to enolase from other insects. Further sequence alignment analysis showed that this *F. occidentalis* enolase shares 77% identity with *P. human corporis* (GeneBank accession No: XP_002430191), 81% identity with *A. pisum* (GeneBank accession No: XP_001948161), and 84% with *A. aegypti* (GeneBank accession No: XP_001653750) enolases. Sixteen phosphorylation sites and thirteen myristoylation sites were identified in this *F. occidentalis* enolase. Both spots 24 and 25 were identified as *F. occidentalis* enolase with a slight change in isoelectric point (Fig. 3.1), which can be due at least in part to the 13 myristoylation sites predicted in this protein found to bind to purified TSWV virions in overlay assays.

The cuticular protein and the endocuticle structural glycoprotein from *F. occidentalis* identified in the overlay assays have a predicted ORF of 159 and 522 nucleotides, respectively. The cuticular protein is predicted to encode for a protein of 53 amino acids while the endocuticle structural glycoprotein should encode for one of 174 amino acids. A Chitin_bind_4 motif was identified in these two proteins; from residues 29 to 32 and 62 to 132 in the cuticular protein and the endocuticle structural glycoprotein, respectively. The *F. occidentalis* cuticular protein shares 52% identity to a predicted protein similar to CG1136 CG1136-PA from the red flour beetle, *Tribolium castaneum* (GeneBank accession No: XP_001809883.1) (Table 3.4). In turn, this predicted protein is similar to cuticular proteins from a number of different insects. Sequence alignment analysis of *F. occidentalis* cuticular protein with that of *A. pisum* (GeneBank accession No: NP_001127760) and *P. humanus corporis* (GeneBank accession No:

XP_002423431) had 7% identity only. In the other hand, the *F. occidentalis* endocuticle structural glycoprotein share 40% identity with that of the body louse, *Pediculus humanus corporis* (GeneBank accession No: XP_002428599). Comparison of the brown planhopper, *Laodelphax striatella*, cuticular protein (GeneBank accession No: KC485263) shown to bound to *Rice stripe virus* with both the cuticular protein and the endocuticle structural glycoprotein of *F. occidentalis* showed that they are 9 and 25% similar, respectively. One to eight phosphorylation sistes, one to eleven myristoylation sites, and none to three N-glycosylation sites were predicted to occur in the *F. occidentalis* cuticular proteins that had entire ORFs (CL4900Contig1_S454, contig15374, and contig14594). Sequence alignment of all the cuticular proteins identified in the virus overlay assay clearly showed that these proteins are not the same. The difference in molecular weight among cuticular proteins identified in spots 19, 20, 21, and 22 (Fig. 3.1) could be because they are differente proteins and/or due to differences in phosphorylation pattern as this posttranslational modification changes the size of proteins. The endocuticle structural glycoprotein identified in the virus overlay assay is predicted to contain 2 phosphorilation sites and 3 N-myristoylation sites, while the one identified in G_N overlay assays is predicted to contain 4 phosphorilation sites and 3 N-myristoylation sites. Sequence alignment of these two endocuticle structural glycoproteins showed that they are not the same proteins. The shifts in isoelectric point and molecular weight observed in the endocuticle structural glycoproteins identified in the G_N overlay assay (Fig. 3.3) can be due to differences in N-myristoylation and phosphorylation, respectively.

The ATP-synt_ab_N and ATP-synt_ab_C motifs (between residues 422 to 431) were identified in the *F. occidentalis* mitochondrial ATP synthase α subunit that is predicted to encode a protein of 555 amino acids from an ORF of 1,665 nucleotides. In this case, the *F. occidentalis* mitochondrial ATP synthase α subunit was most similar to that one of the yellow fever mosquito, *Aedes aegypti* (GeneBank accession No: XP_001655906.1), sharing an 88% identity (Table 3.4). The *Drosophila melanogaster* mitochondrial ATP synthase α subunit (GeneBank accession No: NP_726243) shares an 85% identity to that of *F. occidentalis*. This *F. occidentalis* mitochondrial ATP synthase α subunit has 20 potential phosphorylation sites and 11 potential N-myristoylation sites. Differences in N-myristoylation might explain the shift in isoelectric point observed between the two *F. occidentalis* mitochondrial ATP synthase α subunit identified in G_N overlay assays (Fig. 3.3).

A topological analysis conducted using the HMMTOP software identified one transmembrane domain between amino acids 7 to 23 of the *F. occidentalis* endocuticle structural glycoprotein, while predicting that it is a type I integral membrane protein (having the N-terminus inside the lumen of the endoplasmic reticulum and the C-terminus in the cytoplasm). Furthermore, a signal peptide was identified in the *F. occidentalis* endocuticle structural glycoprotein, which cleavage site was predicted to occur between amino acid residues alanine and leucine at positions 17 and 18, respectively. Transmembrane domains and signal peptides were not identified in cyclophilin, enolase, cuticular protein, and mitochondrial ATP synthase α subunit from *F. occidentalis*. Further analyses to validate the interaction of these *F. occidentalis* proteins with TSWV and to determine their localization within the thrips body are warranted.

Discussion

The function of all six TSWV viral proteins has been extensively studied during the last three decades; the L protein is the RNA-dependent RNA-polymerase (Adkins et al., 1995), the G_N and G_C glycoproteins are the attachment and fusion proteins required to infect the thrips vector (Whitfield et al., 2004; Whitfield et al., 2005b), the NSm is the cell-to-cell movement protein in plants (Storms et al., 1998; Storms et al., 1995), the N is the nucleocapsid protein that encapsidates the genomic RNA (Richmond et al., 1998; Uhrig et al., 1999), and the NSs is a silencing suppressor functional in both plant and insect cells (Bucher et al., 2003; Garcia et al., 2006; Oliveira et al., 2011; Schnettler et al., 2010; Takeda et al., 2002). In contrast, very little is known about the *F. occidentalis* proteins that interact with TSWV and are necessary for the infection of its thrips vector and thus the successful acquisition and transmission of the virus. I have used overlay assays of purified TSWV and recombinant G_N to begin to identify *F. occidentalis* larval proteins resolved by 2-D gel electrophoresis that directly interact with this plant-infecting virus. Five *F. occidentalis* proteins have been identified as candidate proteins that directly interact with TSWV. Using ESI mass spectrometry and bioinformatics, these interacting proteins have been identified as cyclophilin, enolase, cuticular protein, endocuticle structural glycoprotein, and mitochondrial ATP synthase α subunit. I have previously identified cyclophilin, enolase, and mitochondrial ATP synthase α subunit as *F. occidentalis* responsive proteins during TSWV infection of the first instar larvae (Badillo-Vargas et al., 2012).

Cyclophilins, also known as peptidyl-prolyl cis-trans isomerases, are ubiquitous proteins present in all cells of all organisms (eukaryotes and prokaryotes) with roles in several biological processes. They are involved in protein folding and trafficking, muscle differentiation, receptor complex stabilization, cell signaling, detoxification of reactive oxygen species (ROS), RNA processing, spliceosome and RISC assembly, apoptosis, microRNA activity, and immune responses (reviewed in Kumari et al., 2013). The archetypal cyclophilin, cyclophilin A, is cytosolic, while other cyclophilins are present in the endoplasmic reticulum, the mitochondria, or the nucleus (reviewed in Wang and Heitman, 2005). All cyclophilins share a conserved domain designated the cyclophilin-like domain (CLD) (Marks, 1996). However, additional but different domains (e.g. zinc finger domain) are also present in different cyclophilins that are unique to each member of this family of proteins and are associated with subcellular compartmentalization and functional specialization (Arevalo-Rodriguez et al., 2004). Cyclophilin expression has been shown to be induced by both biotic and abiotic stresses including viral infection (An et al., 2007; Bleiber et al., 2005; Chatterji et al., 2009; Kaul et al., 2009; Rits et al., 2008; Yang et al., 2008a). Furthermore, cyclophilins directly interact with virus particles from both animal- and plant-infecting viruses to promote or restrict different aspects of their infection cycles. For example, cyclophilin A is specifically incorporated into newly synthesized virions of the human immunodeficiency virus 1 (HIV-1; *Retroviridae*: *Lentivirus*) which is required for attachment to the host cells and it is also recruited by the GAG polyprotein during HIV-1 infection which promote viral replication (Braaten et al., 1997; Braaten and Luban, 2001; Saphire et al., 1999). Recently, it has been shown that cyclophilin A binds to the viral RNA and replication proteins of *Tomato bushy stunt virus* (TBSV; *Tombusviridae*: *Tombusvirus*) resulting in the inhibition of tombusviral replication in a cell-free assay (Kovalev and Nagy, 2013). Overexpression of cyclophilin A in yeast and plant leaves also resulted in inhibition of TBSV replication *in vivo* (Kovalev and Nagy, 2013). Moreover, cyclophilins have been implicated as a determinant of insect vector competency. In the greenbug, *Schizaphis graminum*, cyclophilins have been identified in vectors of *Cereal yellow dwarf virus* (CYDV; *Luteoviridae*: *Polerovirus*) and have also been found to be involved in the transmission of this plant virus by its aphid vector (Tamborindeguy et al., 2013; Yang et al., 2008b). Previously, I have found this cyclophilin (CL4854Contig1_S454) to be down-regulated in *F. occidentalis* first instar larvae during TSWV infection by a 1.12-fold change (Badillo-Vargas et al., 2012). A sequence alignment using

different cyclophilins from other insects showed that the *F. occidentalis* cyclophilin sequence is most similar (84% identity) with cyclophilin A from the pea aphid, *A. pisum*. Cyclophilin A from *S. graminum* was found to directly interact with purified CYDV in co-immunoprecipitation assay (Tamborindeguy et al., 2013). A direct interaction between TSWV and the *F. occidentalis* cyclophilin A might play a role in the entry into and escape from the midgut as observed in the *S. graminum*-CYDV pathosystem.

Previously, a *F. occidentalis* enolase (CL4706Contig1_S454) was found to be up-regulated in first instar larvae during TSWV infection by 1.40-fold change compared to the virus-free counterparts (Badillo-Vargas et al., 2012). Here, I have found that this enolase also binds to purified TSWV in virus overlay assays. Enolases are essential metalloenzymes that catalyze the conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP) in the glycolytic pathway for energy metabolism and they require the metal ion magnesium to be catalytically active (Brown and Doolittle, 1997). Enolases are found in all organisms and have been shown to be multifunctional proteins that play important roles in many biological and disease processes (Kang et al., 2008). For example, ookinete surface enolase from *Plasmodium berghei* binds the receptor enolase-binding protein for midgut invasion of the mosquito vector (Vega-Rodríguez et al., 2013). Transcripts of an enolase were up-regulated in the small brown planthopper *Laodelphax striatellus* infected by *Rice stripe virus* (RSV; genus *Tenuivirus*) compared to non-infected insects (Zhang et al., 2010). An enolase from the braconid wasp, *Aphidius ervi*, which is injected into the pea aphid *Acyrthosiphon pisum* during parasitization have been suggested to be involved in degradation of host tissues and immune evasion (Falabella et al., 2009). Furthermore, some enolases are matrix metalloproteases which are known to be involved in the cleavage of cell surface receptors, the activation/inactivation of cytokines or chemokines, and the release of apoptotic ligands by degrading all kinds of extracellular matrix proteins such as collagen, elastin, fibronectin, laminin, gelatin, and fibrin among several others (Van Lint and Libert, 2007). Thus, matrix metalloproteases play important roles in cell proliferation and differentiation, as well as in cell adhesion and dispersion, apoptosis, and host defense (Van Lint and Libert, 2007). Matrix metalloproteases have been found to have a role in baculovirus infection of the lepidopteran host by aiding in the remodeling of the basal lamina lining tracheal cells and enabling escape of the virus from the digestive track to establish a systemic infection in the insect host (Means and Passarelli, 2010). Remodeling of the midgut

basal lamina in *F. occidentalis* larval thrips by enolase might explain at least in part the dissemination of TSWV from the midgut into the principal salivary glands observed during the larval stages but not in adult thrips. Further analysis of *F. occidentalis* enolase will enable the characterization of a possible role of this protein in immune evasion, midgut escape or other biological processes relevant to TSWV infection of the thrips vector.

The insect cuticle is a complex, composite material made of chitin filaments embedded in a proteinaceous matrix that form the exoskeleton that provides protection and structural support to these animals (Neville, 1975). The cuticular/cuticle proteins of insects seem to be specific to the type of cuticle that occurs during different stages of insect development (Andersen et al., 1997). There are two types of insect cuticle, the soft cuticle and the stiff cuticle. Here, I have identified 5 cuticular proteins in virus overlay assays and 2 and 9 endocuticle structural glycoproteins in virus overlay assays and G_N-S overlay assays, respectively. All the cuticular proteins and endocuticle structural glycoproteins identified in this study have a Chitin_bind_4 motif. The Chitin_bind_4 motif, also known as the Rebers & Riddiford (R&R) consensus motif, comprises a 35-36 amino acid motif that is present in many insect cuticular/cuticle proteins (Rebers and Riddiford, 1988). Furthermore, an extension of this motif of approximately 68-70 amino acids known as the "extended R&R consensus" has been shown to bind chitin (Iconomidou et al., 1999). The cuticular/cuticle proteins that have either one of these two motifs are classified as the non-cysteine chitin binding domain cuticular proteins (Rebers and Willis, 2001). Using a protein structural model, Hamodrakas *et al.* have predicted that the aromatic side chains within the Chitin_bind_4 motif of cuticle proteins seem to be responsible for the interaction with the polysaccharide chains of chitin (Hamodrakas et al., 2002). Four cuticle proteins from the silk worm *Bombyx mori* that possess the extended R&R consensus motif revealed that this conserved region is indeed responsible for the chitin binding activity (Togawa et al., 2004). The extended R&R consensus motif is widely conserved among cuticular/cuticle proteins mainly of the soft cuticle of many insect species. Thus, the extended R&R consensus is the general chitin-binding domain of cuticle proteins in insects and arthropods (Togawa et al., 2004). Silencing of a cuticular protein containing the R&R consensus motif from the rice brown planthopper, *Nilaparvata lugens*, resulted in a reduced nymphal survival by 26.7% due to incomplete molting (Yan et al., 2013). Interestingly, the transcript of this protein is only expressed in the nymphal stages and its expression peaked in the third instar nymph (Yan et al.,

2013). Recently, a cuticular protein from *L. striatellus* was found to interact with the nucleocapsid protein of *Rice stripe virus* (RSV; genus *Tenuivirus*) in yeast two-hybrid assays and co-immunoprecipitation (Wang et al., 2013). This cuticular protein was detected in the surface of hemocytes and its silencing resulted in lower RSV titer and transmission rates compared to those from not silenced insects (Wang et al., 2013). Csikós *et al.* conducted a detailed study of the trafficking of cuticular proteins from soft cuticle and found that different proteins localized in different organs of the tobacco hornworm *Manduca sexta* and that it was a dynamic process with some cuticular proteins even transported into the hemolymph (Csikos et al., 1999). A topological analysis predicted a transmembrane domain in the endocuticle structural glycoproteins identified in this study. Thus, an endocuticle structural glycoprotein-TSWV interaction may be potentially involved in the attachment and entry of this virus into the midgut of its thrips vector.

Two proteins that bound to G_N-S in overlay assays shown here were identified as mitochondrial ATP synthase α subunit (CL4310Contig1_S454). This protein was previously identified as a responsive protein from *F. occidentalis* first instar larvae being up-regulated by 1.54-fold change during TSWV infection (Badillo-Vargas et al., 2012). The mitochondrial ATP synthase is responsible for the production of the majority of the cellular ATP required by eukaryotes to meet all their energy needs. This holoenzyme is composed of two major functional units, F_O and F_1 (reviewed in Amzel and Pedersen, 1983). The F_O unit is embedded within the mitochondrial inner membrane, while the F_1 unit lies in the mitochondrial matrix region. Protons are directed across the inner membrane by the F_O unit which is hydrophobic in nature. Synthesis of ATP occurs on the F_1 unit which is composed of 5 subunits (α, β, γ, δ, and ε). The ATP synthesis occurs by inducing conformational changes in three catalytic nucleotide binding sites located at the interface between α and β subunits (Abrahams et al., 1994). Disruption of the connection between the F_O and F_1 units abolishes ATP synthesis (Karrasch and Walker, 1999; Rubinstein et al., 2003; Walker and Kane Dickson, 2006). Using a combination of electron and immunofluorescence microscopy, Rojo and colleagues showed that *African swine fever virus* (ASFV; family *Asfarviridae*: *Asfivirus*) induces the migration of mitochondria to the periphery of viral factories (Rojo et al., 1998). Their results suggest a role for the mitochondria in supplying the energy that the virus may require for its morphogenetic processes. A mitochondrial ATP synthase β subunit from the shrimp *Litopenaeus vannamei* bound to *White spot syndrome virus*

(WSSV; *Nimaviridae*: *Whispovirus*) in virus overlay assays (Liang et al., 2010). When recombinant protein was mixed with purified WSSV and then injected intramuscularly into shrimp it resulted in reduced mortality curves by an apparent attenuation of WSSV infection (Liang et al., 2010). Moreover, transcripts of a mitochondrial ATP synthase α subunit have been found to be up-regulated in *L. striatellus* during infection by RSV compared to their naïve counterparts (Zhang et al., 2010). Thus, it is tempting to speculate that different subunits of the mitochondrial ATP synthase complex interact with viruses so they can thrive in their hosts during infection. Identification of two proteins (enolase and mitochondrial ATP synthase α subunit) involved in energy production and metabolism suggests that these biological processes may be required for the successful infection of *F. occidentalis* by TSWV.

In order to validate the interactions observed in overlay assays using either purified TSWV virions or recombinant G_N, I have designed peptide antibodies to four of the *F. occidentalis* interacting proteins (cyclophilin, enolase, endocuticle structural glycoprotein, and mitochondrial ATP synthase α subunit), which have been already generated by GenScript (Piscataway, NJ, USA). These peptide antibodies will be used in immunolocalization studies to determine where these proteins are expressed within *F. occidentalis* larvae and if they co-localize with TSWV virions *in vivo*. Expression of these five *F. occidentalis* interacting proteins in a heterologous insect expression system for additional virus overlay assays or a yeast two-hybrid assay will confirm these protein-protein interactions conclusively.

Materials and Methods

Frankliniella occidentalis cultures and rearing

Our *F. occidentalis* colony, which was established from insects collected on the island of Oahu, HI, is maintained on green bean pods (*Phaseolus vulgaris*) at 22ºC (±2 ºC) using 16-oz clear plastic cups with lids fitted with thrips-proof mesh under laboratory conditions as previously described (Bautista et al., 1995; Ullman et al., 1992). Green beans were incubated with adult thrips for 3 to 4 days to allow females to lay eggs to obtain first instar larvae for the experiments. Adult thrips were then removed using a brush and the green beans were incubated for 24 h at 23ºC, after which emergent larval thrips (0 to 24 h old) were collected in 1.7 mL

nuclease- and protease-free microcentrifuge tubes, flash frozen in liquid nitrogen immediately, and stored at -80ºC.

