ABSTRACT

AGOSTO-SHAW, KAROLYN LU. Fundamental Research Towards Uncovering New Strategies for Management of *Orthotospoviruses* and Thrips Vectors (Under the direction of Dr. Anna E. Whitfield).

Tomato spotted wilt virus (TSWV) and related *Tospovirids* are some of the biggest disease threats to crops worldwide. Efficiently transmitted by nine species of thrips, TSWV continues to cause an economic impact, with new variants able to overcome resistance. Current management strategies focus on host resistance genes, such as *Sw-5*, and use of insecticides to target thrips populations. Problems arising with this disease include the loss of the *Sw-5* by an amino acid change in TSWV NSm protein, and thrips control is difficult due to population resistance to certain chemicals and their small size can also lead to avoiding sprays. Here, we present new techniques for the control of this disease by focusing on both pathogen and vector control. TSWV initial infection site for virus entry is the thrips midgut and the virus attachment protein is the surface glycoprotein, G_N. The specific viral attachment domain of G_N and the gut receptor are both still unknown in this virus-vector system. A random C7C phage display library was used to select for thrips binding peptides through biopanning, from which only one peptide was consistently enriched in multiple screens. The peptide was aligned to G_N, which resulted in a 4- amino acid identity. To test effect of the peptide on TSWV acquisition by thrips, insects were fed on the peptide, followed by TSWV, which resulted in a 57% decrease compared to TBS control. Guts of thrips fed on the peptide were dissected, immunolabeled and observed through confocal microscopy, where the adherence site was pinpointed to the anterior region of the midgut. The thrips binding peptide may be useful for developing a better delivery strategy for antiviral and insecticidal proteins targeting thrips. With rising number of resistance-breaking (RB) strains of TSWV in the US, new methods need to be implemented in fields to control TSWV infections. In attempts to control infections in plants, an

RNA interference strategy was used in tomato plants. Four different hairpins were constructed containing a conserved region of the open reading frame for all five orthotospovirus genes to confer resistance. We have identified four T3 lines with four different constructs that show high level resistance to RB TSWV strains, other TSWV isolates and another species of orthotospovirus, tomato chlorotic spot virus. These plants can be using singly or combined with other resistance genes to obtain broad-spectrum resistance against orthotospoviruses in plant hosts. With further research, these methods could be applied to field to control both virus and vector.

Fundamental Research Towards Uncovering New Strategies for Management of *Orthotospoviruses* and Thrips Vectors

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Plant Pathology

Raleigh, North Carolina

2022

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DEDICATION

To my parents, Konnetta K. Shaw, and Luis A. Agosto Andujar, for all that you have given and dedicated to ensuring my well-being and education. My sisters, Britney, Valerie and Angelynn, for the love and support.

BIOGRAPHY

Karolyn Lu Agosto Shaw born January 20th, 1997 in Caguas, Puerto Rico. Growing up, Ms. Agosto-Shaw enjoyed family time on a farm in Juncos, PR, exploring, camping and hiking around the island; leading to an immersion for ecological impacts of how a small biosystem interacts. She pursed a B.S. degree in Biology at Universidad Ana G. Mendez Recinto de Cupey, in San Juan, PR. During this degree, she had mentorship of multiple faculty and researching on traces of heavy metals from the metropolitan water supply and air. In summer 2018, she had the pleasure joining Louisiana Universities Marine Consortium, Research Experiences for Undergraduates cohort, researching on fungal communities infecting salt marsh plants. This experienced allowed Agosto-Shaw to attend conferences such as 2019 Gulf of Mexico Oil Spill & Ecosystem Science Conference, and Association for the Sciences of Limnology and Oceanography 2019 Aquatic Sciences Meeting, where she partook in the ASLO Multicultural Program. After completion of undergrad, Ms. Agosto-Shaw started a M.S. degree Plant Pathology at NC State University in 2019, focusing on plant virus-vector interactions.

ACKNOWLEDGMENTS

Thanks to all the Agosto-Shaw family and friends for all the encouragement during my degrees and career experiences. To my sisters, Britney, Valerie and Angelynn, for providing mental and emotional assistance. Eduardo Estrada-Rosado, thankyou for being there every day, as a partner, friend, and adventure buddy. To all my friends and family for the love and support.

Special thanks to all who made possible this research thesis and degree, my mentor, Yuting Chen and all the plant-virus-vector lab members for their guidance and assistance. To my graduate committee George Kennedy and Dorith Rotenberg, and particularly, my graduate research advisor and mentor Anna Whitfield, for all the support, guidance and commitment in both career and student development to achieve my goals.

Little to say, thanks to the undergraduate professors Doris Garcia-Crespo, Gabriel Infante-Mendez and Luis Vasquez-Quiñones, and internship mentors Ryann Rossi and Brian Roberts, for being great advisors, mentors, and professional development aide, who made the time and effort to promote, guide and motivate in everything necessary for my educational development.

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CHAPTER 1: Literature Review

Introduction

Plant pathogens cause great devastation to many economically important crops. Plant viruses are small, microscopic infectious particles containing a genome that encodes for proteins that target plant normal function leading to disease and crop loss. Virus infections are determined by factors such as host availability, vector distribution and environmental conditions (McLeish et al., 2019) (figure 1.1). Many plant viruses rely heavily on vectors for transmission from one plant to another. Vectors may serve as conduits for virus entry into plant cells, and oftentimes, contribute to host ranges of the viruses they transmit (table 1.1; Roselló et al., 1996).

Spotted wilt disease was first observed in tomatoes in 1906 (Sakimura, 1962) though was not described until infections were noted in 1915 in Victoria, Australia (Brittlebank, 1919), later spreading to all states. It was not until 1930 that Samuel et al. described the agent as a virus, leading to the name tomato spotted wilt virus (TSWV). Additionally, this report was the first to describe the vector and transmission by *Frankliniella insularis*, the blossom thrips. Years later, the TSWV genome was characterized and placed into the order *Bunyavirales* (reviewed in Montero-Astua et al., 2016).

This vector-borne virus is efficiently transmitted by over nine species of plant infesting thrips (Rotenberg et al., 2015) though in the Northern Hemisphere, the Western flower thrips (WFT), *Frankliniella occidentalis*, is the most efficient species (Roselló et al., 1996). Thrips transmit TSWV during feeding of epidermal cells, but the virus can only be transmitted if acquired during larval stages. Thrips are most active in warm temperatures, leading to increase in populations,

while in colder seasons, thrips will overwinter decreasing the activity of TSWV (Groves et al., 2002; Morsello & Kennedy, 2008).

Following the first incidence of TSWV, cases of diseased plants with the virus started to appear across the world (Roselló et al., 1996). By the 1970's, the U.S. reported cases in Texas and Louisiana, followed by numerous states in the southern U.S., many of which are still heavily affected (Srinivasan et al., 2017; Karavina, 2017). With the intensification of TSWV pressure worldwide, there is an increasing risk to agronomic crops. Reports of TSWV infection have increased and are occurring in areas or on crops previously unreported. For example, in the Dominican Republic, the first case of TSWV in pepper and tomato was reported in 2013 (Martinez et al., 2013).

TSWV can vary in symptoms by host, environmental factors, and isolates (Rosello et al., 1996). Symptoms caused by TSWV infection of plants results in billion-dollar losses annually. Some isolates lead to vigorous symptoms, while others show mild to no symptoms (Gonzalez-Pacheco et al., 2020). The most described signs and symptoms of TSWV infected plants include yellow or brown ringspots, necrosis, and stunting.

Due to the large number of findings worldwide of both TSWV and thrips, it is a high priority crop concern with a limited number of effective control methods. Resistance of the thrips vector to insecticides and virus genomic plasticity are challenges for developing durable virus/vector management strategies. Some of the common control methods include removal of reservoirs and infected plants to decrease inoculum, controlling the vector populations, and using resistant plant varieties. Although these methods may help reduce inoculum in the field and transmission, research continues in developing new strategies to increase crop survivability and production.

Order *Bunyavirales*

Bunyavirales is a viral order composed of over 475 different species that are sorted into 12 different families (Kuhn et al., 2021). These viruses can infect vertebrates, arthropods, and plants. Plant viruses from this order group with the families *Fimoviridae, Phenuiviridae,* and *Tospoviridae*. The family *Tospoviridae* is composed of a single genus, the *Orthotopoviruses*. Although these viruses are exclusive to plant viruses, they do require arthropod vectors to transmit in nature. This order contains viruses that are single stranded, negative-sense or ambisense RNA genomes encapsulated by a nucleocapsid protein (N) and containing a lipid envelope with glycoproteins surrounding it. They contain three RNA segments, denominated Large (L), Medium (M), and Small (S). These can range in the following sizes: L can be 6.3 to12 kb, M 3.5 to 6 kb and S 1 to 2.2 kb (MacLachlan & Dubovi, 2016). The glycoproteins, G_N and G_C, are coded in the M RNA (MacLachlan & Dubovi, 2016; Oliver & Whitfield, 2016), and some can code for a nonstructural protein (NSm), which function has not been fully determined (MacLachlan & Dubovi, 2016). The L RNA sequence contains RNA dependent RNA polymerase sequence and an endonuclease (Olschewski et al., 2020). Meanwhile, the S RNA codes for the N protein and a second non-structural protein (NSs), which interferes with host innate immunity (MacLachlan & Dubovi, 2016).

Even though these viruses are closely related within, they have evolved separately, and only remain close within genera (Briese et al., 2013). Among strains within a genus, species can undergo sequence reassortment leading to new strains when infection of both occur in one host. An example of this would be a combination of L and M from one strain, and S from the other, but rearrangements within one segment are also possible. In the host, virions are not always fully packed, though, when bunyaviruses form a complete structure, these are spherical shape, usually 80 to 120 nm in diameter, with the glycoproteins embedded in the envelope (Briese et al., 2013;

MacLachlan & Dubovi, 2016). These viruses can be deactivated as a response to heat, changes in pH or use of detergents solvents, or disinfectants (MacLachlan & Dubovi, 2016).

Once a bunyavirus enters a host, uncoating occurs and the RNA polymerase is activated to transcribe the mRNA for each segment (L, M and S). Bunyaviruses have been described as “cap-snatching” viruses (Olschewski et al., 2020). The 5 'terminal of bunyaviruses have shown great similarity to the 5' terminal of the RNA from the host cell (Olschewski et al., 2020). These viruses manage to steal this sequence from the host by using the endonuclease, which they encode in their genome, to cut the segment and promote transcription.

Genus *Orthotospovirus*

The genus *Orthotospovirus*, commonly known as tospoviruses, is composed of plant pathogenic viruses that have been placed into the *Bunyavirales* order and the *Tospoviridae* family. All viruses classified contain three negative sense or ambisense RNA segments, as do most viruses in *Bunyavirales*. In addition, all orthotospoviruses are transmitted by arthropods and are made of enveloped spherical particles embedded with glycoproteins. The main vector for all orthotospoviruses identified are different species of thrips (Rotenberg et al., 2015) which can transmit these viruses in a persistent circulative propagative manner.

Since the discovery of the first tospovirus tomato spotted wilt virus (TSWV), in the 1930's, more than 26 species of orthotospoviruses have been described to infect one or more host plants (Kuhn et al., 2021; Montero-Astúa et al., 2016; Rotenberg et al., 2015). All viruses identified have been distributed in five clades based on the N protein amino acid sequence (Oliver & Whitfield, 2016). The distribution of these viruses has been determined in different parts of the world, managing to

infect all continents. Throughout the years, documentation of tospovirus infection has been determined in over 1,000 different host species (Montero-Astúa et al., 2016)

For tospoviruses, the NSm function was described as a cell-to-cell movement protein in plants (Silva et al., 2001). NSm is also involved in long distance movement (Ruark-Seward et al., 2019), leading to a systemic infection. Tospoviruses can induce two different host responses: RNA interference and innate immune response (Oliver & Whitfield, 2016; Zhu et al., 2019). RNA interference is initiated by a Dicer-like endoribonuclease that can cut double stranded RNA during viral replication or any folding. Once chopped into 21-24 nucleotides, the leading strand is used by the RNA-induced silencing complex, or RISC, which can then find complement sequences to degrade. Meanwhile, innate immunity is induced when pathogen effectors are recognized inside the host cell by specific receptors, activating effector triggered immunity (ETI).

Tomato Spotted Wilt Virus

The *Orthotospovirus* species with most interest worldwide due to its high plant host destruction is *Tomato spotted wilt orthotospovirus,* or simply TSWV. This plant infecting *Bunyavirales* uses thrips as vectors to increase the number of plants they infect. They can move from plant to plant by specific protein interactions with the vector (Rotenberg et al., 2015; Whitfield et al., 2015; Whitfield & Rotenberg, 2015). TSWV can infect over 1000 different plant species, increasing economic loss and research to identify management strategies for this disease. The genus was renamed in 2019 from *Tospovirus,* which is derived from Tomato spotted wilt virus (TSWV), to now *Orthotospovirus.*

TSWV segments have been sized to the following ranges: L is 8.9 kb, M is 4.8 kb and S is 2.9 kb (Oliver & Whitfield, 2016). As mentioned for all closely related bunyaviruses, tospovirus

coinfection can lead to segment reassortment (Ruark-Seward et al., 2019), leading to new strains. TSWV has many different strains, which can cause infection of new hosts, new vector competence and can even affect virulence ability. From one host to another, small changes in amino acid sequences in NSm and RdRp demonstrate TSWV ability to adjust to a new host (Ruark-Seward et al., 2019).

Certain viruses can manipulate their vectors to increase transmission efficiency (Groen et al., 2017). The non-structural protein located on the S RNA segment functions to suppress host jasmonic acid (JA) signaling which can lead to better thrips plant preference (Wu et al., 2019). JA is induced by insect herbivory to reduce infestation and feeding. TSWV's ability to influence the host JA pathway increases thrips feeding, which leads to increased transmission for the virus (Wu et al., 2019). Since the virus requires the vector to be transmitted, host manipulation can attract the vector to the infected plants to obtain virus particles and move them to healthy, non-JA signaling plants.

Thysanoptera (Thrips that feed on plants)

The order *Thysanoptera* includes and is exclusive to all thrips species. Thrips are small insects with fringed wings, slender bodies, and asymmetrical mouth parts, consisting of only the left mandible being developed. Over 7,700 species of thrips have been identified, of which 16 species transmit Tospoviruses (Rotenberg et al., 2015). Thrips feeding habits consist of plants and fungi, though some are known to be predators. Species that feed on plants, focus on consuming pollen or sap from epidermal or mesophilic cells. Thrips are good pollinators for plants by the movement of pollen around the flower when feeding, though their plant consumption can affect the overall

health due to a destructive feeding behavior (van de Wetering et al., 1999) resulting in these insects being a pest problem.

Once a female thrips enters a field and manages to reach a favorable crop species, it can become a big problem, since it begins to produce eggs, from which more thrips can emerge and devour the crops. Eggs can be produced with or without being fertilized (Montero-Astúa et al., 2016; Wan et al., 2020). If not fertilized, a haploid egg will result in male thrips emerging. If it is fertilized, or even without being fertilized, but diploid, a female emerges. Since this can occur, the presence of a single female can have a large impact on a whole field.