Total protein extraction from healthy larval thrips and 2-D gel electrophoresis

Total proteins from healthy larval thrips were extracted using the trichloroacetic acid-acetone (TCA-A) extraction method described by Cilia *et al.* (Cilia et al., 2009; Cilia et al., 2011). Briefly, insects were removed from the frozen microcentrifuge tube and placed in a clean, sterile mortar and carefully ground in liquid nitrogen. Using a sterile spatula, the fine powder was transferred into a new 1.7 mL nuclease- and protease-free microcentrifuge tube and 500 µL of the TCA-A extraction buffer (10% of TCA in acetone containing 2% of β-mercaptoethanol) was added. The tubes were inverted ten times by hand and incubated overnight (for at least 12 hours) at -20ºC. The tubes were then spun at 5,000 x *g* for 30 min at 4ºC after which the supernatant was carefully removed. The pellets were washed 3 times with 500 µL of ice-cold acetone without disturbing the pellets. Pellets were air-dried for 20 min at room temperature and then resuspended in 200 µL of Rehydration/Sample Buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM 1,4-dithiothreitol (DTT), 0.2% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue (Bio-Rad, Hercules, CA, USA) using a sterile Kontes pestle (Thermo Scientific, Wilmington, DE, USA). After centrifugation at 12,000 x *g* for 5 min, the protein supernatant was quantified for 2-D gel electrophoresis using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) following the manufacturer's instructions.

One hundred and fifty micrograms of total larval thrips proteins in a final volume of 200 µL of Rehydration/Sample Buffer was applied to an 11-cm IPG strip with a pH 3 to 10 gradient (Bio-Rad) for an overnight passive rehydration at 25ºC. The first and second dimension separation of proteins were performed as described by Badillo-Vargas *et al.* (Badillo-Vargas et al., 2012). Three 2-D gels were run simultaneously for each replicate and used for the overlay (of either purified TSWV virions, recombinant G_N, or healthy plant extract), overlay control (neither purified TSWV virions, recombinant G_N, nor healthy plant extract), and Coomassie Brilliant Blue G-250 staining, respectively. Four replicates were conducted for the virus overlay assay, while two replicates were conducted for the recombinant G_N overlay assay. Only one

experiment was conducted using a healthy plant extract and virus overlay was used as a direct comparison between these two treatments.

TSWV maintenance and purification

We used *F. occidentalis* to periodically passage TSWV (isolate TSWV-MT2) to *Emilia sonchifolia* and maintain a fresh source of virus for the experiments as previously described (Ullman et al., 1993). TSWV was mechanically inoculated onto young *Datura stramonium* plants using thrips-inoculated *Emilia* leaf tissue. Twelve days post inoculation (dpi), leaf tissue from mechanically-inoculated *Datura* plants showing classical tospovirus symptoms were harvested and used for virus purification. Fresh leaf tissue was ground in extraction buffer (0.033 M KH_2PO_4, 0.067 M K_2HPO_4, and 0.01 M Na_2SO_3) using 3 mL of extraction buffer for every gram of fresh weight tissue. The plant material was then filtered through four layers of cheesecloth. The extraction buffer containing extract with plant material was centrifuged at 7 K rpm (7445 *g*) using a Sorvall SLA 1500 rotor (Thermo Scientific) for 15 min at 4ºC. The supernatant was carefully poured off and the pellet was resuspended in 65 mL of 0.01 M sodium sulfite by stirring it for 45 min at 4ºC. The resuspended pellet was allow to stand for 20 min at 4ºC and then centrifuged for 20 min at 8.5 K rpm (8643 *g*) in 50 mL tubes using a Sorvall SS34 rotor (Thermo Scientific). The supernatant was collected and placed into polycarbonate bottles filled up to the neck with 0.01 M sodium sulfite and centrifuged for 33 min at 29.3 K rpm (88205 *g*) in an ultracentrifuge using a 70 Ti rotor (Thermo Scientific) at 4ºC. Following the high speed centrifugation, the supernatant was discarded and the pellet was resuspended in 15 mL of 0.01 M sodium sulfite by stirring at 4ºC for 45 min. The resuspended pellets were then placed into 50 mL tubes and centrifuged at 9 K rpm (9690 *g*) using a Sorvall SS34 rotor at 4ºC for 15 min. The supernatant was spun one more time at 29.3 K rpm (88205 *g*) for 33 min using a 70 Ti rotor using an ultracentrifuge at 4ºC and the pellets resuspended in 4 mL of 0.01 M sodium sulfite by stirring them at 4ºC for 45 min. The resuspended pellets were centrifuged for 15 min at 9 K rpm (9690 *g*) using 50 mL tubes and a Sorvall SS34 rotor at 4ºC. The supernatant was collected and 4 to 5 mL were loaded on top of a sucrose gradient (10 to 40% sucrose) that was centrifuged for 35 min at 21 K rpm (79379 *g*) using a SW28 rotor (Thermo Scientific) in an ultracentrifuge at 4ºC. Following the third high speed centrifugation, the virus bands were visualized by shining

light over the surface of the tube and carefully collected using a pipet. All the virus bands obtained were pooled in polycarbonate bottles filled up to the neck with 0.01 M sodium sulfite and centrifuged for 1 h and 2 min at 29.3 K rpm (88205 *g*) in an ultracentrifuge using a 70 Ti rotor at 4ºC. The supernatant was then removed, the residual liquid dried out with a Kim wipe, and the pellets were resuspended in 100 to 200 µL of 0.01 M sodium sulfite at 4ºC by pipetting. Finally, the purified virus was quantified using the BCA protein assay kit (Thermo Scientific) following the manufacturer's instructions. For virus overlay assays, 25 µg per mL of purified virus was used immediately or aliquots that were maintained in a cryostorage tank (-180ºC) were used before becoming a month old.

Overlay assays

To identify proteins from *F. occidentalis* first instar larvae that could directly interact with TSWV, two different types of overlay assays were conducted, one using purified TSWV virions and another using recombinant G_N glycoprotein expressed in bacterial cells kindly provided by Dr. Thomas L. German at University of Wisconsin – Madison. After electrophoresis, unstained 2-D gels were electroblottted onto Hybond-C Extra nitrocellulose membranes (Amersham Biosciences, Pittsburg, PA, USA) overnight by a constant current of 30 volts at 4ºC in protein transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, and 0.037% SDS) and then used for the overlay assays. First, membranes were blocked in PBST/milk (PBS containing 0.05% Tween 20 and 5% dry milk) for 1 h with slow rotation, immediately followed by the overlay step.

For the virus overlay assays, nitrocellulose membranes containing thrips proteins were incubated overnight at 4ºC with purified virus at 25 µg/mL in PBST/milk. For the G_N overlay assays, 3.5 µg/mL of recombinant glycoprotein in PBST/milk were incubated with the nitrocellulose membrane containing thrips proteins at 4ºC overnight. An overlay control membrane was developed for each overlay assay conducted in which a nitrocellulose membrane containing thrips proteins was incubated with PBST/milk only at 4ºC overnight and thereafter treated exactly the same as the ones that were incubated with purified TSWV virions or recombinant G_N as overlay. Four and two replicates of the virus overlay assay and the G_N overlay assay were conducted, respectively. Following either overlay assay, three 10 min

washes of the membranes with PBST were done at room temperature. Membranes were then incubated for 2 h at room temperature in PBST/milk with a peptide polyclonal rabbit anti-G_N antibody diluted 1:2,000. After five 5 min washes with PBST, membranes were incubated in PBST/milk with HRP-conjugated goat-anti rabbit antibody diluted 1:5,000 for 1 h at room temperature. The secondary antibody was detected using the ECL detection system (Amersham Biosciences).

Moreover, an overlay assay was conducted using a healthy plant extract (mock purification) to identify if plant proteins obtained from virus-free *Datura stramonium* processed through the virus purification method might result in an interaction with thrips proteins in an overlay assay. Nitrocellulose membranes containing thrips proteins were incubated overnight at 4ºC with either healthy plant extract or purified TSWV virions at 25 µg/mL in PBST/milk. Following the overlay step, both membranes were treated exactly the same as described before and images developed using the ECL detection system (Amersham Biosciences).

Collection, ESI mass spectrometry, and identification of thrips proteins detected in overlay assays

To identify the *F. occidentalis* proteins that interacted with purified TSWV virions, protein spots were manually collected from 3 different picking 2-D gels stained with Coomassie Brilliant Blue G-250. Two different picking 2-D gels also stained with Coomassie Brilliant Blue G-250 were used to manually collect the protein spots that interacted with recombinant G_N for identification. The gel pieces containing the protein spots were processed and then subjected to ESI mass spectrometry as previously described by Badillo-Vargas *et al.* (Badillo-Vargas et al., 2012). Generated spectra were used to compare the theoretical fragmentation pattern of *in silico* digested peptides from the *Fo* Seq and the Metazoan non-redundant (nr) protein database of NCBI using MASCOT 2.2 software (www.matrixscience.com) (Matrix Science, Boston, MA, USA). The NCBI database searches were conducted on September 27, 2013. An error tolerance search algorithm and the six possible translation open reading frames were used for protein identification. Furthermore, the following search parameters were also used for protein identification: carbamidomethyl-cysteine as a fixed modification and methionine oxidation as a variable modification, with one missed tryptic cleavage allowed. Protein spots that had Mascot

scores that represented significant matches ($P \leq 0.05$) were considered to be identified. The sequences that matched the *F. occidentalis* larval proteins that bound purified TSWV virions or recombinant G_N were analyzed using the Translate Tool from Expasy (http://web.expasy.org/translate/) to identify the complete ORF and predicted translated protein. A Blastp analysis was conducted on April 3, 2014 using the default parameters to determine the *F. occidentalis* proteins homology to known proteins in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&QUERY=&SUBJECTS). Sequence alignment analysis of *F. occidentalis* proteins with protein sequences from other insects and well characterized organisms was performed using ClustalW2 (www.ebi.ac.uk) to determine their similarity. A topological analysis using the HMMTOP software (http://www.enzim.hu/hmmtop/html/submit.html) was conducted to assign presence/absence and location of predicted transmembrane domains on the candidate thrips proteins. Furthermore, SignalP 4.1 Server was used to identify the presence of signal peptides on the *F. occidentalis* proteins that bound to purified TSWV virions or recombinant G_N. Sequence alignment of the eight cuticular proteins and two endocuticle structural glycoproteins of *F. occidentalis* identified in overlay assays was used to compare if they are or not the same proteins within each group. Finally, Prosite (http://prosite.expasy.org/) was used to identify posttranslational modifications that can account for differences on electrophoretic mobility observed in the overlay assays.

Generation of peptide antibodies against thrips interacting proteins identified in overlay assays

In order to validate the protein-protein interactions observed with the overlay assays of either purified TSWV virions or recombinant G_N, anti-peptide antibodies were designed for *F. occidentalis* cyclophilin, enolase, endocuticle structural glycoprotein, and mitochondrial ATP synthase α subunit. The polyclonal peptide antibodies were all custom made in mouse during this work (GenScript, Piscataway, NJ, USA) (Appendix B). The cyclophilin polyclonal antibody was made against a 14 amino acid peptide (LESFGSHDGKTSKK) from the C-terminus region of cyclophilin, starting at residue 182 from the first methionine. A 14 amino acid peptide (ELRDNDKSQYHGKS) from the N-terminus region of enolase, starting at residue 48 from the

start codon, was used to generate the enolase polyclonal antibody. The endocuticle structural glycoprotein polyclonal antibody also made against a 14 amino acid peptide (VNPDGSFQYSYQTG) starting at residue 57 from the start codon within the N-terminus region of this protein was used to generate a polyclonal antibody against it. Finally, a 14 amino acid peptide (GHLDKLDPAKITDF) from mitochondrial ATP synthase α subunit at residue 494 within the C-terminus region of this protein was used to make a mitochondrial ATP synthase α subunit polyclonal antibody. These four antibodies will be useful tools to conduct co-immunoprecipitation assays and immunolocalization studies to further validate these protein-protein interactions and to characterize their location within the thrips body.

Figures and Tables

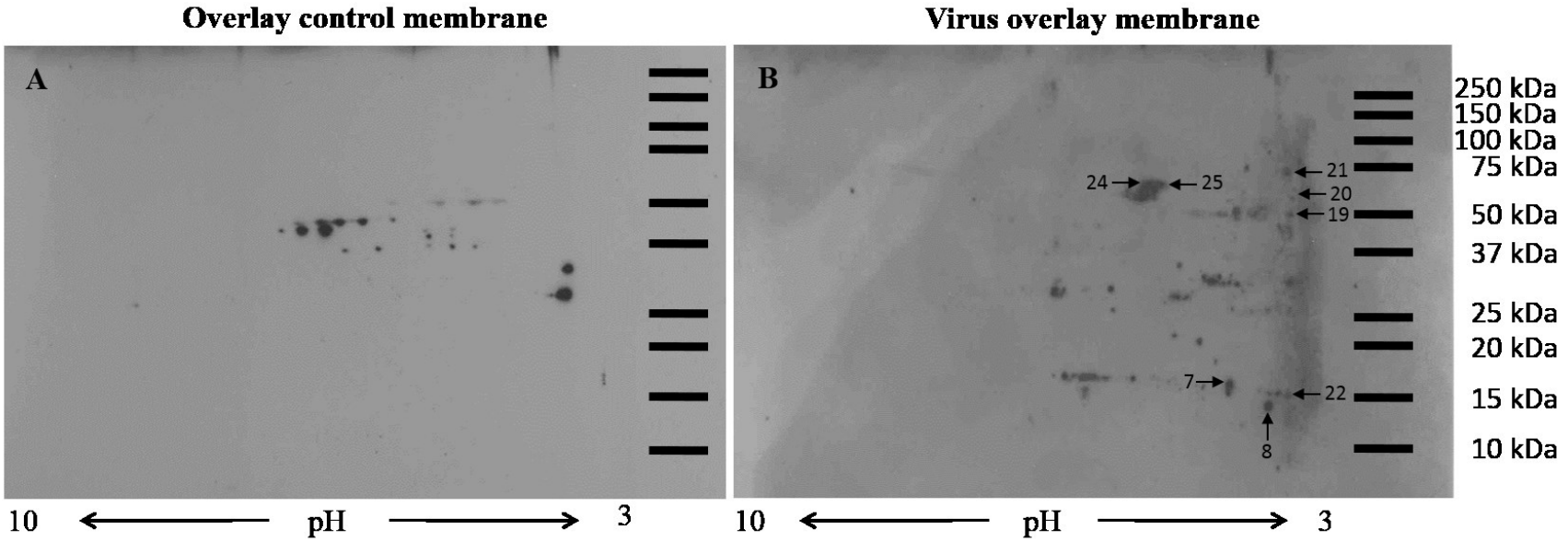

Figure 3.1 Virus overlay assay using purified *Tomato spotted wilt virus* and *Frankliniella occidentalis* first instar larval proteins resolved in two-dimensional (2-D) gels.

One hundred and fifty micrograms of total protein extracted from pooled first instar larvae of *F. occidentalis* were resolved in a 2-D gel and transferred to nitrocellulose membranes that were used for virus overlay assays. Purified TSWV at 25 µg per mL in PBST/milk was incubated overnight on the virus overlay membrane (B) while only PBST/milk was incubated overnight on the no overlay control membrane (A). Following the overlay, both membranes were blocked and treated with a polyclonal peptide rabbit anti-G_N followed by the secondary antibody for detection. Only protein spots that consistently bound to purified TSWV in three (spots 7, 8, 20, 22, and 24) and four (spots 19, 21, and 25) replicates of the virus overlay assay were collected from three individual picking gels and subjected to ESI mass spectrometry for protein identification. Protein spots observed in the no overlay control membrane represent non-specific binding and were not collected for further analysis. Molecular mass (in kilodaltons) is shown on the *y* axis and pI (as pH range) is shown on the *x* axis.

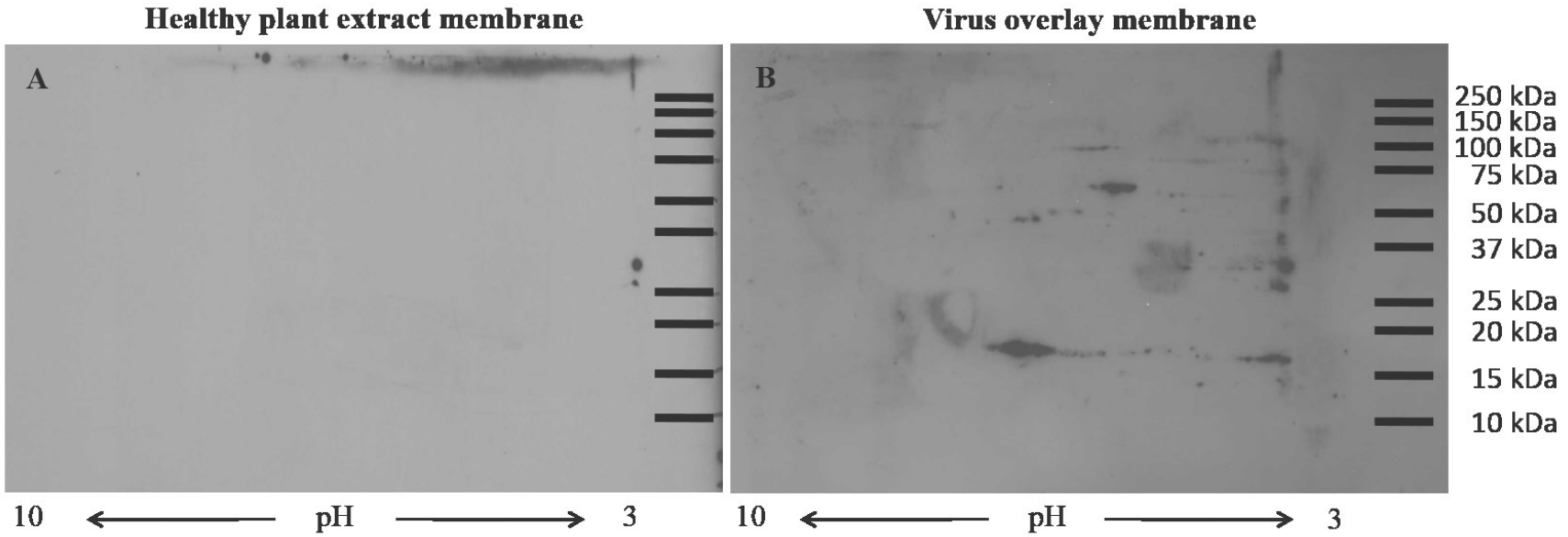

Figure 3.2 Overlay assay using a healthy plant extract and *Frankliniella occidentalis* first instar larval proteins resolved in two-dimensional (2-D) gels.

One hundred and fifty micrograms of total protein extracted from pooled first instar larvae of *F. occidentalis* were resolved in a 2-D gel and transferred to nitrocellulose membranes that were used for overlay assays. Purified TSWV at 25 µg per mL in PBST/milk was incubated overnight on the virus overlay membrane (B) and the same concentration from a healthy plant extract obtained from virus-free *Datura stramonium* plants was incubated overnight on the healthy plant extract membrane (A). Following the overlay, both membranes were blocked and treated with a polyclonal peptide rabbit anti-G_N followed by the secondary antibody for detection. The two protein spots observed when the healthy plant extract was used as overlay represent non-specific binding also observed in the no overlay control membranes. Molecular mass (in kilodaltons) is shown on the *y* axis and pI (as pH range) is shown on the *x* axis.

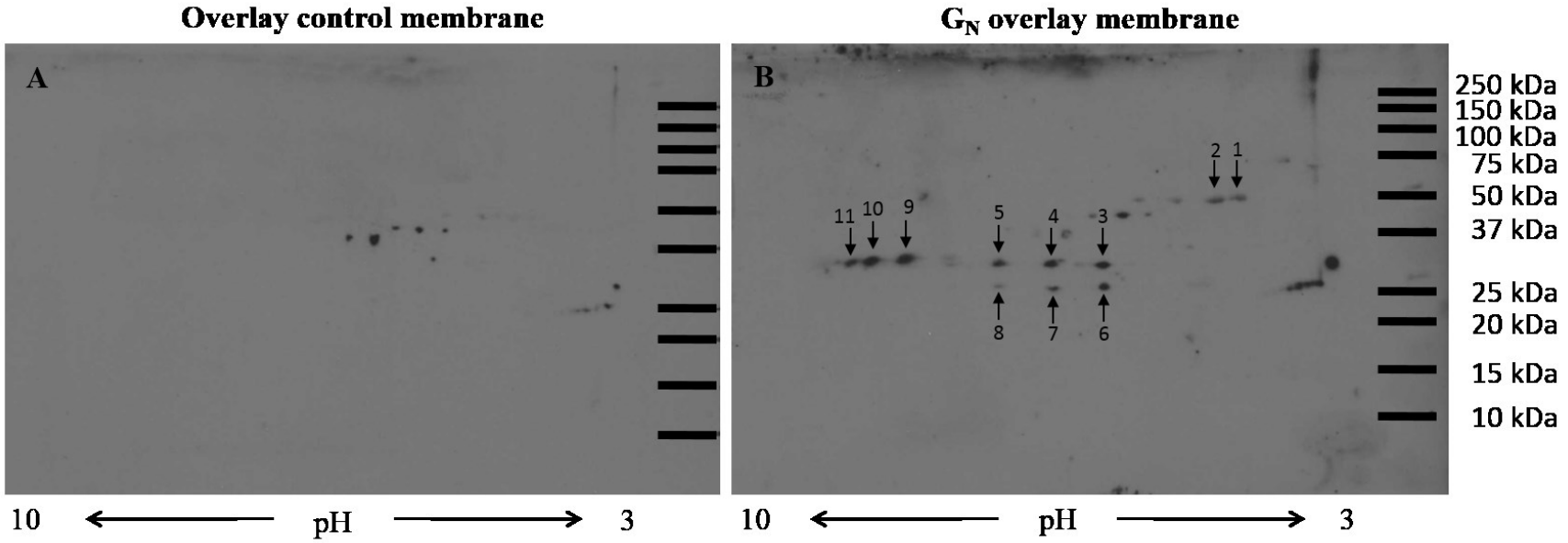

Figure 3.3 Overlay assay using recombinant G_N glycoprotein and *Frankliniella occidentalis* first instar larval proteins resolved in two-dimensional (2-D) gels.

One hundred and fifty micrograms of total protein extracted from pooled first instar larvae of *F. occidentalis* were resolved in a 2-D gel and transferred to nitrocellulose membranes that were used for overlay assays. Recombinant G_N at 3.5 µg per mL in PBST/milk was incubated overnight on the G_N overlay membrane (B) while only PBST/milk was incubated overnight on the no overlay control membrane (A). Following the overlay, both membranes were blocked and treated with a polyclonal peptide rabbit anti-G_N followed by the secondary antibody for detection. Protein spots that consistently bound to the recombinant G_N-S in two (spots 1 through 11) biological replicates of the overlay assay were collected from two individual picking gels and subjected to ESI mass spectrometry for protein identification. Protein spots observed in the no overlay control membrane represent non-specific binding and were not collected for further analysis. Molecular mass (in kilodaltons) is shown on the *y* axis and pI (as pH range) is shown on the *x* axis.

Table 3.1 *Frankliniella occidentalis* proteins that bound to purified *Tomato spotted wilt virus* in virus overlay assays of two-dimensional (2-D) gels.

Spot #	Database searched (sequence matched)	Mascot score	Peptides matched	Peptide sequence	Percent coverage^	Protein ID	Identified motifs	Organism with sequence homology
7*	*Fo* Seq (contig01248)	279	4	R.AQQPYQQYLQNQQFQNYQQR.A R.AAAAPILQYSNDVNPDGSFQYSYQTGDGISAQAAGFTR.N K.DAEAQVVQGSYSYTGPDGVVYTVNYIADENGYR.A K.ALPYYNQQQATYQQQQAAYQR.P	37%	endocuticle structural glycoprotein	Chitin_bind_4	*Pediculus humanus corporis*
	Fo Seq (CL4854Contig1_S454)	363	7	R.VFFDMTVDGQPAGR.I R.ALCTGEQGFGYK.G R.VIPNFMCQGGDFTNHNGTGGK.S R.KFADENFQLK.H K.HTGPGIMSMANAGPNTNGSQFFITTVK.T K.TSWLDNKHVVFGSVIEGMDVVK.K K.HVVFGSVIEGMDVVK.K	29%	cyclophilin	Cyclophilin_ABH	*Nasonia vitripennis*
8*	*Fo* Seq (contig01248)	77	1	K.DAEAQVVQGSYSYTGPDGVVYTVNYIADENGYR.A	10%	endocuticle structural glycoprotein	Chitin_bind_4	*Pediculus humanus corporis*
19±	*Fo* Seq (contig00018)	196	4	R.FGGALGGYNLAQTSQYHIQTDEGPER.Y R.LEDGTVVGTYGWVDADGYLR.L R.PYYPSSTPAVSLVSSTPR.P R.PYYPTSTPAVVSSTPR.P	25%	CG1136 CG1136-PA (similar to cuticular protein)	Chitin_bind_4	*Tribolium castaneum*
	Fo Seq (contig14634)	134	3	R.GYISELPGTYDANSNSVIPEYDGIAVTHNGFR.Y K.AGSFGYVDPFGIR.R R.VIYYNTSPGSGFQVR.K	22%	CG1136 CG1136-PA (similar to cuticular protein)	Chitin_bind_4	*Tribolium castaneum*
	Fo Seq (CL4900Contig1_S454)	113	3	R.GYISELPGTYDASSNSVIPEYDGIAVTHNGFR.Y K.AGSFGYVDPFGIR.R R.VIYYNTSPGSGFQVR.K	10%	CG1136 CG1136-PA (similar to cuticular protein)	Chitin_bind_4	*Tribolium castaneum*
20*	*Fo* Seq (CL1591Contig1_S454)	76	1	K.QESVYTAAQPAISTYK.K	5%	similar to cuticular protein 113	Chitin_bind_4	*Tribolium castaneum*

Spot #	Database searched (sequence matched)	Mascot score	Peptides matched	Peptide sequence	Percent coverage^	Protein ID	Identified motifs	Organism with sequence homology
21±	*Fo* Seq (CL1591Contig1_S454)	89	1	K.QESVYTAAQPAISTYK.K	5%	similar to cuticular protein 113	Chitin_bind_4	*Tribolium castaneum*
22*	*Fo* Seq (CL4854Contig1_S454)	615	9	R.VFFDMTVDGQPAGR.I R.ALCTGEQGFGYK.G R.VIPNFMCQGGDFTNHNGTGGK.S R.KFADENFQLK.H K.FADENFQLK.H K.FADENFQLKHTGPGIMSMANAGPNTNGSQFFITTVK.T K.HTGPGIMSMANAGPNTNGSQFFITTVK.T K.HVVFGSVIEGMDVVK.K K.KVVVADCGQLS	30%	cyclophilin	Cyclophilin_ABH	*Nasonia vitripennis*
24*	*Fo* Seq (CL4706Contig1_S454)	554	17	R.GNPTVEVDLVTELGLFR.A R.AAVPSGASTGVHEALELR.D K.AIDNVNNIIAPELIK.S K.EIDELMLK.L K.LGANAILGVSLAVCK.A K.HIADLAGNTNIILPTPAFNVINGGSHAGNK.L K.LAMQEFMILPTGASSFK.E K.FGLDSTAVGDEGGFAPNILNNK.E K.EGLTLIIDAIAK.A K.VEIGMDVAASEFYK.D K.VEIGMDVAASEFYKDGQYDLDFK.N K.DGQYDLDFKNPNSDK.S K.LTDLYMEFIK.E K.EFPMVSIEDPFDQDHWDAWTTITGK.T K.TNIQIVGDDLTVTNPK.R K.VNQIGSVTESIQAHLLAK.K R.SGETEDTFIADLVVGLSTGQIK.T	47%	unknown (similar to enolase)	Metal_binding	*Dendroctonus ponderoase*
	Fo Seq (contig12136)	96	2	R.QGDVVQGSYSLVEPDGSR.R R.TVEYTADPVNGFNAVVHK.D	24%	cuticular protein	Chitin_bind_4	*Pediculus humanus corporis*
	Fo Seq (contig14594)	102	1	R.TVDYTADPVNGFNAVVR.K	3%	cuticular protein	Chitin_bind_4	*Pediculus humanus corporis*
	Fo Seq (CL504Contig1_S454)	113	2	K.AAVAVDTDYDPNPSYNYAYDIHDSLTGDAK.S R.TVEYTADPVNGFNAVVHK.E	19%	cuticular protein	Chitin_bind_4	*Pediculus humanus corporis*

Spot #	Database searched (sequence matched)	Mascot score	Peptides matched	Peptide sequence	Percent coverage^	Protein ID	Identified motifs	Organism with sequence homology
25±	*Fo* Seq (CL4706Contig1_S454)	407	15	R.GNPTVEVDLVTELGLFR.A R.AAVPSGASTGVHEALELR.D K.AIDNVNNIIAPELIK.S K.HIADLAGNTNIILPTPAFNVINGGSHAGNK.L K.LAMQEFMILPTGASSFK.E K.FGLDSTAVGDEGGFAPNILNNK.E K.EGLTLIIDAIAK.A K.VEIGMDVAASEFYK.D K.VEIGMDVAASEFYKDGQYDLDFK.N K.DGQYDLDFKNPNSDK.S K.LTDLYMEFIK.E K.EFPMVSIEDPFDQDHWDAWTTITGK.T K.TNIQIVGDDLTVTNPK.R K.VNQIGSVTESIQAHLLAK.K R.SGETEDTFIADLVVGLSTGQIK.T	43%	unknown (similar to enolase)	Metal_binding	*Dendroctonus ponderosa*
	Fo Seq (CL504Contig1_S454)	99	1	K.AAVAVDTDYDPNPSYNYAYDIHDSLTGDAK.S	12%	cuticular protein	Chitin_bind_4	*Acyrthosiphon pisum*

* = *Frankliniella occidentalis* proteins that bound to purified *Tomato spotted wilt virus* in three virus overlay assays.

± = *Frankliniella occidentalis* proteins that bound to purified *Tomato spotted wilt virus* in four virus overlay assays.

^ = Highest percent coverage obtained among the three picking gels used to collect protein spots for identification using ESI mass spectrometry.

Table 3.2 *Frankliniella occidentalis* proteins that bound to a recombinant G_N glycoprotein in overlay assays of two-dimensional (2-D) gels.

Spot #	Database searched (sequence matched)	Mascot score	Peptides matched	Peptide sequence	Percent coverage^	Protein ID	Identified motifs	Organism with sequence homology
1	*Fo* Seq (CL4310Contig1_S454)	595	12	R.AAELSSILEER.I K.NIQADEMVEFSSGLK.G K.GMALNLEPDNVGIVVFGNDK.L K.GMALNLEPDNVGIVVFGNDKLIK.E R.TGAIVDVPVGDDLLGR.V K.TALAIDTIINQQR.F K.YTIIVAATASDAAPLQYLAPYSGCAMGEYFR.D K.HALIIYDDLSK.Q R.EAYPGDVFYLHSR.L R.EVAAFAQFGSDLDAATQQLLNR.G K.QGQYVPMAIEEQVAVIYCGVR.G K.IVTDFLASFNAASK	27%	mitochondrial ATP synthase α subunit	ATP-synt_ab_N; ATP-synt_ab_C	*Aedes aegypti*
2	*Fo* Seq (CL4310Contig1_S454)	302	5	K.GMALNLEPDNVGIVVFGNDK.L R.TGAIVDVPVGDDLLGR.V R.VVDALGDAIDGK.G K.HALIIYDDLSK.Q K.IVTDFLASFNAASK	12%	mitochondrial ATP synthase α subunit	ATP-synt_ab_N; ATP-synt_ab_C	*Aedes aegypti*
3	*Fo* Seq (CL4382Contig1_S454)	633	9	R.SSVVSQSVPVVSK.T K.SVPQYQQQYQTVSQYQSVPQYQQQVVVK.S K.SVPQYQQQVVVK.S K.SAPVYSQVHHVVEQQAAPVLLR.H R.TAFVPQYDSVSVSASAQPK.Y K.ILSQVQEFDPAGIYR.V R.VNFQTENGIQSAETGSVK.D R.ASGAHLPQVPEEIQR.S R.SLELNAAQPQKYDQDGNLVSQF	43%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*
4	*Fo* Seq (CL4382Contig1_S454)	137	3	R.SSVVSQSVPVVSK.T K.SVPQYQQQYQTVSQYQSVPQYQQQVVVK.S R.VNFQTENGIQSAETGSVK.D	15%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*
5	*Fo* Seq (CL4382Contig1_S454)	169	6	R.SSVVSQSVPVVSK.T K.SVPQYQQQVVVK.S R.VNFQTENGIQSAETGSVK.D R.ASGAHLPQVPEEIQR.S R.AAAEHGVAIVCPDTSPR.G K.ACQAVNMPVVLQMR.E	22%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*

Spot #	Database searched (sequence matched)	Mascot score	Peptides matched	Peptide sequence	Percent coverage^	Protein ID	Identified motifs	Organism with sequence homology
6	*Fo* Seq (CL4382Contig1_S454)	1,154	11	R.SSVVSQSVPVVSK.T K.SVPQYQQQYQTVSQYQSVPQYQQQVVVK.S K.SVPQYQQQVVVK.S K.SAPVYSQVHHVVEQQAAPVLLR.H R.HVEQEIPAYQSVQHVHQPVYQSVQHVAAHHVAAPVVSR.T R.TAFVPQYDSVSVSASAQPK.Y K.ILSQVQEFDPAGIYR.V R.VNFQTENGIQSAETGSVK.D R.VNFQTENGIQSAETGSVKDIQAK.D R.ASGAHLPQVPEEIQR.S R.SLELNAAQPQKYDQDGNLVSQF	53%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*
7	*Fo* Seq (CL4382Contig1_S454)	413	8	R.SSVVSQSVPVVSK.T K.SVPQYQQQYQTVSQYQSVPQYQQQVVVK.S K.SVPQYQQQVVVK.S R.TAFVPQYDSVSVSASAQPK.Y R.VNFQTENGIQSAETGSVK.D R.VNFQTENGIQSAETGSVKDIQAK.D R.ASGAHLPQVPEEIQR.S R.SLELNAAQPQKYDQDGNLVSQF	74%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*
8	*Fo* Seq (CL4382Contig1_S454)	355	7	R.SSVVSQSVPVVSK.T K.SVPQYQQQYQTVSQYQSVPQYQQQVVVK.S K.SVPQYQQQVVVK.S R.VNFQTENGIQSAETGSVK.D R.VNFQTENGIQSAETGSVKDIQAK.D R.ASGAHLPQVPEEIQR.S R.SLELNAAQPQKYDQDGNLVSQF	29%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*
9	*Fo* Seq (CL4382Contig1_S454)	155	2	R.VNFQTENGIQSAETGSVK.D R.VNFQTENGIQSAETGSVKDIQAK.D	5%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*
10	*Fo* Seq (CL4382Contig1_S454)	130	1	R.VNFQTENGIQSAETGSVK.D	4%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*
11	*Fo* Seq (CL4382Contig1_S454)	150	7	R.SSVVSQSVPVVSK.T K.SVPQYQQQYQTVSQYQSVPQYQQQVVVK.S K.SVPQYQQQVVVK.SR.VNFQTENGIQSAETGSVK.D R.TAFVPQYDSVSVSASAQPK.Y R.VNFQTENGIQSAETGSVK.D R.VNFQTENGIQSAETGSVKDIQAK.D R.ASGAHLPQVPEEIQR.S	13%	endocuticle structural glycoprotein	Chitin_bind_4	*Harpegnathos saltator*

^ = Highest percent coverage obtained among the three picking gels used to collected protein spots for identification using ESI mass spectrometry.

Table 3.3 Features of full-length genes of cyclophilin, enolase, cuticular protein, endocuticle structural glycoprotein, and mitochondrial ATP synthase α subunit from *Frankliniella occidentalis* and their predicted translated proteins selected for further characterization.

Putative gene	Sequence matched	Predicted ORF (nt)	Predicted protein (aa)	Identified motifs
cyclophilin	CL4854Contig1_S454	618	206	Cyclophilin_ABH
enolase	CL4706Contig1_S454	1,302	434	Metal_binding
cuticular protein	CL4900Contig1_S454	159	53	Chitin_bind_4
endocuticle structural glycoprotein	contig01248	522	174	Chitin_bind_4
mitochonrial ATP synthase α	CL4310Contig1_S454	1,665	555	ATP-synt_ab_N; ATP-synt_ab_C

Table 3.4 Sequence analysis of cyclophilin, enolase, cuticular protein, endocuticle structural glycoprotein, and mitochondrial ATP synthase α subunit from *Frankliniella occidentalis* against similar proteins from other insects and well characterized organisms.