The specificity of a vector is determined by the transmission protein that the virus might contain, and for which the vector has a receptor. In recent years, progress was made on characterizing proteins related to the virus-vector interactions, leading to the identification of the viral attachment protein, G_N, that is required for thrips transmission (Rotenberg et al., 2015; Sin et al., 2005; Whitfield et al., 2004). Badillo-Vargas et al. (2019) identified six different thrips interacting proteins with homology to proteins involved in TSWV transmission cycle at the first-instar larvae stage. More research on these proteins could lead to an effective form of control, decreasing the use of pesticides.

TSWV Vector - WFT

With their small size of 1 to 4 mm, thrips are good tospoviruses vectors. WFT, *Frankliniella occidentalis*, are used to model transmission of TSWV, since these are found around the world and can transmit more than one species of tospoviruses. Thrips are a distinguished vector not only because of their small size and insecticide resistance, but because they manage to reach uninfected plants by their jumps and flights. A thrips may be specific to one or more tospoviruses, which

sometimes increases the chances of transmission of different strains (Wijkamp et al., 1995). These vectors can transmit TSWV and other tospoviruses in a persistent, propagative, circulative manner.

Persistent viruses are acquired during longer feeding and can take minutes to hours to acquire. Because these viruses circulate the insect body, there is a latent period before the insects can transmit, in some insects taking hours, but can reach up to weeks in others, and the virus can be retained for days or life. For these viruses, protein interactions require stronger bonds, due to the necessity for the virus to pass tissues to reach salivary glands to be transmitted. Most interactions first occur in either the midgut or hindgut. These viruses can also be subdivided into propagative and non-propagative. Propagative viruses replicate in the vector, which increases the viral inoculum, leading to more particles transmitted, and some persistent viruses can replicate in more than one tissue. Once thrips acquire TSWV by feeding on infected plants, they remain vectors for life.

Ren et al. (2020) demonstrated how flower shapes can attract thrips, but colors and volatile odors at short distances can have a stronger impact on their preferences. In thrips without viral infections, behaviors observed are different from that of infected thrips. For TSWV (X. Wu et al., 2019), it was shown that it manages to manipulate plant responses to infection, resulting in more feeding visits from thrips. Although these responses are manipulated in the plant, TSWV infection leads to behavioral changes in thrips that contribute to virus transmission.

TSWV can influence infected thrips copulation patterns. Wan et al. (2020) published important data on how TSWV can affect sexual behavior, the ratio of females to males that emerge, and the time of offspring development. When neither female nor male was infected, normal walking and circling was observed; versus when both were infected, leading to the female moving faster before

and the male after copulation, with signs of the female inducing detachment and resistance during intercourse. In cases where both male and female where TSWV infected, led to higher numbers of offspring. Development time for males was shorter than females, though infection had different results by thrips species and by time for males, though longer development time was observed in infected females. Their results suggest that TSWV infection can increase male development in lower time frames increasing transmission and less plant destruction (van de Wetering et al., 1999).

Temperature and whether changes can have a great impact on plant growth. This can influence the available host for TSWV infection, leading to infection of weeds or annual plants. Thrips take advantage of available plants located in specific areas they can reach in certain times (Chappell et al., 2013). Many plants serve as TSWV hosts and or reservoirs, leading to spread by thrips onto new hosts that emerge or develop in different times of the year (Chappell et al., 2013). Weeds serves as reservoirs for the virus which they can acquire year-round and spread to perennials and seasonal crops. In some plants, symptoms can be different, and others might not express any symptoms.

Molecular interactions with TSWV

To acquire TSWV, thrips must feed on infected plants during larval stages. The uptake commences with sucking plant sap from mesophyll and epidermal cells, which then flows through the alimentary canal and reaches the midgut (MG). In the MG, receptors are needed to enter epithelium cells to cross to the hemolymph and reach salivary gland (SG) receptors (Rotenberg et al., 2015; Rotenberg & Whitfield, 2018). Once TSWV reaches the SG, TSWV can be transmitted to new plants. Adult thrips spread the virus better, because they have wings, making it easy to reach uninfected plants.

Thoughts regarding ineffective acquisition by adults that does not lead to transmission of TSWV include the in internal proximity of the SG in larvae versus adults (Rotenberg et al., 2015). In adults, physiological changes prevent close contact of SG and MG. Though in larval stages, the approximation of these tissues to the MG allows TSWV to pass to the SG, where replication can occur, leading to transmission to the host. The reorganization of the tissues that occur between the larval stages in adults Results in the inability of the virus to reach the SG and be transmitted.

Molecular Research on G_N

In many studies (Sin et al., 2005; Whitfield et al., 2004), glycoproteins on the TSWV envelope, G_N and G_C, have been shown to be important for WFT transmission. The acquisition of TSWV is dependent on the interaction between a viral protein and a thrips receptor. These activities are believed to take place in the route TSWV uses inside thrips using the G_N protein on the TSWV envelope. Other bunyaviruses (Wu et al., 2017) have been found to use the G_N to adhere to vector receptors.

A soluble form of G_N in excess (Whitfield et al., 2008) was enough to decrease virus abundance in WFT and overall transmission onto new plants. These studies (Montero-Astúa et al., 2014; Sin et al., 2005; Whitfield et al., 2004, 2008), demonstrate that G_N is an important component needed for transmission, though receptors to which G_N may bind have not been characterized. A possible G_N receptor was located in thrips MG by using a soluble form of G_N which localized in the MG of WFT (Whitfield et al., 2004). Since a receptor is needed for TSWV to enter the epithelium cells, the adherence of soluble G_N competes with TSWV G_N, resulting in decrease in virus abundance in thrips and decrease of infection when placed on plants. Whitfield et al. (2014) used the soluble G_N

to transformed plants to express soluble G_N, which thrips acquired and overall transmission of TSWV decreased (Montero-Astúa et al., 2014).

Virus-vector control strategies

With changes to our climate, we see effects on crop emergence and pathogen movement (McLeish et al., 2019). For TSWV and thrips, this usually means warmer winters with thrips activity increasing earlier and virus affecting as soon as plants are introduced in fields (Chappell et al., 2013). These small insects are difficult to control due to their ability to rapidly develop resistance to insecticides. This makes control of thrips complex, but being excellent tospoviruses vectors also, means that controlling the virus is difficult. With viral evolution and increase in host diversity new strategies are focusing on engineering techniques for resistance (figure 1.7, Zhao et al, 2020), including the use of RNAi.

RNA interference (RNAi) functions by using dsRNA from replicating viruses, secondary structures within their genome or dsRNA genome. dsRNA is cleaved by DICER proteins in the plant and incorporates one strand into the RNA-induced silencing complex (RISC) while the other strand is degraded by Argonaute proteins. RNAi confers resistance by transporting small interfering RNA (siRNA) into neighboring cells where they can be replicated by host RdRp and move to other cells to target complementary strands. Current research is focused on the use of RNAi as means to control viral diseases in plants by integrating the virus sequence into the plant genome to form secondary RNA structures and directly activate RNAi.

Due to viruses being difficult to manage, other research alternatives focus on targeting the vector. Among the options, various chemicals such as imidacloprid, sulfoxaflor, and flonicamid, and some oils for instance: rosemary, peppermint, garlic and canola, to be used as an insecticide have been

investigated, but few have had positive results. Renkema et al. (2020) identified various diamide chemicals with potential use for early control of thrips population on strawberries in Florida, but also requires routine sprays during the season with various diamide products.

Fu et al. (2020) found that flower injection in banana plants lowered the thrips population. Imadacloriorid and Spirotetramat injections were used (Fu et al., 2020) to lower the banana thrips second instar larvae without harming banana crop production. Even though these chemicals are directly injected into the flower, leading to the crop, the bananas, and flowers themselves can lose the chemicals by degradation, and after 1 week, concentrations and thrips mortality rate are extremely lowered. Since thrips are highly attracted to flower shapes (Ren et al., 2020), injection of insecticide to plant tissue could lead to potential increase in other thrips species mortality.