***F. occidentalis* protein**	**Sequence matched**	**Percent identity**	**Protein matched**	**Organism with sequence homology**	**Accession #**	***E*-value**
cyclophilin	CL4854Contig1_S454	88	cyclophilin	*Nasonia vitripennis*	XP_001607048.1	5e-103
		84	cyclophilin A	*Acyrthosiphon pisum*	BAH70637	3e-97
		73	cyclophilin A	*Homo sapiens*	NP_066953	2e-85
		62	cyclophilin	*Pediculus humanus corporis*	XP_002425817	8e-95
		46	cyclophilin H	*Acyrthosiphon pisum*	XP_001943358	1e-50
		37	cyclophilin B	*Acyrthosiphon pisum*	NP_001156707	1e-50
enolase	CL4706Contig1_S454	87	unknown (similar to enolase)	*Dendroctonus ponderosae*	AEE62848.1	0.0
		84	enolase	*Aedes aegypti*	XP_001653750	0.0
		81	enolase	*Acyrthosiphon pisum*	XP_001948161	0.0
		77	enolase	*Pediculus human corporis*	XP_002430191	0.0
cuticular protein	CL4900Contig1_S454	52	CG1136 CG1136-PA (similar to cuticular protein)	*Tribolium castaneum*	XP_001809883.1	0.088
		9	cuticular protein	*Laodelphax striatella*	KC485263	NS
		7	cuticular protein	*Acyrthosiphon pisum*	NP_001127760	NS
		7	cuticular protein	*Pediculus human corporis*	XP_002423431	NS

***F. occidentalis* protein**	**Sequence matched**	**Percent identity**	**Protein matched**	**Organism with sequence homology**	**Accession #**	***E*-value**
endocuticle structural glycoprotein	contig01248	40	endocuticle structural glycoprotein	*Pediculus humanus corporis*	XP_002428599	1e-38
		25	cuticular protein	*Laodelphax striatella*	KC485263	2e-10
mitochondrial ATP synthase α subunit	CL4310Contig1_S454	88	mitochondrial ATP synthase α subunit	*Aedes aegypti*	XP_001655906.1	0.0
		85	mitochondrial ATP synthase α subunit	*Drosophila melanogaster*	NP_726243	0.0

NS = No significant similarity found

Chapter 4 - Development of RNA interference tools for functional genomic assays and control strategies of *Frankliniella occidentalis*

Abstract

The insect order Thysanoptera is exclusively comprised of small insects commonly known as thrips. The western flower thrips, *Frankliniella occidentalis* Pergande, is an important agricultural pest amongst thysanopterans due to the direct feeding damage it causes to hundreds of plant species and indirectly by transmitting several tospoviruses efficiently. *F. occidentalis* has a worldwide distribution and several populations from different geographical areas have developed resistance against many insecticides, making the control of thrips and the viruses they transmit more challenging. Currently, RNA interference (RNAi) methods have not been developed for any member of the insect order Thysanoptera. The overall goal of this work was to develop effective RNAi tools for functional genomic assays and new control strategies of *F. occidentalis*. The B subunit of the vacuolar ATP synthase (vATPase-B) holoenzyme, which is involved in the transport of solutes across plasma membranes and the regulation of the pH in the lumen of various organelles and organs in metazoans, was selected as the RNAi target gene in this study. vATPase-B double-stranded RNA (dsRNA) generated using *in vitro* transcription was injected into adult female thrips to investigate its effectiveness for eliciting RNAi in *F. occidentalis*. Gene expression analysis using real-time quantitative reverse transcriptase-PCR (qRT-PCR) showed that injection of vATPase-B dsRNA resulted in a target gene transcript reduction of 23% at two days post injection (dpi). Nonetheless, the moderate but significant reduction in vATPase-B transcript level resulted in two observable phenotypes. There was a significant reduction in adult female thrips survival caused by the injection of vATPase-B dsRNA compared to insects injected with either GFP dsRNA or DEPC-H_2O and with non-injected insects as well. Furthermore, injection of vATPase-B dsRNA had a negative effect on adult female thrips fertility as there was a significant reduction of offspring produced by females injected with this dsRNA compared to control insects injected with GFP dsRNA or DEPC-H_2O and also with their non-injected counterparts. Overall, my findings are the first to demonstrate

that injection of thrips with dsRNA with identical sequence to a *F. occidentalis* gene does trigger silencing of the target gene and have the potential to negatively impact both thrips survival and fertility. The development of RNAi tools for *F. occidentalis* to target essential genes suggests that RNAi-based strategies have the potential and can be further exploited not only to study gene function in any thrips species but also to impair the life cycle of thrips, which ultimately might enable the design of control strategies to target thrips populations and tospovirus transmission in field crops and greenhouses.

Introduction

The insect order Thysanoptera is comprised of at least 7,400 described species of small insects commonly known as thrips. The order, which is named from the Greek words thysanos and pteron that mean fringed wings, belongs to the Hemipteroid assemblage (also known as the true bugs that includes aphids, leafhoppers, planthoppers, treehoppers, whiteflies, scale insects, cicadas, shield bugs, assassin bugs, seed bugs, flower bugs, and lice as well). Thysanopterans share some common but unique characteristics such as a postembryonic remetaboly development with two non-feeding pupal stages (Heming, 1973), a haplodiploid genetic system where males are haploid and females are diploid (Crespi, 1992; Jacobson et al., 2013), and asymmetrical mouthparts composed of a resorbed right mandibule and a fully developed left mandibule that form the stylet used to feed from cells (Heming, 1980). However, they display a great diversity with respect to morphological structures, behavioral characteristics, and food preferences. For example, some thrips species feed on other insects or mites (predators) while others feed on fungal spores (fungivores). Moreover, a large number of thrips species feed on a variety of plants (phytophagous), many of which are of economic importance such as tomato, peanut, potato, peppers, corn, wheat, and several ornamental plants. Phytophagous thrips are piercing-sucking insects that feed by emptying plant cells of their cytoplasmic and other cellular contents (Childers and Aachor, 1991; Chisholm and Lewis, 1984; Hunter and Ullman, 1989; Hunter and Ullman, 1992), thus, they are considered insect pests (Morse and Hoddle, 2006; Reitz, 2009). Furthermore, fourteen thrips species are known vectors of plant-infecting viruses in the genus *Tospovirus* (Riley et al., 2011). High fecundity combined with a short generation time, high locomotory activity, a predisposition to parthenogenesis, a female-biased sex ratio, preference

for concealed spaces, a wide range of plant hosts, and the habit of puncturing epidermal and mesophyll plant cells are some behavioral characteristics shared by most thrips vector species (Moritz et al., 2001).

Tospoviruses belong to the virus family *Bunyaviridae* and are exclusively transmitted by a limited but specific species of thrips. Once a tospovirus is acquired by an efficient vector, the thrips will transmit the virus for its entire life span due to the persistent propagative nature of their interaction. Currently, thrips and tospoviruses are increasing in importance worldwide and control strategies to effectively manage them are urgently needed. The western flower thrips, *Frankliniella occidentalis* Pergande, is an economically important pest in field crops and greenhouses and it is the most efficient vector of *Tomato spotted wilt virus* (TSWV), the type species of the genus *Tospovirus*. Control of thrips and tospoviruses relies heavily on the use of insecticides. However, several populations of *F. occidentalis* from different geographical areas have developed resistance against many insecticides, making more challenging the control of tospoviruses and their thrips vectors (Bielza, 2008; López-Soler et al., 2008; Robb et al., 1995). Two cytochrome P450 genes, CYP6EB1 and CYP6EC1, were found to be over-expressed in acrinathrin-resistant *F. occidentalis*, suggesting that an increased level of detoxification is at least partially involved in the resistance to insecticides (Cifuentes et al., 2012). Moreover, chemical control of thrips may not always reduce tospovirus transmission (Culbreath et al., 2003; Mandal et al., 2012; Morse and Hoddle, 2006; Reitz, 2009). A recombinant G_N glycoprotein from TSWV fed exogenously to *F. occidentalis* larvae was found to bind thrips guts and block TSWV acquisition and transmission (Whitfield et al., 2004; Whitfield et al., 2008). Recently, transgenic tomato plants expressing the recombinant G_N have been shown to interfere with TSWV acquisition by *F. occidentalis* larvae (Montero-Astúa et al., 2014). Thrips fed on these transgenic plants as larvae also had lower viral loads and transmission rates as adults suggesting that transgenic plants are a promising option for thrips and tospovirus management (Montero-Astúa et al., 2014). Nevertheless, additional strategies that can be alternated or used simultaneously with G_N-S transgenic plants in integrated pest management programs can strengthen thrips and tospovirus control.

Eukaryotic organisms, including insects, possess a common mechanism for the regulation of endogenous gene expression and antiviral defense responses known as RNA interference (RNAi) in animals and post-transcriptional gene silencing (PTGS) in plants. This cellular

process is triggered by the presence of double-stranded RNA (dsRNA), which is then recognized by the protein Dicer. Dicer-like proteins are endonucleases that upon recognition of dsRNAs cleave these molecules into 21-23 nucleotides to produce short interfering RNAs (siRNAs). These siRNAs are loaded into the RNA interference silencing complex (RISC) that uses the guide (antisense) strand to degrade messenger RNA in a sequence-specific manner resulting in gene silencing. Thus, RNAi is not only a research tool to ascertain the biological function of virtually any gene in an insect species, but silencing of essential genes in insects has become an attractive alternative to develop new strategies to control insect pests. Whyard and associates stated that the main challenge for most insect molecular biologists today is to find easy and reliable methods of dsRNA delivery (Whyard et al., 2009). Injection of dsRNA directly into the insect's hemocoel is the most common method of delivery. For example, injection of C002 dsRNA into the pea aphid, *Acyrthosiphon pisum*, resulted in the down-regulation of the corresponding transcript and in a reduced survival of aphids on fava bean plants (Mutti et al., 2006; Mutti et al., 2008). Beet armyworm, *Spodoptera exigua*, injected with chitin synthase dsRNA displayed larval abnormalities such as disorder in the insect cuticle and no expansion of the trachea epithelial wall (Chen et al., 2008). Similarly, injection of the red flour beetle, *Tribolium castaneum*, with chitin synthase dsRNA resulted in disruption in molting, cessation of ingestion, and reduction in larval size (Zhu et al., 2005). More recently, injection of vATPase-B and vATPase-D dsRNA into the corn planthopper, *Peregrinus maidis*, not only increased insect mortality but also reduced fecundity (number of eggs oviposited in corn leaves) in a significant manner (Yao et al., 2013).

Plant-mediated RNAi has been proposed as a promising and environmentally-sound approach to control insect pests of important commodities (Price and Gatehouse, 2008). Western corn rootworm, *Diabrotica virgifera virgifera*, fed on transgenic corn plants expressing vATPase-A dsRNA caused significant less feeding damage in a growth chamber assay (Baum et al., 2007). Similarly, transgenic plants expressing cytochrome P450 (CYP6AE14) dsRNA reduced the levels of the corresponding transcript in the midgut of the cotton bollworm, *Helicoverpar armigera*, retarded larval growth, and impaired larval tolerance to the cotton metabolite gossypol (Mao et al., 2007). Green peach aphid, *Myzus persicae*, fed on *Nicothiana benthamiana* or *Arabidopsis thaliana* transiently or constitutively expressing Rack-1 or C002 dsRNAs had a 60% reduction of the corresponding transcript and also produced less progeny

(Pitino et al., 2011). Plant-mediated RNAi can thus be exploited as a control strategy to minimize the damage caused by insect pests and to reduced their populations (Baum et al., 2007; Huvenne and Smagghe, 2010; Mao et al., 2007; Zhu, 2013). Furthermore, careful design of dsRNAs from coding or non-coding regions of essential genes can lead to the development of control strategies that are tailored to specific species in order to combat insect pests without affecting non-target organisms (Whyard et al., 2009). Interestingly, delivery of dsRNAs against three different genes (hexose transporter, carboxypeptidase, and trypsin-like serine protease) using transgenic plants to feed the brown planthopper, *Nilaparvata lugens*, resulted in reduction of their expression in the midgut but did not lead to increased mortality (Zha et al., 2011). Thus, irrespective of the delivery method used, proof of concept to demonstrate that RNAi operates in an insect species by down-regulation of a target gene and by negatively affecting the normal developmental cycle of a pest is of utmost importance before generating transgenic plants to devise control strategies that can be deploy in greenhouses or the field.

The highly conserved enzyme, vacuolar ATP synthase (vATPase) has been one of the most attractive targets in the development of RNAi technologies for insect pests using injection and ingestion from either artificial diets or transgenic plants. vATPase is a holoenzyme involved in the transport of solutes across plasma membranes and the regulation of the pH in the lumen of various organelles and organs in metazoans (Nelson et al., 2000). The enzyme is present in nearly all epithelial tissues of insects and more specifically, it plays an essential role in nutrient uptake and ion balance in the insect's digestive track (Wieczorek et al., 2000; Wieczorek et al., 2009). The vATPase holoenzyme is composed of two functional domains, V0 and V1. The V0 domain, which is composed of five different subunits (a-e), is the membrane-bound domain of the holoenzyme and function in proton translocation against their own translocation gradient (Forgac, 1998). The V1 domain, which is dynamic as it assembles and disassembles on the cytoplasm of the epithelial cells, is composed of eight different subunits (A-H) and is responsible for the hydrolysis of ATP into ADP and phosphate to produce energy to pump protons and transport solutes across plasma membranes (Cipriano et al., 2008; Jefferies et al., 2008). Several vATPase subunits have been selected as RNAi targets in insect pests. For example, knockdown of vATPase-B in the model insect *Drosophila melanogaster* resulted in death of all flies tested (Davies et al., 1996). Using artificial diets amended with vATPase-A or vATPase-D dsRNAs to feed several coleopteran species, Baum and colleagues showed a negative impact on larval

growth and survival (Baum et al., 2007). Furthermore, feeding of *D. virgifera virgifera* on transgenic corn roots expressing vATPase-A dsRNA resulted in a significant reduction in feeding damage compared to the damage caused by insects maintained in control plants (Baum et al., 2007). Silencing of vATPase-E by oral ingestion of dsRNA-ammended diets resulted in an increased mortality of *D. melanogaster*, *Manduca sexta*, *T. castaneum*, and *A. pisum* (Whyard et al., 2009). Feeding of three vATPase-E dsRNAs targeting different regions of this gene reduced the transcript level in a 41, 55, and 48% in *N. lugens* (Li et al., 2011). vATPase-B and vATPase-E transcript levels were reduced by 81 and 59%, respectively, in the Colorado potato beetle, *Leptinotarsa decemlineata*, after oral delivery of the corresponding dsRNA encapsulated in heat-inactivated bacteria sprayed on potato leaves (Zhu et al., 2011). Wuriyanghan and associates demonstrated that oral delivery of both dsRNA and siRNA through artificial diets induce silencing of a vATPase gene in the potato/tomato psyllid *Bactericerca cockerelli* (Wuriyanghan et al., 2011). Similarly, feeding of dsRNA against vATPase-A through sucrose solutions resulted in a significant transcript knockdown in the yellow fever mosquito *Aedes aegypti* (Coy et al., 2012). Silencing of vATPase-B and vATPase-D in *P. maidis* resulted in increased mortality and reduced fecundity when the corresponding dsRNA was either ingested from artificial diets or directly injected into the insect's hemocoel (Yao et al., 2013).

Currently, RNAi methods have not been developed for any member of the insect order Thysanoptera. Here, I have developed and used an injection method to deliver vATPase-B dsRNA directly into the hemocoel of adult female thrips to elicit RNAi as proof of concept that this cellular response occurs in *F. occidentalis*. Delivery of vATPase-B dsRNA into adult females resulted in a significant reduction (1.3-fold decrease) of this transcript in insects injected with dsRNA against the target gene compared to those injected with GFP dsRNA in relation to those injected with DEPC-H_2O. Furthermore, injection of adult female thrips with vATPase-B dsRNA resulted in significant increased mortality and decreased fertility compared to non-injected insects or insects injected with DEPC-H_2O or GFP dsRNA. Adult female thrips injected with vATPase-B dsRNA, GFP dsRNA, and DEPC-H_2O produced an average of 1.0, 3.4, and 5.6 larvae per female, respectively, while non-injected adult female thrips produced an average of 6.7 larvae per female. Overall, my findings demonstrate that injection of adult female thrips with dsRNA with identical sequence to a *F. occidentalis* gene does trigger silencing of the target gene and have the potential to negatively impact both thrips survival and fertility. These results

suggest that an RNAi-based strategy has the potential and can be further exploited to 1) study gene function in any thrips species and 2) impair thrips survival and/or fertility, which ultimately will aid in the control of thrips populations and tospovirus transmission in field crops and greenhouses.

Results

Knockdown of vATPase-B in dsRNA-injected adult female thrips

Adult female thrips were directly injected with 80 ng of vATPase-B dsRNA to attempt to knockdown vATPase-B gene expression in *F. occidentalis*. Gene expression analysis using real-time quantitative reverse transcriptase-PCR (qRT-PCR) revealed that there was a significant 1.3-fold decrease ($P = 0.004$) in the relative abundance of vATPase-B in adult female thrips injected with vATPase-B dsRNA compared to those injected with GFP dsRNA at 2 days post injection (dpi) (Fig. 4.1). This fold change represents a 23% reduction in the relative abundance of vATPase-B mRNA in insects injected with the dsRNA designed against this target gene in *F. occidentalis*.

Mortality of adult female thrips injected with vATPase-B dsRNA

Survival of adult female thrips injected with vATPase-B dsRNA was determined for 14 days and compared to the survival of three control groups, injected with GFP dsRNA or DEPC-H_2O and to non-injected insects. The average results from three independent biological replicates, all of which followed a very similar trend, are shown in Figure 4.2. In general, the vATPase-B dsRNA treatment resulted in a reduction in the proportion of live female thrips over time compared to all the control groups. Overall, survivorship of adult female thrips in the vATPase-B treatment group was significantly lower than that of insects injected with either GFP dsRNA ($P < 0.0001$) or DEPC-H_2O ($P < 0.0001$) and to non-injected insects ($P < 0.0001$) as well. Survivorship in the GFP control group was significantly lower than that of the non-injected group ($P < 0.0001$) but was not significantly different when compared to the DEPC-H_2O-injected group ($P = 0.3337$). Furthermore, survivorship in the DEPC-H_2O-injected group was significantly lower from that of the non-injected group ($P < 0.0001$). Survivorship in the DEPC-

H_2O control group was significantly lower to that of the non-injected control group at 5 dpi (P = 0.03), 6 dpi (P = 0.03), 13 dpi (P = 0.05), and 14 dpi (P = 0.02). There were no significant differences in adult female thrips survival between the GFP and DEPC-H_2O control groups at any time point. Interestingly, survivorship in the GFP control group was significantly lower when compared to the non-injected control group at 5 dpi and 6 dpi (P = 0.02 both time points) and was significantly lower again at 10 dpi (P = 0.03), 12 dpi (P = 0.05), and 14 dpi (P = 0.02). The survivorship in the vATPase-B treatment group began to decline significantly at 6 dpi (P = 0.05) and continued declining significantly until the end of the experiment (P = 0.02 at 7 dpi, P = 0.003 at 8 dpi, P = 0.001 at 9 dpi, P = 0.004 at 10 dpi, P = 0.003 at 11 dpi, P = 0.005 at 12 dpi, P = 0.005 at 13 dpi, and P = 0.01 at 14 dpi) when compared to the GFP control group. When compared to the DEPC-H_2O-injected control group, the survivorship in the vATPase-B treatment group began to decline significantly at 2 dpi (P = 0.03) and continued declining significantly until 14 dpi (P = 0.01). A significant reduction in survival between the vATPase-B treatment group and the non-injected control group started at 2 dpi (P = 0.003) and continued until 14 dpi (P < 0.0001). The results indicate that injection of adult female thrips has a slightly negative effect on insect survival as the proportion of live insects injected with GFP dsRNA or DEPC-H_2O was significantly lower than their non-injected counterparts. However, these two treatments were not significantly different when compared to each other but were significantly different from the vATPase-B dsRNA treatment, indicating that the vATPase-B target gene silencing had an effect on thrips survival.

Fertility of adult female thrips injected with vATPase-B dsRNA

To determine if vATPase-B dsRNA has an effect on the fertility of *F. occidentalis*, adult female thrips were allowed to oviposit in green beans and the number of emergent larvae was recorded for 14 days for the vATPase-B dsRNA treatment and the control groups (GFP dsRNA, DEPC-H_2O, and non-injected). Figure 4.3 shows the average results from three independent biological replicates of the fertility bioassay. The vATPase-B dsRNA treatment resulted in a reduction in the number of offspring produced over the course of the 14-day experiment compared to all the control groups. Overall, the number of offspring produced in the vATPase-B treatment group was significantly lower than that of insects from the GFP dsRNA (P = 0.0031),

DEPC-H_2O ($P = 0.0001$), and non-injected ($P < 0.0001$) control groups. Fertility in the GFP control group was not significantly different to the DEPC-H_2O-injected control group ($P = 0.0927$) but was significantly lower than that of the non-injected control group ($P = 0.0437$) while there was no significant difference between the DEPC-H_2O-injected and the non-injected control groups ($P = 0.7248$). The number of larvae produced in the GFP dsRNA control group was significantly lower to those produced in the DEPC-H_2O-injected control group only at 5 days ($P = 0.02$), while it was significantly lower to those produced in the non-injected control group at 5 days (and $P = 0.009$) and from 9 days until the end of the experiment ($P = 0.05$ and $P = 0.02$, respectively). However, there were no significant differences in the production of offspring between the DEPC-H_2O and the non-injected control groups at any time point. Production of offspring in the vATPase-B treatment group was significantly lower at 6 ($P = 0.0006$), 10 ($P = 0.02$), 11 ($P = 0.002$), 12 ($P = 0.0002$), 13 ($P = 0.001$), and 14 days ($P = 0.008$) when compared to the GFP dsRNA control group. Furthermore, the number of larvae produced in the vATPase-B treatment group was significantly reduced when compared to those produced in the DEPC-H_2O-injected and non-injected control groups beginning at 4 days ($P = 0.05$ and $P = 0.02$, respectively) until the end of the experiment ($P = 0.0008$ and $P = 0.0002$, respectively). These results suggest that GFP dsRNA has a negative impact in insect fertility compared to the injection of DEPC-H_2O, which was not significantly different from the non-injected control. However, the number of offspring produced by adult female thrips injected with vATPase-B dsRNA was significantly reduced than those from the GFP dsRNA treatment, indicating that the vATPase-B target gene silencing has an effect in thrips survival.

Discussion

F. occidentalis is a cosmopolitan insect pest and vector of several tospoviruses of agricultural importance worlwide. Additional molecular tools are needed to gain a better understanding of the general biology of this insect pest as well as to dissect the molecular basis of vector competency in the *F. occidentalis*-TSWV interaction. In the last few years, RNAi has become a powerful strategy to ascertain gene function of specific genes in insects. Insects belonging to the orders Blattodea (Cruz et al., 2006; Maestro and Belles, 2006; Pueyo et al., 2008), Coleoptera (Baum et al., 2007; Tomoyasu and Denell, 2004; Zhao et al., 2011; Zhu et al.,

2011), Diptera (Coy et al., 2012; Dzitoyeva et al., 2001; Walshe et al., 2009; Zhang et al., 2010), Hemiptera (Araujo et al., 2006; Jaubert-Possamai et al., 2007; Mutti et al., 2006; Shakesby et al., 2009), Hymenoptera (Amdam et al., 2003; Deshwal and Mallon, 2014; Ratzka et al., 2013), Isoptera (Hamilton and Bulmer, 2012; Zhou et al., 2008), Lepidoptera (Mao et al., 2007; Turner et al., 2006), Neuroptera (Konopova and Jindra, 2008), and Orthoptera (Dong and Friedrich, 2005; Nakamura et al., 2008) have been suitable for RNAi studies. Successful RNAi methods have also been established for mites (Kamau et al., 2013; Khila and Grbic, 2007; Kwon et al., 2013) and ticks (Kocan et al., 2009; Soares et al., 2005). Currently, there are no RNAi methods developed for any member of the insect order Thysanoptera and the functionality of this biological process remains unexplored within thrips. Here, I have established a dsRNA injection method to directly deliver vATPase-B dsRNA into the hemocoel of *F. occidentalis* to assess the RNAi effects in 1) the relative abundance of the gene transcript, 2) insect survival, and 3) insect fertility. *In vitro* synthesized vATPase-B dsRNA injected into adult female thrips resulted in a significant 1.3-fold knockdown (23% reduction) in the relative abundance of vATPase-B compared to the GFP dsRNA-injected counterparts at 2 dpi. Knockdown of the vATPase-B transcript resulted in increased mortality (proportion of live females) and reduced fertility (number of offspring). My findings are the first to demonstrate that injection of adult female thrips with dsRNA with identical sequence to a *F. occidentalis* gene does trigger silencing of the target gene and has the potential to negatively impact both thrips survival and fertility.

The main challenge to establish RNAi tools for a specific insect species lie in finding a reliable method of dsRNA delivery (Whyard et al., 2009). Oral delivery of dsRNA in artificial diets not only was unsucsessful to elicit RNAi in *F. occidentalis* but was also detrimental to the development of thrips (Appendix A). Injection delivery of dsRNA has been widely used in many insect species and it has proven to be one of the most effective methods for systemic gene silencing and to determine the efficacy of target genes for control strategies (Siomi and Siomi, 2009). The advantages of using the injection delivery method is that the dsRNA concentration is known by the researcher, all injected-insects received the same exact amount of dsRNA, less dsRNA is needed compared to the amount needed in oral delivery methods, and several organs can be potentially targeted at once. The injection delivery method has allowed the knockdown of target genes in insects and has been useful to investigate if there is a phenotype associated to the gene silencing that can be further exploited as a control strategy. For example, injection of

peptidoglycan recognition protein (PGRP12) dsRNA into the green rice leafhopper, *Nephotettix cincticeps*, resulted in high mortality of fourth instar, fifth instar, and adult females (Tomizawa and Noda, 2013). Although the dsRNA injection delivery method causes a direct physical damage to the insect cuticle when poked with a needle, this method remains suited for functional analyses to screen target genes for selecting the best candidates to be targeted in plant-mediated RNAi strategies for pest control. Practical application of RNAi as a biopesticide in the field will rely on application of dsRNA on plant surfaces or the constitutive expression of dsRNA in transgenic plants. However, assessment of the effect of target genes in survival and fertility by injection delivery of dsRNA can be used as the initial step before embarking on the time-consuming process of generating transgenic plants. Here, I established and used a dsRNA injection method to deliver vATPase-B dsRNA into the hemocoel of *F. occidentalis* females. The significant transcript reduction of vATPase-B (23% reduction) at 2 dpi clearly demonstrates that injection of dsRNA can elicit an RNAi response in *F. occidentalis*. Thus, delivery of dsRNA by injection is an excellent tool for study gene function in adult *F. occidentalis* females.

The conserved vATPase subunits are essential genes and effective targets for RNAi in insects (Baum et al., 2007; Li et al., 2011; Whyard et al., 2009; Yao et al., 2013). The multiple vATPase subunits form two domains, V0 and V1, which assemble into the functional holoenzyme that is involved in the transport of protons and solutes across plasma membranes and the regulation of pH in various organs in animals (Cipriano et al., 2008; Jefferies et al., 2008; Nelson et al., 2000). Silencing of several vATPase subunits in different insect species through ingestion or injection has been documented to increase insect mortality in *Diabrotica virgifera virgifera* (Baum et al., 2007), *Bactrocera dorsalis* (Li et al., 2011), *Bemisia tabaci* (Upadhyay et al., 2011), *Manduca sexta* (Whyard et al., 2009), and *Bactericerca cockerelli* (Wuriyanghan et al., 2011)). Nine vATPase subunits (A, B, C, D, E, F, G, H, and d) were identified in a partial *F. occidentalis* transcriptome generated previously (Badillo-Vargas et al., 2012; Rotenberg and Whitfield, 2010). vATPase-B was selected as the target gene in my efforts to develop RNAi tools for functional genomic assays and control strategies of *F. occidentalis*. In the model insect, *Drosophila melanogaster*, a single gene encodes the vATPase-B (*vha55*) which is specifically expressed in the midgut, hindgut, rectum, larval brain, larval salivary glands, Malpighian tubules, testes, accessory gland, duct, bulb, and ovaries (Allan et al., 2005). In this study, injection of vATPase-B dsRNA resulted in significantly higher mortality compared to the control groups

(injected with GFP dsRNA or DEPC-H_2O and non-injected). Similarly, silencing of vATPase-B and vATPase-D in the corn planthopper, *Peregrinus maidis*, resulted in an increased mortality (Yao et al., 2013). As was the case with adult *F. occidentalis* females, the mortality caused by either vATPase-B or vATPase-D silencing in *P. maidis* was significant beginnig at 6 dpi (Yao et al., 2013), despite the fact that adults and not inmature stages were injected in this study.

Bohrman and Braun have reported that the vATPase enzyme is abundant in apical and lateral follicule cell membranes, nurse cell membranes, and the oolema, and it is involved in vesicle acidification and yolk processing necessary for follicule growth (Bohrmann and Braun, 1999). In this study, *F. occidentalis* females injected with vATPase-B dsRNA had significantly lower fertility rates (number of offspring produced) compared to females injected with either GFP dsRNA or DEPC-H_2O and to non-injected insects as well. Knockdown of vATPase subunits B and D resulted in a reduction of *P. maidis* reproductive capacity (i.e., egg production) that could have been at least in part due to the deformed ovarioles with abnormal fat tissues and few mature oocytes (Yao et al., 2013). The aforementioned results support the role of vATPase-B in thrips fertility and the reduction of offspring production observed here during silencing of this target gene. Silencing of a different gene, arginine kinase, also increased adult mortality and greatly reduced fecundity and fertility of the striped flea beetle *Phyllotreta striolata* (Zhao et al., 2008). Thus, selection of essential insect genes that have a negative effect in survival and fertility of an agricultural pest seem to be promising targets for plant-mediated RNAi strategies that will reduce insect population and their reproductive capacities.

This study represents the first report of RNAi in *F. occidentalis* by injection of dsRNA. The increased mortality and reduced fertility caused by the vATPase-B transcript knockdown supports the potential of devising plant-mediated RNAi strategies using transgenic plants that express hairpins of essential genes from thrips to control this important agricultural pest. Such tools can be applied in field and greenhouse settings to control thrips. Furthermore, knockdown of essential genes in thrips that compromises their normal development might also have important implications in the spread of the plant viruses that they transmit. Additionally, RNAi tools for *F. occidentalis* represent useful functional genomic tools to study the role of thrips genes during the insect vector interaction with tospoviruses. A number of *F. occidentalis* candidate genes identified as responsive or interacting proteins during *Tomato spotted wilt virus* infection of the insect vector can be further studied if RNAi tools can be used with larval thrips,

which are the developmental stages that acquired the virus for successful transmission as adults. However, the injection delivery of dsRNA described here represents a useful tool to study important aspects of the life cycle of thrips during adulthood such as genes involved in insecticide resistance (e.g. P450) and reproduction (e.g. vitellogenin) that could be exploited as management strategies to control this insect pest and consequently tospovirus transmission.

Materials and Methods

Frankliniella occidentalis rearing

F. occidentalis were reared on green bean pods (*Phaseolus vulgaris*) and maintained in 16-oz clear plastic deli cups with lids fitted with thrips-proof mesh at 22ºC (±2 ºC) under laboratory conditions as previously described (Bautista et al., 1995; Ullman et al., 1992). In order to synchronize insect development to obtain adult female thrips for dsRNA injection, adult thrips were given access to green bean pods for 3 to 4 days for females to oviposit. All adult thrips were then removed and the green beans were incubated at 23ºC for around 12 to 15 days, after which young adult female thrips (~ 3 days after adult eclosion) were collected and used in injection experiments.

dsRNA synthesis

To generate dsRNA for injection experiments, total RNA was extracted from a pool of ~200 healthy larval thrips with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previously described (Badillo-Vargas et al., 2012). One microgram of *F. occidentalis* total RNA was used for cDNA synthesis using a Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Following cDNA synthesis, the vATPase-B sequence fragment was amplified by PCR using gene-specific primers ligated to the 26 bp T7 RNA polymerase promoter and 1 µL of cDNA as template. The vATPase-B-specific primers to synthesize dsRNA (Table 4.1) were designed using the E-RNAi Webservice from The German Cancer Research Center (http://www.dkfz.de/signaling/e-rnai3/) and the CL1922Contig1_S454 sequence from the *Fo Seq* database generated previously (Badillo-Vargas et al., 2012). The GFP sequence fragment was amplified from the pSITE-2NB vector (Chakrabarty et al., 2007) by PCR

using gene-specific primers described before (Yao et al., 2013). Using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA), 1 µg of PCR product (500 bp for vATPase-B and 305 bp for GFP) was used as the template for the *in vitro* synthesis of dsRNA following the manufacturer's instructions. The dsRNA was precipitated overnight using 100% ethanol at -20ºC, washed 3 times with 70% ethanol, and resuspended in RNase-fee water. The quantity and quality of the dsRNA were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and by 2% agarose gel electrophoresis, respectively. The *in vitro* synthesized vATpase-B and GFP dsRNA was used as the template for RT-PCR and the amplified products were sequenced to verify their nucleotide composition before conducting the injection of adult female thrips.

dsRNA injection of adult female thrips

Young adult female thrips (~ 3 days after adult eclosion) were starved for 1 h prior to the injection experiments to deliver dsRNA directly into the insect's hemocoel. In order to determine the efficiency of RNAi in adult female thrips by injection, four treatments were used in the experiment: non-injected female thrips or female thrips injected with DEPC-H_2O, dsRNA of GFP, or dsRNA of vATPase-B. Thirty adult female thrips per treatment were injected using a Nanoinjector II (Drummond Scientific, Broomall, PA, USA) for the gene expression analysis. Adult female thrips were immobilized by placing them on the sticky side of Scotch tape and immediately injected with 12 nL of dsRNA (80 ng per insect) of each dsRNA solution at 1 nL/sec piercing only once through the stretchable membrane between the second and third tergites of the abdomen. Injected female thrips were then placed by treatment in plastic shot cups containing a piece of green bean with lids fitted with thrips-proof mesh and incubated at 22ºC (±2 ºC) under laboratory conditions in a completely randomized design. The experiment included four treatments with two experimental replicates per treatment and a total of four independent biological replicates were conducted for the gene expression analysis. For the experiments to assess the effect of RNAi in the survival and fertility of adult female thrips, 24 insects were injected per treatment and they were maintained individually in plastic shot cups having a piece of green bean. Three independent biological replicates of the injection experiment to assess adult female thrips survival and fertility were conducted, which consisted of

four treatments (non-injected or injected with H_20, dsRNA of GFP and dsRNA of vATPase-B) in a completely randomized block design.

Gene expression analysis to assess vATPase-B silencing in adult female thrips

To determine if RNAi can be used for functional genomic assays of *F. occidentalis*, I conducted four biological replicates of the injection experiment and used those insects for gene expression analysis. Following the injection of adult female thrips, two samples per treatment (non-injected or injected with H2O, dsRNA of GFP, or dsRNA of vATPase-B) consisting of 6 adult female thrips each were collected in 1.7 mL nuclease-free microcentrifuge tubes at 2 days post injection (dpi), flash frozen immediately, and stored at -80ºC. TRIzol reagent (Invitrogen) was used to extract total RNA from the pooled female thrips as described before (Badillo-Vargas et al., 2012). One microgram of *F. occidentalis* total RNA was used to generate cDNA for each sample using the Verso cDNA Synthesis Kit (Thermo Scientific) and the RT enhancer component to remove any traces of genomic DNA contamination. Gene-specific primers (Table 4.1) for the target gene (*F. occidentalis* vATPase-B) were designed using Beacon7 software while those for the internal reference (*F. occidentalis* actin gene) were as described by Boonham *et al.* (Boonham et al., 2002). Eight microliters of undiluted cDNA template were used in a 10 µL SYBR Green Supermix reaction (Bio-Rad, Hercules, CA, USA) and the real-time qPCR was performed in a 2-step amplification with 40 cycles of 95ºC for 30 s and 56ºC for 30 s using a Bio-Rad CFX96 real-time thermocycler (Bio-Rad). The relative expression method described by Pfaffl was used to calculate the relative abundance of the target RNA (vATPase-B) in the dsRNA treatments (dsRNA of GFP and dsRNA of vATPase-B) compared to the injection control (DEPC-H_2O) (Pfafll, 2001).

Assessment of survival and fertility of adult female thrips

Survival and fertility bioassays using adult female thrips after injection were conducted to determine if RNAi methods (specifically using dsRNA of vATPase-B) have the potential to cause insect mortality and decrease the production of offspring and might then be applied as a control strategy to manage *F. occidentalis*. Three biological replications of this experiment were conducted an 24 insects were injected per treatment (insects injected with DEPC-H2O, dsRNA

of GFP, or dsRNA of vATPase-B) and individually placed in plastic shot cups containing a piece of green bean that served as food source and as oviposition substrate. Furthermore, 24 non-injected adult female thrips were also individually placed in plastic shot cups as a fourth treatment. Twice a week new pieces of green beans were added into the shot cups and the old ones were kept to allow larval eclosion from eggs laid in them. Using a dissecting scope, adult female thrips survival and the number of offspring generated by each female in the four treatments were recorded daily for 14 days.