An up-and-coming idea for control of pathogens, is the use of peptides. Rudolph et al. (2003) used a yeast two-hybrid assay to engineer a 29-aa peptide derived from TSWV nucleocapsid protein (N) domain that interacts with the host, used to interfere with virus accumulation in plants. The modified plants to carry such peptides, resulted in healthy and symptomless, while control plants symptoms lead to host death (Rudolph et al., 2003). This approach could lead to an increase in host survivability by the use of similar viral sequences with means to block virus infection.

Arthropod control research

Many plant viruses have been tackled by resistance genes, crop rotation, virus free material, or vector control. Though these strategies are effective, with time or changes in certain strains, host defense can be lost, and new methods are needed to decrease infection. Bonning et al. (2014) used the Pea enation mosaic virus (PEMV) CP as means for vector control by fusing it to spider toxin from *Hadronyche versuta*, resulting in aphid paralysis after entering the aphid hemolymph

(Bonning et al., 2014). This is only one example of a toxin that has been used to attack arthropod vectors, but other toxins, such as *Bacillus thuringiensis*, better known as Bt toxin was effective against certain Lepidopterans, Hemipterans, Coleopterans and Dipterans species (Chougule et al., 2013).

New research in pest controls is looking at possible peptides that can work as a biocontrol for plant pathogens (Heck & Brault, 2018). The use of peptides could increase the ability of certain toxins to enter other tissues in the vectors, making tackle possible. When discussing measures for plant virus control, in some cases, it is effective to apply a control strategy against the vector (Groen et al., 2017) to target transmission routes, lowering the overall virus that enters new plants. These strategies could be the future of vectored pathogen control.

Conclusion

TSWV causes great devastation to our food source in the US and globally. We currently rely on resistant varieties to increase crop production, but TSWV resistant variants continue to emerge (de Oliveira et al., 2018; Ciuffo et al., 2005; Batuman et al. 2017; López et al., 2011). Because thrips have a short life cycle, high fecundity, and great adaptability (Roselló et al., 1996), management of thrips populations can be difficult because controlling populations with insecticides leads to small effects in fields. Current research still focuses on understanding the complete transmission cycle of TSWV by thrips. Since there are so many unknowns in the G_N sequence needed for transmission and the receptor within thrips, I am taking a different approach to identify thrips interacting sequences providing insights into controlling thrips populations. In chapter 2, a focus on using a phage display library, was investigated to identify peptides binding to thrips. With these

peptides, attempts were made to block viral entry in thrips guts; meanwhile, localization of the peptide was also identified.

Another focus in research is breeding new varieties of resistant plants. The problem with resistance breeding is that it is very time consuming, and resistance can be lost in little time (Thompson and Zilj, 1996). In chapter 3, a transgenic approach is used to compare resistance in two different strains of TSWV, one from North Carolina, and a second from California showing resistance breaking to the Sw-5 resistance gene. We hope to promote more efficient plant immunity in less time by using a hairpin strategy insert in tomato plants. Both Chapter 2 and 3 focus on testing virus-vector control methods that have previously been applied to other systems; our strategy plans to decrease virus movement and infection and increase crop production.

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Tables and Figures

Table 1.1. TSWV disease incidence on different crops through several countries and the year published (from Roselló et al., 1996, table 2).

Country or continent	Crop	Incidence	Reference
Canada	Ornamental plants	Epidemic in character	Berling et al. (1992)
USA (Hawaii)	Lettuce	Losses of 50–90%	Cho et al. (1986, 1989)
	Papaya	Important losses	Cho et al. (1986, 1989)
	Pineapple	Important losses	Cho et al. (1986, 1989)
Brazil	Pepper	Losses of 49–69%	Boiteux et al. (1993a)
	Papaya	Important losses	Berling et al. (1992)
	Pineapple	Important losses	Berling et al. (1992)
Europe	Broads beans	Can totally destroy the crop	Berling et al. (1992)
	Lettuce	Can totally destroy the crop	Berling et al. (1992)
	Pepper	Can totally destroy the crop	Berling et al. (1992)
Spain	Egg plant	Notable losses	Jordá (1992)
	Celery	Notable losses	Jordá (1992)
	French bean	Notable losses	Jordá (1992)
	Artichoke	Notable losses	Jordá (1992)
Senegal and India	Peanut	A threat to the crop	Berling et al. (1992)
Malaysia	Chili pepper	Important losses	Shukor et al. (1989)

Table 1.2. Thrips vectors of Orthotospoviruses (obtained from Rotenberg et al., 2015).

Table 1

Tospovirus species and tentative tospovirus species and the thrips documented to transmit them.

Tospovirus	*Thrips Vector*
Alstroemeria necrotic streak virus, AlNSV[a]	*Frankliniella occidentalis*
Bean necrotic mosaic virus, BeNMV[a]	*Unknown*
Calla lily chlorotic spot virus, CCSV[a]	*Thrips palmi*
Capsicum chlorosis virus, CaCV[a]	*Ceratothripoides claratris* *Thrips palmi* *Frankliniella schultzei*
Chrysanthemum stem necrosis virus, CSNV[a]	*Frankliniella occidentalis* *Frankliniella intonosa* *Frankliniella schultzei*
Groundnut bud necrosis virus, GBNV	*Frankliniella schultzei* *Scirtothrips dorsalis* *Thrips palmi*
Groundnut chlorotic fan-spot virus, GCFSV[a]	*Scirtothrips dorsalis*
Groundnut ringspot virus, GRSV	*Frankliniella occidentalis* *Frankliniella schultzei* *Frankliniella gemina*
Groundnut yellow spot virus, GYSV	*Scirtothrips dorsalis*
Hippeastrum chlorotic ringspot virus, HCRV[a]	*Unknown*
Impatiens necrotic spot virus, INSV	*Frankliniella occidentalis* *Frankliniella fusca* *Frankliniella intonosa*
Iris yellow spot virus, IYSV	*Frankliniella fusca* *Thrips tabaci*
Melon severe mosaic virus, MSMV[a]	*Unknown*
Melon yellow spot virus, MYSV[a]	*Thrips palmi*
Pepper necrotic spot virus, PCSV[a]	*Unknown*
Polygonum ring spot virus, PolRSV	*Dictyothrips betae*
Soybean vein necrosis virus, SVNV[a]	*Neohydatothrips variabilis*
Tomato chlorotic spot virus, TCSV	*Frankliniella occidentalis* *Frankliniella schultzei* *Frankliniella intonosa*
Tomato necrotic ringspot virus, TNRV	*Ceratothripoides claratris* *Thrips palmi*
Tomato necrosis virus, TNeV	Unknown
Tomato spotted wilt virus TSWV	*Frankliniella occidentalis* *Frankliniella fusca* *Frankliniella schultzei* *Frankliniella intonosa* *Frankliniella bispinosa* *Frankliniella cephalica* *Frankliniella gemina* *Thrips setosus* *Thrips tabaci*
Tomato yellow (fruit) ring virus, TYRV[a]	*Thrips tabaci*
Tomato zonate spot virus, TZSV[a]	*Frankliniella occidentalis*
Watermelon bud necrosis virus, WBNV	*Thrips palmi*
Watermelon silver mottle virus, WSMoV	*Thrips palmi*
Zucchini lethal chlorosis virus, ZLCV	*Frankliniella zucchini*

[a] Tentative tospovirus species that have been described but have not been approved by the International Committee on Taxonomy of Viruses (ICTV).

Figure 1.1. Required interactions for disease to occur. Vector-borne diseases require the presence of a vector to transmit the disease to a susceptible host. If environmental conditions are not appropriate for the host, even with the vector and the pathogen, dis disease will not occur.