Statistical analyses

Generalized linear model analyses were conducted using SAS v9.2 (SAS Institute, Cary, NC, USA) in order to determine if there were significant effects on gene expression, survival, and fertility caused by the injection of vATPase-B dsRNA into adult female thrips. For the gene expression analysis of the target gene transcript, the relative abundance of vATPase-B in the dsRNA treatments (dsRNA of GFP and dsRNA of vATPase-B) compared to the injection control (DEPC-H_2O) was calculated using the relative expression method developed by Pfaffl (Pfaffl, 2001). The relative abundances of the two dsRNA treatments (dsRNA of GFP and dsRNA of vATPase-B) were then analyzed using the SAS procedure PROC GLIMMIX with a gamma distribution. The LSMEANS statement was used to generate least square differences and p-values to determine if there were significant differences between the dsRNA treatments and between the four independent experiments. For the survival data (proportion of live female thrips), the PROC GLIMMIX procedure with a beta distribution and a logit-link function including the RANDOM statement, with shot cups containing the female thrips measured over time, and the LSMEANS statement were used accordingly. The analysis was performed as a factorial design with the statistical model consisting of two main effects (dsRNA treatment and time) and the interaction term (dsRNA treatment*time), and it included an autoregressive structure of the data. Type III analysis was performed to test the significance of the treatment main effect. For the offspring count data, a value of 1 was added to all the results across the four treatments to eliminate the zeros (no offspring were observed for the first few days of the experiment as it takes between 2 or 3 days since oviposition from larvae to hatch) to obtain convergence of the algorithm used to fit the data. A regression analysis using the negative

binomial with an invertible linearizing log-link function was performed using the SAS procedure GENMOD for the offspring count data. The analysis, which was performed as the factorial design described above, also included an autoregressive structure of the data and the REPEATED statement, with shot cups containing the viable offspring measured over time. Least square differences and p-values were generated using the LSMEANS statement to compare treatment means.

Figures and Tables

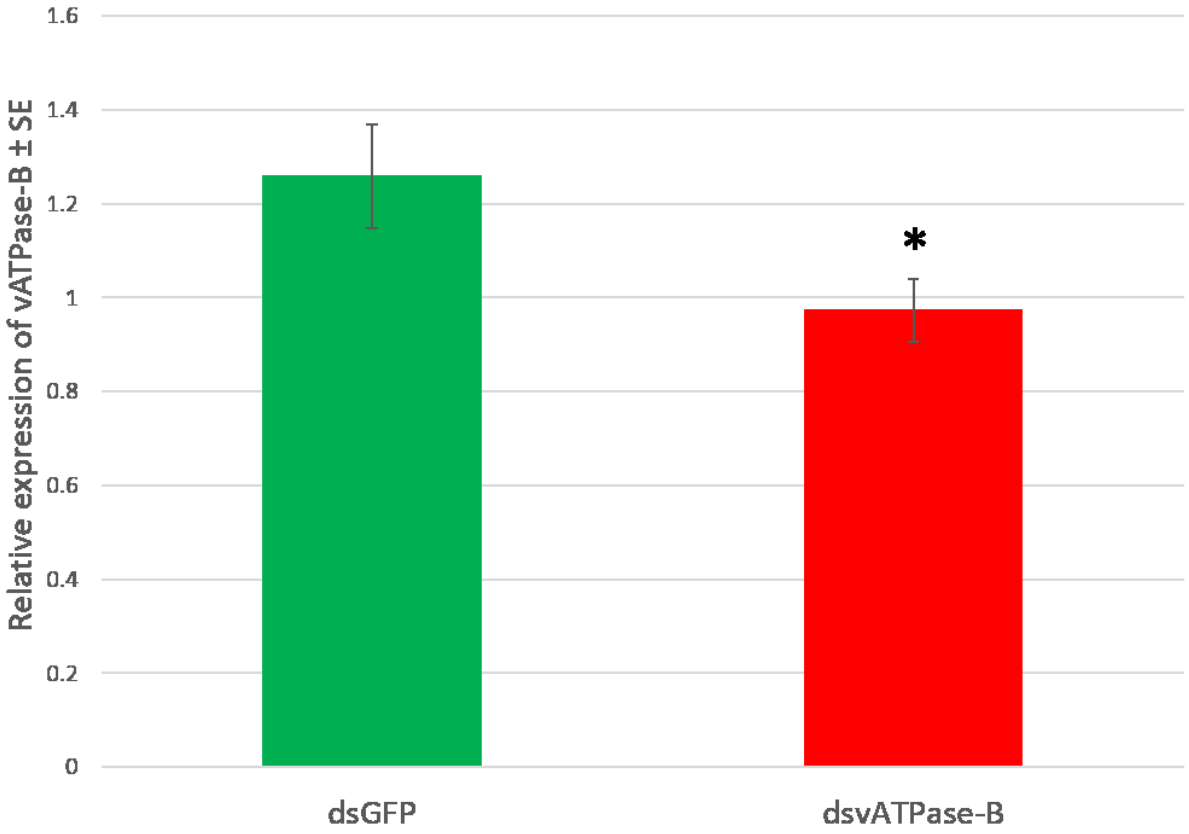

Figure 4.1 Reduction in the relative abundance of vATPase-B transcript in adult *Frankliniella occidentalis* females by injection of dsRNA.

Adult *F. occidentalis* females were injected with 12 nL of dsRNA (80 µg/insect) of vATPase-B or GFP. Groups of six pooled female thrips were collected at 2 days post injection and gene expression of vATPase-B was analyzed by real-time quantitative reverse transcriptase-PCR relative to DEPC-H_2O-injected insects using *F. occidentalis* actin as the internal reference gene. Each bar represent the mean and standard error (SE) of the mean of two experimental replicates (samples) for four independent biological replicates. The asterisk (*) indicates that treatment means differ significantly ($P < 0.05$).

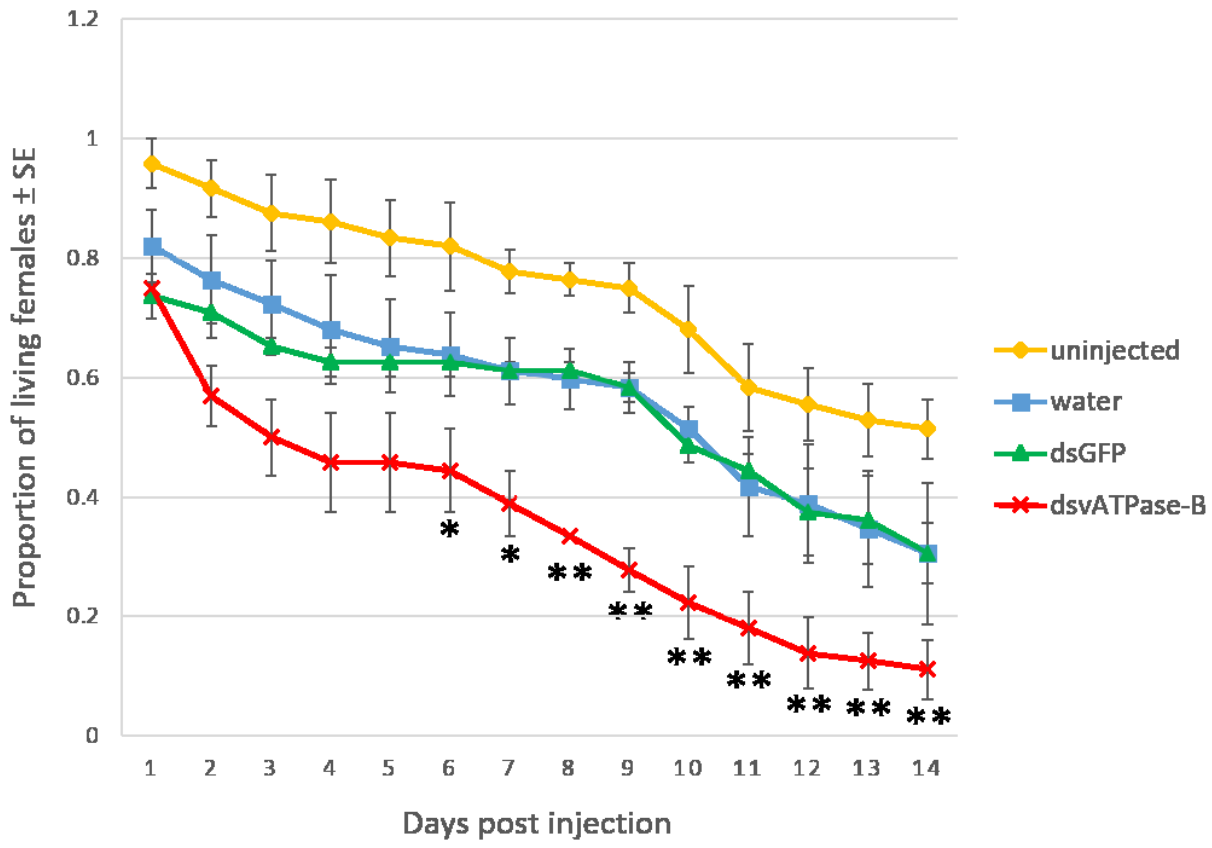

Figure 4.2 Adult *Frankliniella occidentalis* female mortality after injection of vATPase-B dsRNA.

Adult *F. occidentalis* females were injected with 12 nL of dsRNA (80 µg/insect) of vATPase-B or GFP or DEPC-H_2O. Non-injected female thrips were also included to assess the survival rate (proportion of live females) of undamaged insects. Twenty four adult females per treatment were maintained separately in shot cups containing a green bean piece as food source, which were monitored repeatedly for 14 days post injection. Three independent biological replicates of the experiment were conducted. A single asterisk (*) indicates that treatment means differ significantly at a $P < 0.05$ from the GFP dsRNA control group while a double asterisk (**) indicates that treatment means differ significantly at a $P < 0.01$ from GFP dsRNA control group.

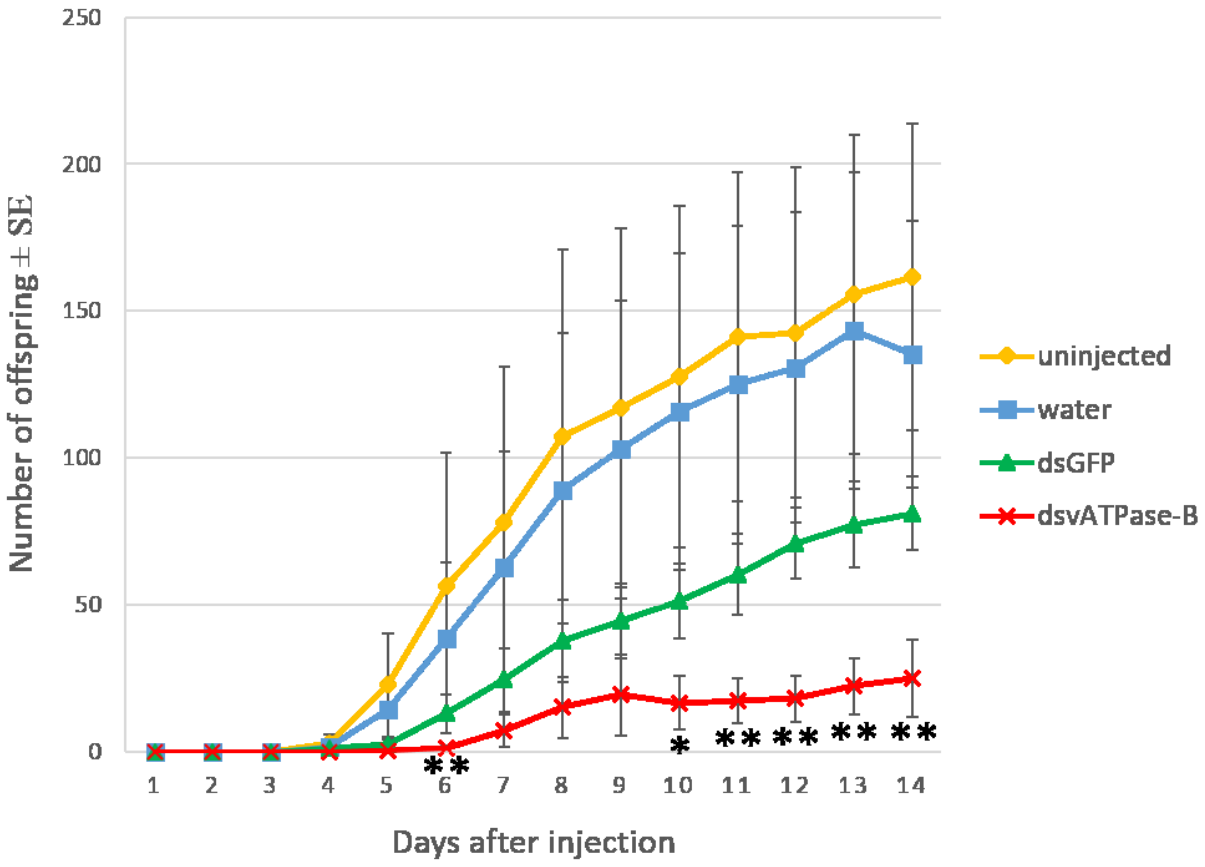

Figure 4.3 Fertility of adult *Frankliniella occidentalis* females after injection with vATPase-B dsRNA.

Adult *F. occidentalis* females were injected with 12 nL of dsRNA (80 µg/insect) of vATPase-B or GFP or DEPC-H_2O. Non-injected female thrips were also included to assess the fertility rate (number of offspring produced) of undamaged insects. Twenty four adult females per treatment were maintained separately in shot cups containing a green bean piece as food source and oviposition substrate. Individual cups were monitored repeatedly for 14 days post injection to assess the production of offspring. Three independent biological replicates of the experiment were conducted. A single asterisk (*) indicates that treatment means differ significantly at a $P < 0.05$ from the GFP dsRNA control group while a double asterisk (**) indicates that treatment means differ significantly at a $P < 0.01$ from GFP dsRNA control group.

Table 4.1 Primer sequences used for dsRNA synthesis and real-time quantitative PCR.

Gene	Primer use	Sequence (5' to 3')	Product length	Primer efficiency
GFP	dsRNA synthesis	T7F: GGATCCTAATACGACTCACTATAGGGGTGACCACCCTGACCTAC F: GTGACCACCCTGACCTAC T7R: GGATCCTAATACGACTCACTATAGGGTTGATGCCGTTCTTCTGC R: TTGATGCCGTTCTTCTGC	305 bp	
vATPase-B	dsRNA synthesis	T7F: GGATCCTAATACGACTCACTATAGGGGGACCTTTGGTGATTTTGGA F: GGACCTTTGGTGATTTTGGA T7R: GGATCCTAATACGACTCACTATAGGGAACACAGATTTGCCTGGGAC R: AACACAGATTTGCCTGGGAC	500 bp	
actin	qPCR	F: GGTATCGTCCTGGACTCTGGTG R: GGGAAGGGCGTAACCTTCA	105 bp	93%
vATPase-B	qPCR	F: CAGATTCCTATCCTTACTATGC R: TGTGAAGTTGTCGGTCTA	106 bp	99%

Chapter 5 - General discussion and future perspectives

Summary of results and products

Viruses in the family *Bunyaviridae* have been and continue to be a threat to human health, animal husbandry, and plant production worldwide (de Oliveira et al., 2012; Guler et al., 2012; Herder et al., 2012; McMullan et al., 2012; Walter and Barr, 2011). Their success as viral pathogens is due at least in part to the persistent, non-lethal infection of their arthropod or rodent vectors that specifically transmit them (German et al., 1992). The type species of the genus *Tospovirus* within the virus family *Bunyaviridae*, *Tomato spotted wilt virus* (TSWV), has been increasing in importance and significance since its initial discovery and description in Australia in 1915 (Brittlebank, 1919). TSWV is one of the ten most devastating plant viruses known and it poses a high risk to food security worldwide by infecting hundreds of plant species (Scholthof et al., 2011). Unfortunately, there are no treatments to cure viral infections in plants. Once a virus infects a plant the only viable alternative is to remove and destroy the plant in an attempt to reduce the source of virus inoculum and hence its secondary spread. The only options to manage plant viruses are to prevent or avoid infection using host resistant varieties, cultural practices, or in some cases applying insecticides for plant viruses that are dependent of arthropod vectors for transmission. Thus, novel control strategies that target a specific plant virus or even its insect vector are highly desired.

Like other bunyaviruses, TSWV is transmitted in a persistent propagative fashion by its vectors (Ullman et al., 1993; Wijkamp et al., 1993). The western flower thrips, *Frankliniella occidentalis* Pergande, is one of the most efficient vectors of tospoviruses (Riley et al., 2011). TSWV is the best characterized tospovirus with respect to interactions with its most efficient vector, *F. occidentalis*. It has been well established that TSWV must be acquired by larval thrips in order to be transmitted later by adult thrips (Lindord, 1932; van de Wetering et al., 1996). Both males and females are able to transmit the virus although there is a tendency for males to be more efficient transmitters of TSWV than their female counterparts (Rotenberg et al., 2009; Stafford et al., 2011; van de Wetering et al., 1999). Interestingly, adult thrips that feed on virus

infected plants do acquire TSWV but it is restricted to the midgut tissues and does not reach the salivary glands, thus, this virus infection represents a dead end (de Assis Filho et al., 2004; Ullman et al., 1992). Furthermore, the virus it is not transovarially transmitted and offspring produced by virus-infected parents are virus-fee (Wijkamp et al., 1996). TSWV binds to and enters the thrips midgut, replicates in the midgut epithelial cells and surrounding muscle tissue, and then journeys to and replicates in salivary gland tissues for transmission to occur. Moreover, the replication strategy, genome organization, and role of viral proteins for TSWV have been extensively studied. However, despite the importance of *F. occidentalis* as an insect pest and vector of tospoviruses, the thrips molecules that interact with and respond to TSWV during the infection of the insect vector remain poorly understood. Thus, the work in this doctoral dissertation aimed to use proteomic tools and functional genomic methods to study thrips and tospoviruses and to begin to dissect the molecular bases of the *F. occidentalis*-TSWV interaction. The specific goals of my research were to 1) identify larval thrips proteins that were differentially expressed due to virus infection, 2) identify larval thrips proteins that directly interact with purified TSWV virions or a TSWV glycoprotein (G_N), and 3) develop RNA interference (RNAi) tools for functional genomic assays and control strategies of *F. occidentalis*. To achieve these goals, I compared the protein profiles of TSWV infected and non-infected larval thrips using two-dimensional (2-D) gel electrophoresis and identified differentially expressed proteins using mass spectrometry. The findings of my experiments indicate that there are indeed changes at the protein level in *F. occidentalis* larvae due to TSWV infection. Moreover, larval thrips proteins that interacted directly with either purified TSWV virions or recombinant G_N shown previously to play a role in viral attachment to thrips guts were identified by overlay assays of 2-D gels. Finally, my findings are the first to demonstrate that injection of adult female thrips with double-stranded RNA (dsRNA) with identical sequence to a *F. occidentalis* gene does trigger silencing of the target gene transcript and have the potential to negatively impact thrips survival and fertility.