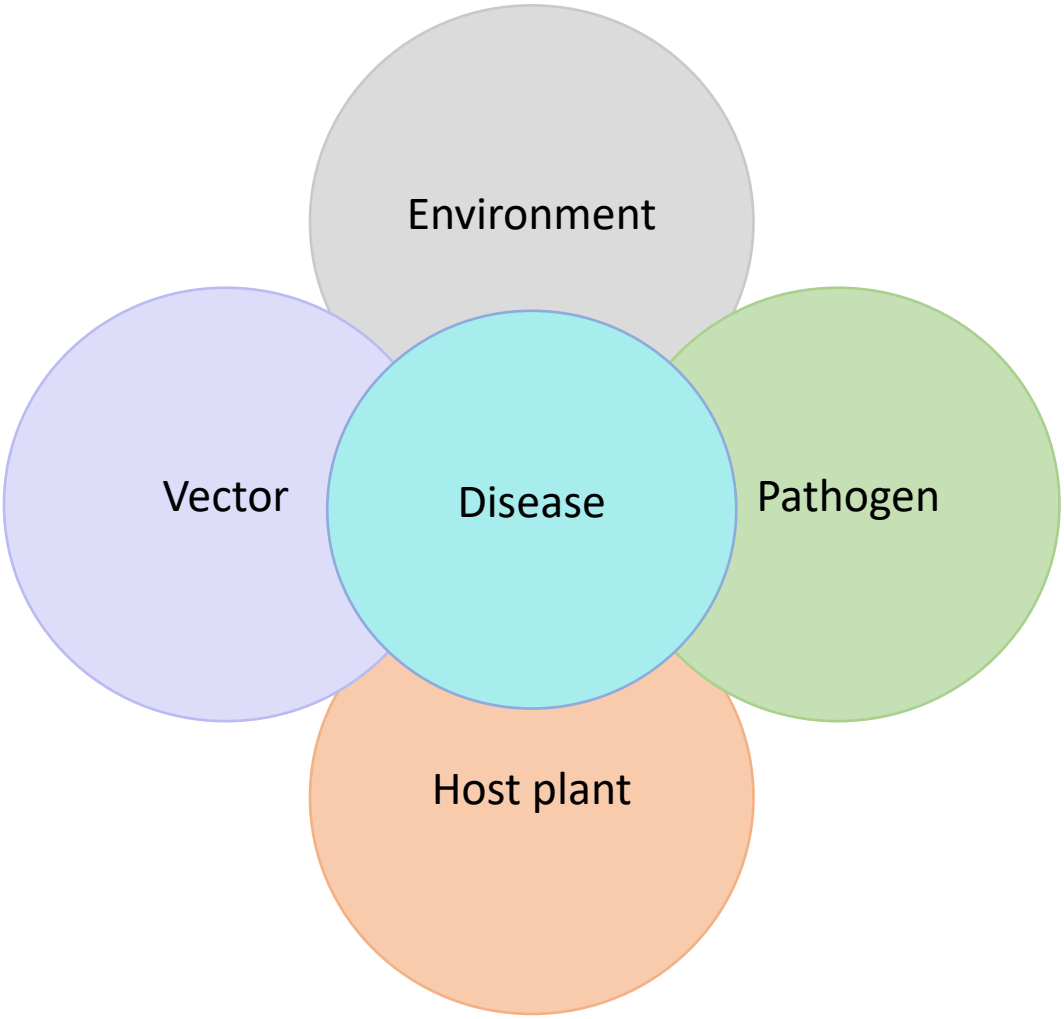
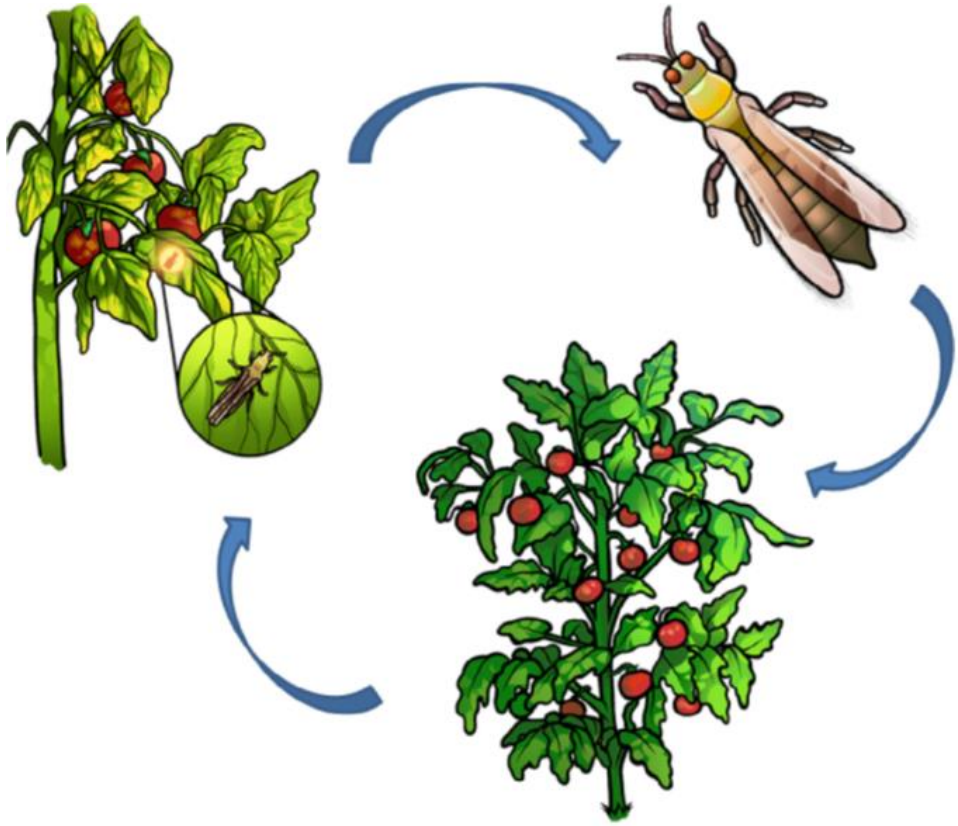


Figure 1.2. TSWV cycle from host to vector (from Whitfield and Rotenberg, 2015). Thrips acquire TSWV from infected tomato host plants, for example. The virus will need a latent period in the vector, where it passes the midgut and enters the salivary glands to



Whitfield, A. E., & Rotenberg, D. (2015). *Current Opinion in Insect Science*, *8*, 79–87

Figure 1.3. TSWV viral structure (from Rotenberg et al., 2015). Composed of an envelope to protect three genomic segments Large sized 8.9 kb, Medium is 4.8 kb and Small is 2.9 kb. Surrounding the envelope are two glycoproteins (GN and GC), which are useful during vector transmission.

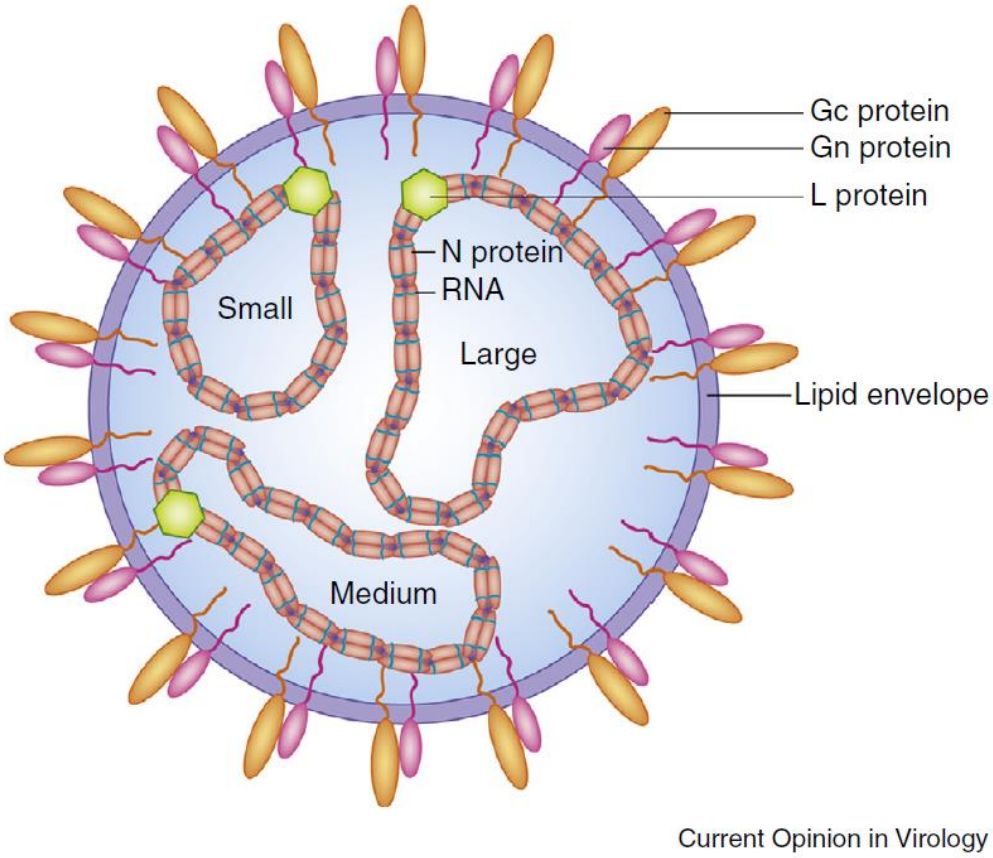


Figure 1.4. Use of reservoirs as means to survive colder seasons (from Chappell et al., 2013). In the Spring, thrips become active with the warmer temperatures and host plants and other plants are introduced. TSWV also becomes active by thrips transmission. With the overlapping period of winter-summer reservoirs, TSWV can remain in the field through the seasons. In Summer, host plants and summer reservoirs serve as inoculum sources for thrips. In the fall, colder temperatures lead to absence of host, but inoculum is present in the summer reservoirs. Winter reservoir plants start sprouting and thrips can transmit TSWV to these plants before the Winter when the thrips will overwinter. TSWV utilizes reservoir plants to remain in the field year-round.

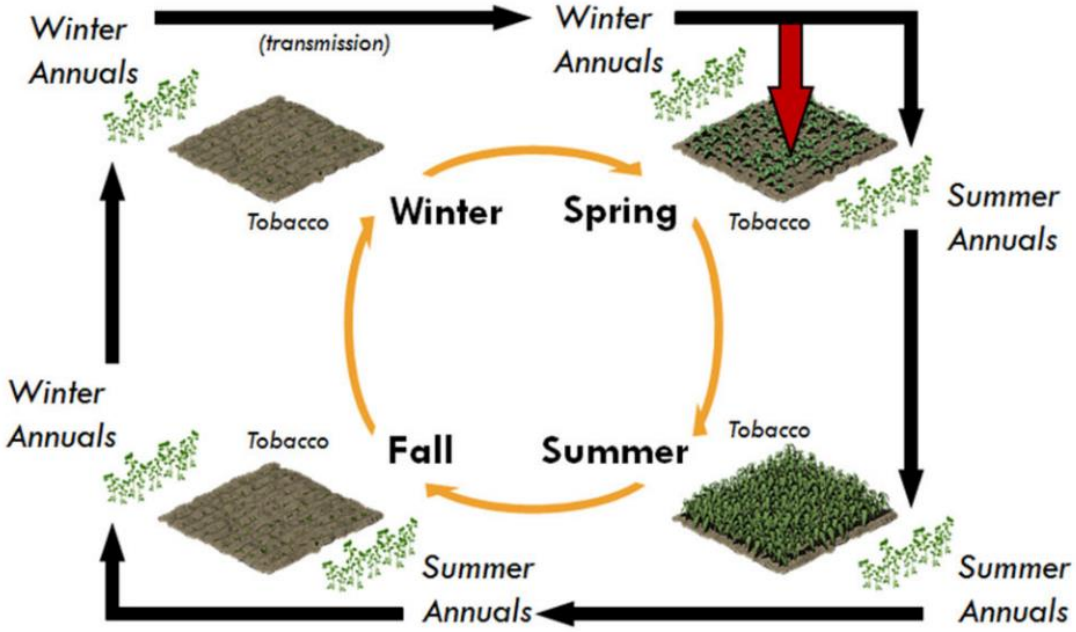


Figure 1.5. Thrips life & transmission cycle (from Rotenberg et al., 2015). Thrips have six stages in the life cycle: egg, 1st and 2nd instar larvae, pre-pupa and pupa and adult. To acquire TSWV and be transmissible, thrips need to feed on virus-infected plants during larval stages. Transmission can occur in larval stages. Adults are the better transmitters because they have wings and tend to move longer distances than larvae.