My doctoral work focused on gaining a better understanding of the thrips proteins that directly interact with TSWV and those that respond to virus infection as well as developing tools that can be used to further characterize the specific role of these proteins in different steps of the virus-vector interaction. The studies were all conducted using *F. occidentalis*, a plant pest of agricultural significance on its own and an important vector of TSWV. In this work, I have

identified a number of proteins from thrips that are putatively involved, either interacting or responding, in the infection process of the insect vector by a plant-infecting virus transmitted in a persistent propagative manner. I have also shown proof of concept that the RNAi mechanism operates in *F. occidentalis* to silence target gene transcripts, and this has the potential to be used as a control strategy to manage thrips damage and tospovirus transmission.

The major contributions of this dissertation are summarized in detail per chapter:

Proteomic analysis of *Frankliniella occidentalis* and differentially expressed proteins in response to *Tomato spotted wilt virus* infection.

Using 2-D gel electrophoresis coupled with mass spectrometry, proteomic tools for thrips were developed. As a first step, these tools were used to characterize the proteome of healthy larval thrips. Furthermore, these tools were applied to conduct an analysis of differentially expressed proteins of *F. occidentalis* larvae during TSWV infection to identify putative proteins that respond to the virus infection of the insect vector. The major results of this chapter were:

- Fifty two percent of the 194 proteins spots clearly resolved from healthy first instar larvae were identified using the *Fo* Seq and the Metazoan non-redundant (nr) protein database from NCBI. However, a higher proportion of peptides (47%) matched the *Fo* Seq while a lower proportion (23%) matched sequences from the Metazoan database. These results clearly show the value of the *F. occidentalis* transcriptome to identify proteins from this insect species. The 48% of proteins that were not identified might represent proteins that are species-specific or unique to the insect order Thysanoptera.
- There were 15 proteins identified from healthy first instar larvae that were functionally annotated as proteins associated with cell killing or the innate immune response suggesting that they might have multifunctional roles or that there is a basal level of expression of defense-related genes ready to combat pathogen infection such as the invasion by TSWV.
- An increase of TSWV acquisition efficiency by *F. occidentalis* larvae up to 90% was obtained when using *Emilia sonchifolia* plants 12 days after mechanical inoculation using thrips-inoculated *E. sonchifolia* up to a month after virus

transmission by the insect vector. Higher and more consistent acquisition rates of TSWV by thrips represent a big improvement in the study of this pathosystem.

- Twenty six protein spots that displayed differential abundances in response to TSWV infection were identified. Interestingly, 62% of these protein spots were down-regulated by TSWV infection demonstrating a complex response by the thrips vector to a virus transmitted in a persistent propagative manner.
- Electrospray ionization (ESI) mass spectrometry allowed the identification of 37 proteins within the 26 spots that were differentially expressed between TSWV infected and non-infected larval thrips. This moderate but marginally significant change in protein profile represent the first set of candidate proteins with putative roles in this virus-vector interaction.
- Thirty two percent of these proteins had gene ontology assignments representing biological roles associated with the infection cycle of other plant- and animal-infecting viruses and antiviral defense responses. Among the 14 proteins in the category of response to stimuli, there were 9 proteins clearly associated with innate immune defenses that might be implicated in the antiviral response of the thrips vector during TSWV infection. The down-regulation of 5 of these proteins suggests that there might be a controlled regulation of viral replication that culminates in the successful transmission of TSWV without compromising the well-being of the insect vector.

Identification of *Frankliniella occidentalis* proteins that directly interact with *Tomato spotted wilt virus*.

Thrips proteins that putatively interact with TSWV were initially identified using overlay assays of *F. occidentalis* larval proteins resolved by 2-D gels. The membranes containing the larval thrips proteins were either overlayed with purified TSWV virions or a TSWV glycoprotein (G_N) produced in bacterial cells. Protein spots that were consistently observed in 3 or 4 replicates of the virus overlay assay or 2 replicates of the recombinant G_N overlay assay were identified using ESI mass spectrometry and Mascot searches of the *Fo* Seq and the Metazoan nr protein database from NCBI. The major findings obtained for this chapter were:

- Using mass spectrometry, four unique *F. occidentalis* proteins were identified as putative interactors with purified TSWV virions; these proteins were cyclophilin, enolase, cuticular protein, and endocuticle structural glycoprotein.
- Two unique *F. occidentalis* proteins were identified as putative interactors with recombinant G_N. One of these proteins, endocuticle structural glycoprotein, was also identified using the virus overlay assay, while the other one, mitochondrial ATP synthase α subunit was exclusively identified in overlay assays using the recombinant G_N.
- The *F. occidentalis* cyclophilin, enolase, and mitochondrial ATP synthase α subunit proteins, identified here as putative interactors with either purified TSWV virions or recombinant G_N, were also identified as responsive *F. occidentalis* proteins in the differentially expression analysis. Both enolase and mitochondrial ATP synthase α subunit were up-regulated by TSWV infection while cyclophilin was down-regulated.
- These interacting proteins might play roles in attachment and entry (cuticular protein and endocuticle structural glycoprotein), endocytosis/exocytosis (cyclophilin and mitochondrial ATP synthase α subunit), and escape from different tissues (enolase) for transmission to occur.
- The identification of these 5 thrips proteins has lead to the design of additional strategies to further validate the protein-protein interactions between the thrips proteins and TSWV (by expression of the thrips proteins in an heterologous insect expression system for additional overlay assays) and to determine the localization of these proteins within thrips larvae to predict their possible role in the infection of the insect vector and identify if there is colocalization with TSWV particles (by immunohistochemistry of larval thrips tissues using antibodies generated against the thrips candidate proteins).

Development of RNA interference tools for functional genomic assays and control strategies of *Frankliniella occidentalis*.

The lack of thrips tissue culture and transgenesis methods to maintain thrips cells on media and to make transgenic thrips, respectively, has been and continues to be a major barrier to understanding the general biology of thrips as well as to dissect the molecular basis of vector competency in the *F. occidentalis*-TSWV interaction. An alternative approach is to use RNAi for functional genomic assays where dsRNA will trigger silencing of a target gene transcript in thrips to assess its biological function, effects in the insect's life cycle, and interaction with the tospovirus they transmit. Currently, there are no RNAi methods developed for any member of the insect order Thysanoptera. Delivery of dsRNA directly into the thrips body by injection was explored here using *F. occidentalis* adult female thrips. The main results and conclusions from these experiments were:

- Overall, there was a 23% reduction of the vATPase-B transcript in adult female thrips injected with vATPase-B dsRNA in comparison to those injected with GFP dsRNA relative to insects injected with DEPC-H_2O. Statistical analysis showed that this reduction is significant ($P = 0.04$). This reduction in vATPase-B transcript level resulted in two observable phenotypes, increased mortality and reduced fertility.
- Three biological assays showed that there was a significant increase in insect mortality caused by vATPase-B dsRNA compared to the GFP dsRNA-injected ($P < 0.0001$), DEPC-H_2O-injected ($P < 0.0001$), and non-injected ($P < 0.0001$) control groups.
- Injection of adult female thrips with vATPase-B dsRNA had a negative effect in insect fertility, decreasing the number of viable offspring produced in a significant manner. Overall, the number of viable offspring produced by adult female thrips injected with vATPase-B dsRNA differed from those produced by the GFP dsRNA-injected ($P = 0.0031$), DEPC-H_2O-injected ($P = 0.0001$), and non-injected ($P < 0.0001$) adult female thrips significantly.
- This dsRNA delivery strategy can then be potentially used to ascertain the biological role of genes in *F. occidentalis* and most likely other thrips species and suggests that plant-mediated RNAi has the potential to be used as a control

strategy to manage these insect pests in field crops and greenhouses. Transmission of TSWV and other tospoviruses might be disrupted or completely abrogated if plant-mediated RNAi strategies are used to silence essential genes of their thrips vectors.

General discussion

Development of proteomic approaches using 2-D gel electrophoresis to study thrips-tospovirus interactions was an attractive strategy to begin to dissect the molecular interplay between TSWV and its most efficient insect vector, *F. occidentalis*. TSWV and *F. occidentalis* could be considered the model pathosystem amongst thysanopterans and the plant viruses they transmit. The identification of 37 responsive candidate proteins from larval thrips during virus infection together with the identification of 5 interacting candidate proteins from larval thrips that are putatively involved in the infection process of the insect vector provide new insights into the molecular basis of this important interaction. The differential expression (62% down- and 38% up-regulated) of these *F. occidentalis* proteins during TSWV infection suggests that there is a complex response of larval thrips during its interaction with this plant virus transmitted in a persistent propagative manner. Proteins such as cyclophilin, enolase, vacuolar ATP synthase, mitochondrial ATP synthase, apolipoprotein, thioredoxin-dependent peroxidase, stress induced phosphoprotein-1, glyceraldehyde-3-phosphate dehydrogenase, and heat shock proteins just to mention a few are proteins that have been implicated in the infection process of other plant- and animal-infecting viruses. For example, vacuolar ATP synthase has been identified as a host factor required for virus entry into the host cells during the infection of negative-sense RNA viruses including members of the *Bunyaviridae* family (Hollidge et al., 2012; Panda et al., 2011). Moreover, the conservation of insect proteins such as cyclophilin and cuticle proteins amongst insect vectors that transmit plant viruses along the persistent transmission pathway might be considered biological signatures of this type of transmission as they have also been identified as biomarkers for vector competency in whiteflies and several aphid species that transmit begomoviruses and luteoviruses, respectively (Cilia et al., 2012). Cyclophilin A and B from *S. graminus* have been shown to bind to CYDV-PAV using a combination of assays and mass spectrometry approaches (Tamborindeguy et al., 2013). Interestingly, the Chitin_bind_4 motif

detected in the cuticular protein and endocuticle structural glycoprotein identified in the overlay assays conducted here is also present in a cuticular protein of the brown planhopper, *Laodelphax striatella*, shown to bind the ribonucleocapsid protein of *Rice stripe virus* (RSV) *in vitro* and *in vivo* (Wang et al., 2013). Silencing of this protein in the brown planthopper resulted in a reduced viral load and transmission of RSV by its insect vector (Wang et al., 2013). These results suggests that this motif could be important in the attachment of TSWV to a receptor in the thrips midgut as it has been documented that this motif is present in proteins expressed in gut tissues (Rebers and Willis, 2001; Talbo et al., 1991). Ultimately, this work has generated a suite of candidate genes (42 proteins total) that could enable the development of novel strategies to control thrips damage and tospovirus transmission.

Finally, the development of RNAi tools for functional genomic assays and control strategies of *F. occidentalis* show a significant reduction (1.3-fold decrease that represents a 23% knockdown) of the target gene transcript level at two days post injection, an increased mortality, and a reduced fertility (number of viable offspring produced by females). Similar results were found with other insect vector (*Peregrinus maidis*) of a different plant virus (*Maize mosaic virus*; *Rhabdoviridae*: *Nucleorhabdivirus*), when vATPase-B or vATPase-D dsRNA was injected or orally delivered through artificial diets to the planthoppers (Yao et al., 2013). My findings provide clear evidence that RNAi is functional in a member of the insect order Thysanoptera and supports the concept that RNAi can be exploited not only to study the role of thrips genes but also can be translated into control strategies that can be deployed in field and greenhouse settings. Plant-based RNAi strategies in which thrips can acquired dsRNAs directly from epidermal and mesophyll plant cells in natural scenarios are the next step in the war against thrips and tospoviruses. Silencing of responsive or interacting candidate genes identified here might potentiate the negative impact of the silencing effect if this causes TSWV to be pathogenic to *F. occidentalis* by disarming the thrips innate immune response. Thus, my work has opened new research avenues that warrant careful examination to devise control strategies against thrips and tospoviruses, which might also have implications in the management of other plant-infecting viruses transmitted by arthropod vectors.

Future research and perspectives

The identification of the *F. occidentalis* responsive and interacting candidate proteins and the development of RNAi strategies to determine their specific role in the biological processes underlying vector competency and the ability to transmit TSWV efficiently is just the beginning of the effort to uncover the intricacies of the *F. occidentalis*-TSWV interaction. The results obtained throughout this work provide a suite of candidate genes that need to be studied in detail in order to better understand their role during the infection of the insect vector and to develop sustainable and efficient pest and virus control strategies. Possible research avenues that are critical to be followed up based on the results showed herein to achieve these goals are listed below.

- Conduct functional assays of *F. occidentalis* responsive proteins to determine their role in the TSWV infection of the thrips vector. Alternatively, the expression of these same proteins can be monitored in non-vector thrips of TSWV to determine if their expression, or lack thereof, plays a role in vector competency of this plant-infecting virus.
- The proteomic approach used here can be applied to study other thrips vector species and their response to different tospoviruses (e.g. *Thrips tabaci* and *Iris yellow spots virus*) to see if a similar suite of proteins are responsive in a different thrips-tospovirus interaction as it has been observed amongst insect vectors (aphids and whiteflies) of other plant viruses (luteoviruses and begomoviruses) transmitted along the persistent circulative pathway (Cilia et al., 2012).
- Validation of the *F. occidentalis* interactive proteins in their ability to bind either purified TSWV virions or recombinant G_N using a heterologous insect expression system for additional overlay assays and antibodies for localization studies or co-immunoprecipitation are of high priority.
- Characterization of the cuticular protein and endocuticle structural glycoprotein that have the Chtin_bind_4 domain as putative receptors of TSWV in thrips is of great interest as it might unlock a novel strategy to control tospovirus transmission by possibly abolishing the early step of virus acquisition by the insect vector. This can potentially lead to the discovery of tospovirus receptors in

other thrips vector species and the design of a universal strategy to block the virus acquisition amongst thysanopteran-tospovirus interactions.

- Test the functional genomic assay developed here to target different essential genes in thrips to increase the negative effect in the insect life history traits such as feeding, development, survival, fecundity, and fertility. Thus, several of them can be stacked in management strategies to control these insect pests and the viruses they transmit.
- Development of a plant-based RNAi strategy to deliver dsRNA against essential thrips target genes is of great interest as it can be used to silence genes in thrips larval stages that might be more susceptible to the knockdown effect and are the developmental stages that can acquire TSWV for an efficient transmission during adulthood.

The research directions listed here are just a few examples of biological questions and hypotheses that need to be further studied in order to obtain a better picture of the intrinsic interactions between thrips and tospoviruses. New genomic approaches (e.g. RNA-sequencing) using whole insects at different life stages as well as analyzing the expression profile of specific organs comparing virus-free and virus-infected treatments promise to increase our understanding of insect molecules that are important during virus infection of the thrips vector. Moreover, recent findings and ongoing research are demonstrating that basic research can indeed be translated into new strategies with potential to be applied in the field to control plant-infecting viruses and their arthropod vectors, which will have a positive impact in food production and food security worldwide (Bonning et al., 2014; Chougule et al., 2013; Montero-Astúa et al., 2014; Whitfield et al., 2014).

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Appendix A - Assessment of a feeding strategy to orally deliver double-stranded RNA to elicit RNA interference in *Frankliniella occidentalis* larvae using an artificial diet and feeding chamber

Introduction

Thrips and tospoviruses are increasing in importance worldwide and innovative control strategies to efficiently manage the pest damage and virus transmission are highly desired. Furthermore, functional genomic tools to conduct studies to assign functional roles to thrips proteins that might be biologically significant during different steps of the virus-vector interaction (e.g. entry, replication, spread, and transmission) are urgently needed. Currently, there are no RNA interference (RNAi) methods for insects within the order Thysanoptera. To begin to assess the efficacy of different delivery methods, I have performed a number of feeding assays using an artificial diet and feeding chamber to deliver double-stranded RNA (dsRNA) orally to *Frankliniella occidentalis* first inastar larvae.

Results

There was no discernible vATPase-B silencing in *F. occidentalis* first inastar larvae fed vATPase-B dsRNA through an artificial diet at 2, 4, or 6 days post feeding (dpf) compared to larvae fed on GFP dsRNA-amended diets (Fig. A.1). Placing a *Datura stramonium* leaf disc on top of the artificial diets amended with dsRNA did not resulted in silencing for any of the time points analyzed here (Fig. A.2). Despite the fact that the average relative expression ratios (RERs) from the two samples of insects that fed on GFP dsRNA (5.01) or vATPase-B dsRNA (2.55) at 4 dpf seem to indicate a reduction in the vATPase-B transcript level, the individual RERs of each individual GFP dsRNA (0.83 and 9.19) or vATPase-B dsRNA (0.69 and 4.41) sample indicate that the apparent silencing is really an artifact of the high variation among samples fed on the same dsRNA treatment. Moreover, using liposomes-dsRNA complexes to deliver dsRNA through artificial diets to *F. occidentalis* first instar larvae did not reduce the

vATPase-B transcript relative expression (Fig. A.3) nor did the addition of a *D. stramonium* leaf disc on top of the liposomes-dsRNA-amended diets (Fig. A.4). It is worth mentioning that in some instances I observed an increase of vATPase-B relative expression in *F. occidentalis* larvae fed on vATPase-B dsRNA compared to those fed on GFP dsRNA (6 dpf in Fig. A.1, 2 dpf in Fig. A.2, 2 dpf in Fig. A.3, 6 dpf in Fig. A.4, etc.). The biological or molecular bases of this increase are not known, however, feeding for several consecutive days on an artificial solution that lacks the essential nutrients needed by thrips clearly compromised insect development as the thrips did not develop past the first instar larval stage as long as they remained feeding on the diets. This detrimental effect on thrips development caused by extensive feeding on the artificial diet made it difficult to assess RNAi in *F. occidentalis* first instar larvae using this delivery method.

Discussion

Using artificial diets to orally administer dsRNA to silence target genes has been done with a number of different insect species. For example, 1.0 µg/µL of nitrophorin 2 dsRNA ingested by the second instar nymphs of the triatomine bug *Rhodnius prolixus* in a feeding solution reduced gene expression by 42±10% (Araujo et al., 2006). This reduction resulted in a 4-fold increase on plasma coagulation of saliva from knockdown bugs compared to that from control bugs (Araujo et al., 2006). It has been shown that feeding vATPase-A or vATPase-D dsRNAs through artificial diets to several coleopteran species negatively impacted larval growth and survival (Baum et al., 2007). Feeding of a diet amended with aquaporin dsRNA at 1.0 µg/µL to pea aphids, *Acyrthosiphon pisum*, reduced the corresponding transcript by more than 2-fold within 24 h and this knockdown resulted in elevated osmotic pressure of the aphid's hemolymph (Shakesby et al., 2009). dsRNA and siRNA (short interfering RNA) of a vATPase gene orally administered using an artificial diet resulted in transcript silencing in the potato/tomato psyllid *Bactericerca cockerelli* (Wuriyanghan et al., 2011). Similarly, feeding of dsRNA against vATpase-A through sucrose solutions resulted in a significant transcript knockdown in the yellow fever mosquito *Aedes aegypti*, at 12, 24, and 48 h after feeding (Coy et al., 2012). Furthermore, oral delivery of vATPase-E dsRNA using artificial diets resulted in an increased mortality of *Drosophila melanogaster*, *Manduca sexta*, *Tribolium castaneum*, and *A.*

pisum (Whyard et al., 2009). Ingestion of artificial diets amended with either vATPase-B and vATPase-D dsRNA induced silencing of the corresponding transcripts in the corn planthopper, *Peregrinus maidis*, which increased mortality, reduced fecundity, and affected the normal development of the wing's elytra and adult female's ovaries compared to insects fed diets amended with GFP dsRNA (Yao et al., 2013).