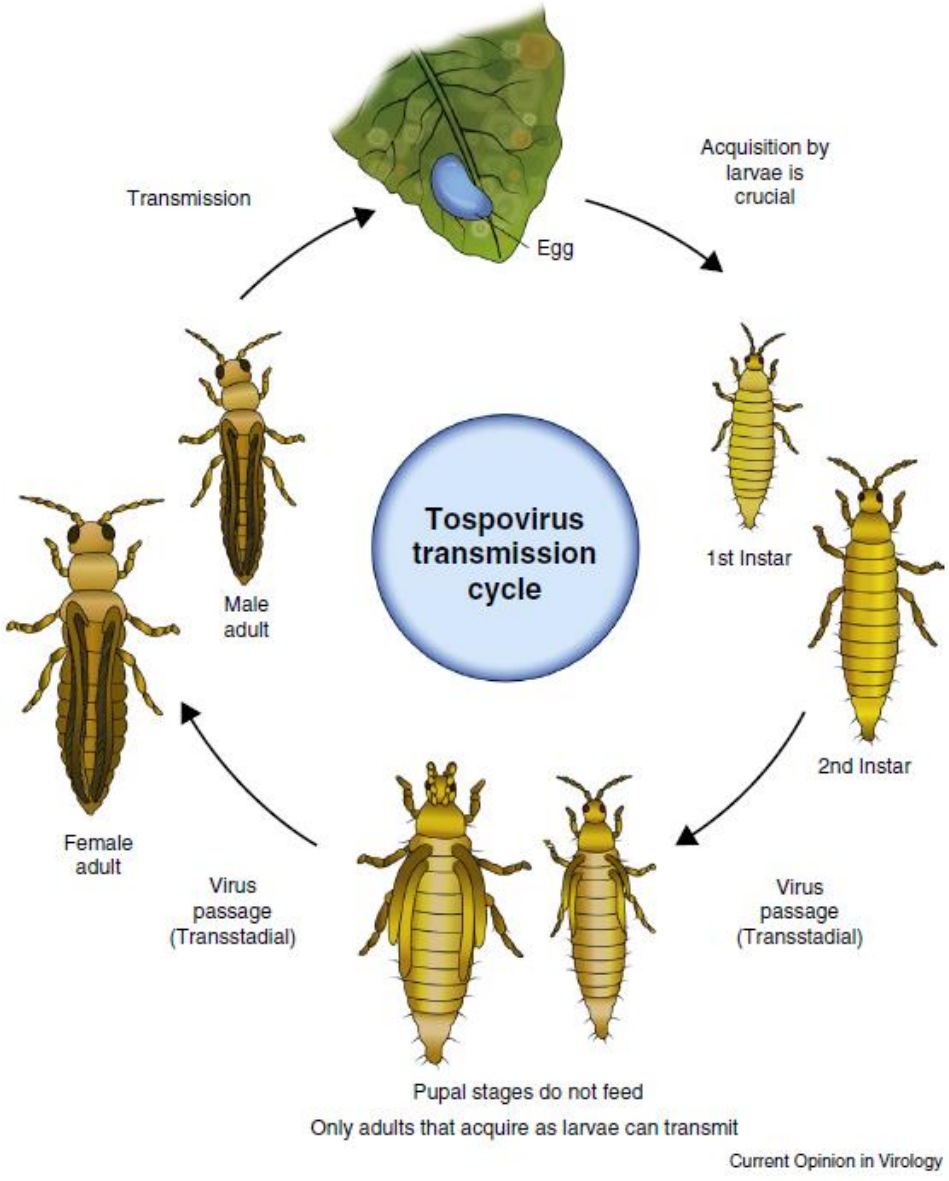
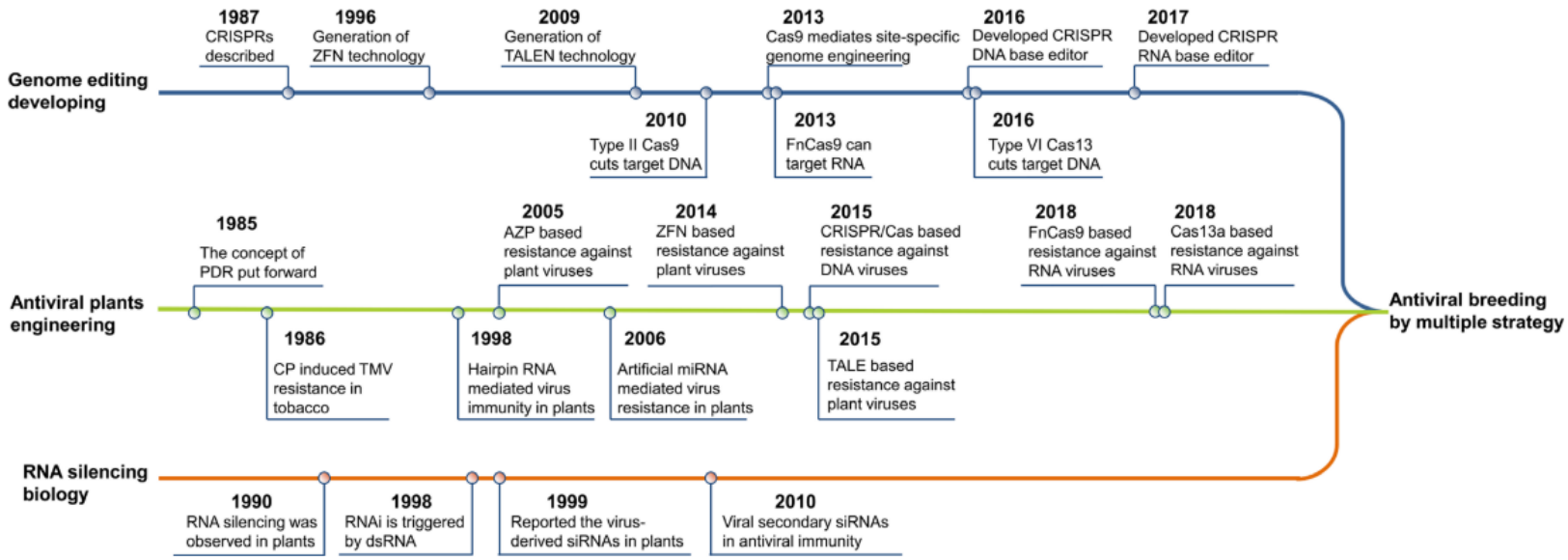


Figure 1.6. Tomato spotted wilt virus (TSWV) symptoms on mechanically inoculated (a, b and c) tomato plants and on Emilia sonchifolia. Plants were grown and kept under greenhouse conditions inoculated at different time points and by different strains of TSWV, a. inoculated with Hawaii strain, b. with North Carolina, and c. and d. with California resistance breaking; the plant tissue shown in c. is a TSWV resistant plant.

Figure 1.7. Engineering viral resistance through the years (obtained from Zhao et al., 2020). Timeline of methods being used in plant resistance using genome editing, antiviral plant engineering and RNA silencing. Researchers continue investigating these techniques in search to promote better produce.



CHAPTER 2: Identification of peptides that bind to Western flower thrips, *Frankliniella occidentalis*.

Abstract

Tomato spotted wilt virus (TSWV) is a plant pathogen with a wide host range, decreasing crop value worldwide. The most efficient and widespread vector of TSWV is the Western flower thrips (WFT), *Frankliniella occidentalis*. Thrips acquire TSWV during feeding on infected plants, with the viral entry site found in the midgut. TSWV has an envelope with two glycoproteins, G_N and G_C, which G_N has been shown to be involved in TSWV entering the midgut cells. Thrips receptors and viral glycoprotein attachment domains remain unknown. To address this deficit in knowledge of TSWV-thrips molecular interactions, we used a phage display library to identify 7-mer peptides that bind to the *F. occidentalis* guts in feeding assay. A single peptide was obtained from two independent biopanning replicates from which 24 total plaques were sequenced, indicating this peptide enrichment was due to strong interactions. Comparison with TSWV G_N sequence revealed four identical amino acid sequence to the 7-mer. To evaluate the effect the 7-mer peptide had on TSWV acquisition, first instar larvae were fed on the peptide and TSWV infected leaves for 5hr acquisition access period (AAP). The peptide resulted in a 57% decrease in the number of insects with detectable virus, but no difference in virus titer. Finally, using antibodies, the peptide localized in the thrips anterior region of the midgut, the same binding site of TSWV. The strong interaction of this peptide with WFT could help to reveal the TSWV attachment site in G_N and be used to develop antiviral and insecticidal thrips-targeting proteins.

Introduction

The order *Bunyavirales* contains the family *Tospoviridae,* a virus family that is composed of over 26 species of enveloped plant viruses with negative and ambisense, single-stranded RNA genomes (Kuhn et al., 2021; Rotenberg et al., 2015). Among the species, tomato spotted wilt virus (TSWV) infect over 1,000 different host species. Sixteen different species of thrips, order *Thysanoptera*, efficiently transmit orthotospoviruses when acquired during larval stages (reviewed in Rotenberg et al., 2015). Among these, Western flower thrips (WFT), *Frankliniella occidentalis,* are commonly found worldwide and they are the most efficient TSWV-transmitters (Wijkamp et al., 1995). Some thrips that feed on plants are classified as pests because they feed on flowers and leaf tissue, affecting crop growth and development, while also transmitting TSWV, decreasing the economic value of many ornamentals, fiber, and food crops.

TSWV is transmitted by thrips in a persistent circulative-propagative manner. During feeding of host plants, thrips acquire TSWV and deposit virus during saliva injection (Roselló et al., 1996). Short and long feeding probes into the cell, lead to TSWV ingestion (Stafford et al 2011). In the midgut lumen, important virus-vector interactions take place, where TSWV may bind to receptors to cross to the midgut epithelium. Here, TSWV replicates and passes through the basal lamina to exit the gut tissues. TSWV particles must reach the salivary glands, where replication occurs once again before being injected during non-ingestion probes into the host plant. When thrips acquire TSWV particles at larval stages, they will remain vectors for the remaining life cycle.