Using an artificial diet to orally deliver 1 µg/µL of vATPase-B dsRNA to *F. occidentalis* first instar larvae did not result in reduction of the relative expression of the corresponding transcript when compared to those fed on GFP dsRNA-amended diets. Even when a leaf disc was placed on top of the artificial diet amended with naked dsRNA or when liposomes with or without a leaf disc where used to attempt to increase ingestion and possibly uptake, silencing of vATPase-B was negligible. Thus, the oral delivery using the artificial diet described here (70% serum-free media and 10% sucrose in ddH_2O) is not an efficient method to silence target genes in *F. occidentalis* larvae. A different artificial diet or transient expression of hairpin constructs from plants are alternative approaches to orally administer dsRNA of target genes to *F. occidentalis* larvae. Ultimately, the constitutive expression of hairpin constructs in transgenic plants will provide a source of constant supply of dsRNA and/or siRNA that can be evaluated to determine the efficacy of using RNAi to control thrips damage and tospovirus transmission in field crops and greenhouse production.

Materials and Methods

Emergent larval thrips (0 to 24 h old) were brushed into small plastic shot cups that were then covered with a stretched layer of parafilm. Twenty five microliters of an artificial diet containing 70% serum-free media and 10% sucrose in double-distilled water (ddH_2O) were placed on top of the stretched layer of parafilm. Diets were amended with 1.0 µg/µL of either GFP dsRNA or vATPase-B dsRNA, while a control diet was not-amended. The diets were then sandwiched by a second layer of stretched parafilm and the feeding chambers were incubated at 22ºC (±2 ºC) under laboratory conditions for the duration of the entire experiment. The three treatments (diet only, diet-dsGFP, and diet-dsvATPase-B) were duplicated by using a second feeding chamber with the exact same treatments. All diets were replenished every 24 h for a 6 day period. Ten larval thrips were pooled per feeding chamber to obtain two samples per

treatment in an experiment. The two samples per treatment were collected at 2, 4, and 6 dpf. Following the same indications specified here, this experiment was conducted using few modifications; naked dsRNA, naked dsRNA covered by a leaf disk, dsRNA packaged into liposomes, or dsRNA packaged into liposomes covered by a leaf disk. *F. occidentalis* total RNA was extracted from the pooled inects using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previously described (Badillo-Vargas et al., 2012). One microgram of *F. occidentalis* total RNA was used to generate cDNA for each sample using a Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions and the RT enhancer component was added to remove any traces of genomic DNA contamination. Gene-specific primers for the target gene (*F. occidentalis* vATPase-B) and for the internal reference gene (*F. occidentalis* actin) were used to quantify gene expression in each sample. Eight microliters of undiluted cDNA template were used in a 10 µL SYBR Green Supermix reaction (Bio-Rad, Hercules, CA, USA) and the real-time qPCR was performed in a 2-step amplification with 40 cycles of 95ºC for 30 s and 56ºC for 30 s using a Bio-Rad CFX96 real-time thermocycler (Bio-Rad). Relative abundance of the target RNA (vATPase-B) in the dsRNA treatments (diet-dsGFP and diet-dsvATPase-B) compared to the control (diet only) were calculated using the relative expression method (Pfaffl, 2001).

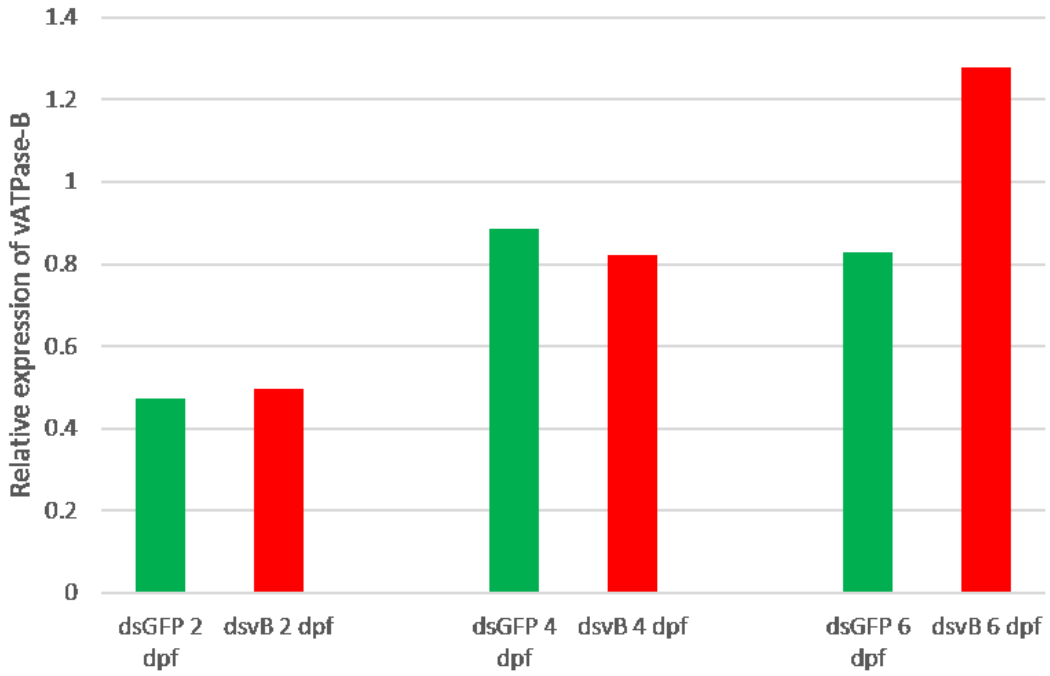

Figure A.1. Silencing of vATPase-B in *Frankliniella occidentalis* first instar larvae fed on double-stranded RNA-amended diets.

Young first instar larval thrips (0 to 24 h old) were allowed to feed on artificial diets amended with vATPase-B dsRNA or GFP dsRNA as a negative control for up to six days. Larval thrips that fed on diet only were used as the calibrator group to calculate relative expression of vATPase-B in the dsRNA treatments. All diets were replenished every 24 h during a 6 day period. Insects were collected at 2, 4, and 6 days post feeding on the diets for real-time quantitative reverse transcriptase-PCR analysis of gene expression. vATPase-B transcript abundance was normalized to *F. occidentalis* actin transcripts. Each bar represents the mean of n = two experimental replicates each consisting of 10 pooled insects.

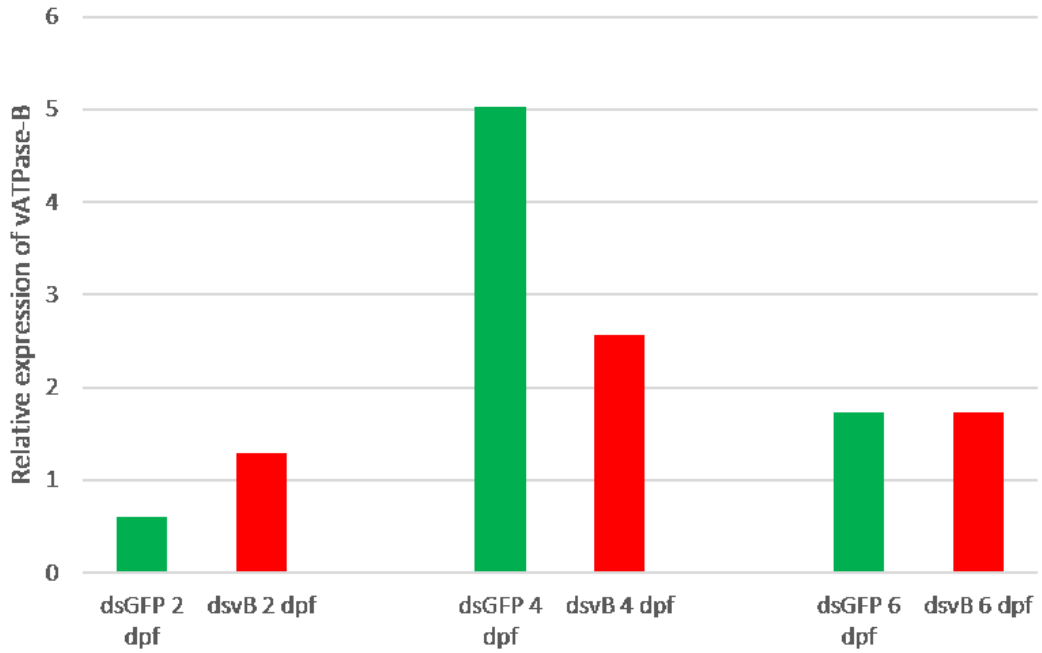

Figure A.2. Silencing of vATPase-B in *Frankliniella occidentalis* first instar larvae fed on double-stranded RNA-amended diets covered by a *Datura stramonium* leaf disc.
Young first instar larval thrips (0 to 24 h old) were allowed to feed on artificial diets amended with vATPase-B dsRNA or GFP dsRNA as a negative control for up to six days. Larval thrips that fed on diet only were used as the calibrator group to calculate relative expression of vATPase-B in the dsRNA treatments. All diets were covered by a *Datura stramonium* leaf disc and both diets and leaf discs were replaced daily for a 6 day period. Insects were collected at 2, 4, and 6 days post feeding on the diets for real-time quantitative reverse transcriptase-PCR analysis of gene expression. vATPase-B transcript abundance was normalized to *F. occidentalis* actin transcripts. Each bar represents the mean of n = two experimental replicates each consisting of 10 pooled insects.

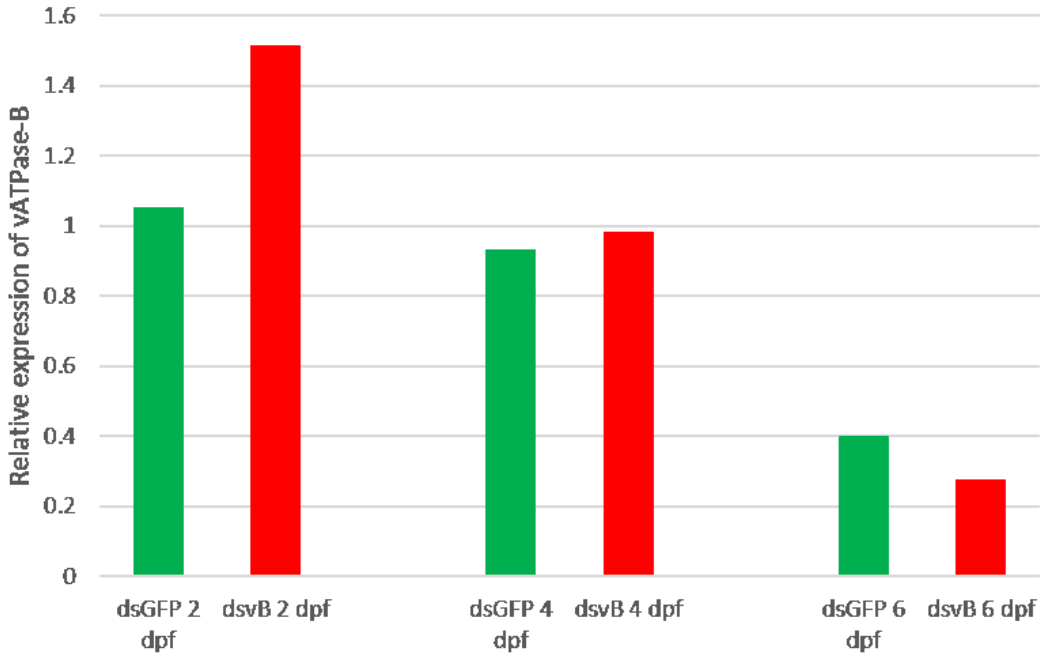

Figure A.3. Silencing of vATPase-B in *Frankliniella occidentalis* first instar larvae fed on liposomes-double-stranded RNA complexes through diets.

Young first instar larval thrips (0 to 24 h old) were allowed to feed on artificial diets amended with liposome-vATPase-B dsRNA-complexes or liposome-GFP dsRNA-complexes as a negative control for up to six days. Larval thrips that fed on diet only plus liposomes were used as the calibrator group to calculate relative expression of vATPase-B in the dsRNA treatments. All diets were replenished every 24 h during a 6 day period. Insects were collected at 2, 4, and 6 days post feeding on the diets for real-time quantitative reverse transcriptase-PCR analysis of gene expression. vATPase-B transcript abundance was normalized to *F. occidentalis* actin transcripts. Each bar represents the mean of n = two experimental replicates each consisting of 10 pooled insects.

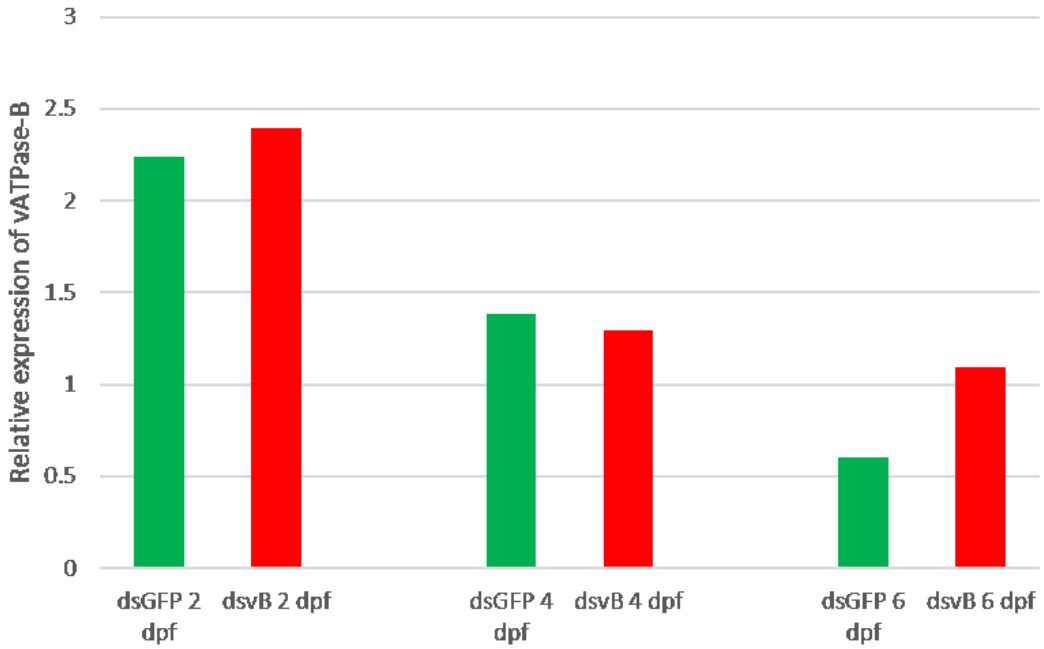

Figure A.4. Silencing of vATPase-B in *Frankliniella occidentalis* first instar larvae fed on liposomes-double-stranded RNA complexes through diets covered in chocolate.

Young first instar larval thrips (0 to 24 h old) were allowed to feed on artificial diets amended with liposome-vATPase-B dsRNA-complexes or liposome-GFP dsRNA-complexes as a negative control for up to six days. Larval thrips that fed on diet only plus liposomes were used as the calibrator group to calculate relative expression of vATPase-B in the dsRNA treatments. All diets having empty or dsRNA-packaged liposomes were covered by a *Datura stramonium* leaf disc and both diets and leaf discs were replaced daily for a 6 day period. Insects were collected at 2, 4, and 6 days post feeding on the diets for real-time quantitative reverse transcriptase-PCR analysis of gene expression. vATPase-B transcript abundance was normalized to *F. occidentalis* actin transcripts. Each bar represents the mean of n = two experimental replicates each consisting of 10 pooled insects.

Appendix B - *Frankliniella occidentalis* transcript and protein sequences corresponding to the candidate interacting proteins selected for further characterization

This appendix provides the nucleotide and amino acid sequences of the *Frankliniella occidentalis* genes and proteins that correspond to the candidate interacting proteins identified in overlay assays that have been selected for further characterization. Bases in red indicate the start and stop codons for the messenger RNA of these 5 genes. Amino acid residues highlighted in aquamarine correspond to the peptides used to generate custom made antibodies in mouse. Further characterization of these candidate interacting proteins in *F. occidentalis* promises to open new research avenues to better understand thrips-tospovirus interactions and develop new control strategies to manage these agriculturally important pathosystems.

Cyclophilin

>CL4854Contig1_S454

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GTGATTGAGGGCATGGATGTCGTGAAGAAGTTGGAATCCTTCGGCTCCCACGATGGC
AAGACCTCCAAGAAGGTGGTTGTTGCTGACTGTGGCCAGCTGTCA**TGA**

Met A L A L R S I R H S L S R P L L G Q F A C R T L P V S L C L P A V R N L C E A Met S
L P R V F F D Met T V D G Q P A G R I V I E L R S D V V P K T A E N F R A L C T G E Q

G F G Y K G S I F H R V I P N F Met C Q G G D F T N H N G T G G K S I Y G R K F A D E
N F Q L K H T G P G I Met S Met A N A G P N T N G S Q F F I T T V K T S W L D N K H V
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Enolase

>CL4706Contig1_S454

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Y K D G Q Y D L D F K N P N S D K S Q W L S G E K L T D L Y Met E F I K E F P Met V S
I E D P F D Q D H W D A W T T I T G K T N I Q I V G D D L T V T N P K R I Q Met A V D
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Cuticular protein

>CL4900Contig1_S454

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Met A T V G F P S C G F T N K N R F S C P L D Q R S F T Y Met T R G K L G I E H Y Y L
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Endocuticle structural glycoprotein

>contig01248

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Met K L E L V A L C L T V G S A L A L Q I P Q Y R A Q Q P Y Q Q Y L Q N Q Q F Q N Y
Q Q R A A A A P I L Q Y S N D V N P D G S F Q Y S Y Q T G D G I S A Q A A G F T R N L
G I K D A E A Q V V Q G S Y S Y T G P D G V V Y T V N Y I A D E N G Y R A Q G A H L
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Mitochondrial ATP synthase α subunit

>CL4310Contig1_S454

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N A A S K Stop