Thrips and TSWV protein-protein interactions are important determinants for infection of the vector. The TSWV virion has two glycoproteins (G_N and G_C) on the lipid envelope, which have been found to be involved in acquisition for virus transmission (Whitfield et al., 2004; 2008). The glycoprotein G_N has been functionally distinguished for virus binding to midgut cells, while G_C

allows the genetic material to enter cells (Whitfield et al., 2004 and 2005; Guardado-Calvo et al., 2016). In past research, a soluble form of G_N blocked viral entry into thrips midgut (Whitfield et al., 2008). Although the role of G_N has been characterized for this process, the specific binding region remains unknown. New approaches to find specific regions of G_N that can bind to thrips midguts continue to possibly identify peptides to block virus transmission or deliver insecticidal proteins to thrips and they may aid in finding virus receptors in thrips.

Phage display libraries have been previously used to identify peptides that can bind to receptors in different insect-pathogen systems (Rudolph et al., 2003; Sera, 2017) where research led to identifying similarities between the pathogen and the peptide. In 2001, Gosh et al. presented the phage display library as an effective approach to find a 12-aa gut and salivary binding peptide in female *Anopheles gambiae* and *Anopheles stephensi* mosquito's gut. When the causal agent of malaria, the parasite *Plasmodium*, was fed to females after the 12-aa peptide, competition between the parasite proteins and the peptide for an unknown receptor decreased the parasites' ability to complete the dissemination route in the mosquito vector and thereby transmission to a host. Similarly, Liu et al. (2010) identified a 12-aa peptide using a phage display library that could bind to the gut epithelium of aphids, the peptide was given the designation of GBP3.1. When compared to Pea enation mosaic virus (PEMV) coat protein (CP), GBP3.1 had a similar sequence from the BG-BH loop on the major CP. Through an inhibition assay, GBP3.1 was tested and found to interact with receptors that could disrupt PEMV from binding and entering the insect's hemocoel. This research led to identifying protein sequences required for vector acquisition of the viral particles.

Liu et al. (2010) and Gosh et al. (2001) are examples of the application of the phage display library to discover proteins involved in pathogen acquisition by arthropod vectors. Here, we used a phage

display library approach to screen peptides that attach to WFT to identify TSWV G_N attachment proteins. Candidate peptides were aligned to TSWV glycoproteins, used in viral inhibition assays and guts labeled with antibodies to identify binding sites. This approach will help uncover new information on TSWV G_N regions required for virus transmission. Identifying binding peptides with effects on TSWV acquisition can lead to insights on new vector control strategies, including developing toxin fused proteins to directly target this difficult to control vector.

Materials and Methods

Thrips Colony

A colony of virus-free *F. occidentalis* (originated from Oahu, HI, Bautista et al., 1995) were maintained on green bean pods (*Phaseolus vulgaris*) in 16oz plastic deli containers with leak-proof lids under a controlled environment, at room temperature, feeding twice a week (on Mondays and Thursdays) and eggs collected each feeding day. To generate first instar larvae for phage feeding, acquisition and immunolabeling experiments, beans were incubated with adult thrips for 3 days to allow the insects to oviposit. Thrips were removed and the beans were incubated at 23°C for 16 h. Larval thrips (0 to 16 h old) were then harvested by gentle brushing of beans onto a sheet and tapped into a microcentrifuge tube for experiments.

Phage Display Library

A Ph.D.™-C7C Phage Display Peptide Library Kit was purchased from New England BioLabs (NEB, Ipswich, MA). This kit was used to identify peptide-peptide interactions within the WFT by using phages with random 7-mer sequences of amino acids displayed on the M13 phage attachment protein, gene III. The kit provides *Escherichia coli* strain ER2738, which is used for

biopanning and is grown on Luria broth (LB) plate with tetracycline as followed in the Ph.D. Phage Display Libraries manual from New England BioLabs (NEB, Ipswich, MA). The kit also provides -96 and -28 gIII sequencing primer for sequencing plaque ssDNA to identify the random peptide sequences inserted into gene III (M13 attachment protein).

Biopanning

Biopanning was performed to identify candidate peptides by selecting those that specifically bind to a target and enriching for the strongest protein-protein interactions. This process is divided into multiple steps: i) thrips feeding of phages expressing a random peptide on its surface, ii) elution of the binding phages, and iii) amplification of phages (Figure 2.1). Phage feeding solution was prepared on microcentrifuge caps using 170µL of Tris-buffered saline (TBS, pH 7.5) with 7% (w/v) sucrose and 50µL from the C7C-Phage display library stock and parafilm. Two hundred 16hr age-synchronized, 1^{st} instar thrips were collected in replicate in microcentrifuge tubes and starved for 1 hr prior to adding the feeding solution for 3 hrs. After feeding, the thrips in each tube were ground in 150µL of cold TBS with 1mg/ml BSA with a RNase-Free Disposable Pellet Pestles (Fisher Scientific, Hampton, NH) at room temperature and mixture combined, followed by three wash steps with 500µL of TBS and 1% (v/v) Tween-20 to remove any un-bound phages. To elute the bound phage, 200µL of 0.2M Glycine-HCl (pH 2.2) with 1mg/ml BSA was used to resuspend grounded thrips and rotated for 15 min. Mixture was then neutralized with 30µL of 1M Tris-HCl (pH 9.1) and stored at 4 ˚C. An overnight culture of ER2738 was diluted 1:100 in 20mL of LB to obtain optical density (OD) < 0.01, and 20uL of phages were added to amplify phages for 4.5hrs with shaking at 250rpm at 37 ˚C in a 250mL Erlenmeyer flask. Culture was transferred into 50mL falcon tubes and centrifuged at 12,000 x g for 10 min at 4 ˚C, transferred again to a clean tube and

centrifuged again to remove cells. Phages were precipitated using ¼ volume of cold 20% PEG/ 2.5M NaCl overnight at 4 ˚C. The next day, phages were centrifuged at 12,000 x g for 15 min at 4 ˚C and resuspended in 1mL of cold TBS. To purify the phages for further rounds of biopanning, phages were precipitated again in ¼ volume of cold 20% PEG/ 2.5M NaCl for 1hr on ice and centrifuged at 14,000 rpm for 10 min at 4 ˚C. Pellet was resuspended in 200µL of TBS and centrifuged to remove any remaining cell debris. To titer phages, plates were prepared containing LB + agar and 50mg/mL of IPTG and 40mg/mL of X-gal. An overnight ER2738 was re-suspended (1:100) in LB and grown at 225rpm at 37˚C to a 0.5 OD (~2.5 hrs). Five µL of phages were diluted in series in 45µL of LB (eluted phages were diluted 10^{-1} to 10^{-5}, while amplified phages were diluted 10^{-6} to 10^{-11}). Two hundred µL of ER2738 (OD 0.5) were incubated with 10µL of each phage dilution for 10-15 min and the full mixture was added to 3mL of top agar (LB + 7% agar) to pour over LB-IPTG/X-gal plates. For further biopanning rounds, 10^{-10}-10^{-11} dilution plates were used to estimate the amount of phage in TBS needed based on the number of plaques. We performed two independent bioreplicates composed of three rounds of biopanning.

ssDNA purification and PCR

ssDNA purification was performed on single blue plaques selected from LB with IPTG/X-gal titer plates with <100 blue colonies. Single colonies were grown and purified as directed in the Ph.D. Phage Display Libraries manual (pages 13-15) for ssDNA purification. Phages grown from plaques in 1mL of LB were obtained by centrifugation at 14,000 rpm for 30 seconds. Supernatant was used to precipitate phages in 200µL of 20% PEG/ 2.5M NaCl. To precipitate ssDNA, phage pellet was suspended in 100µL of iodide buffer and 250µL of ethanol were combined for 10 min, followed by a wash step using 500µL of 70% ethanol and elution using nuclease free water. To verify ssDNA in each plaque sample, polymerase chain reaction (PCR) was used to amplify the

gene III region containing the insert (forward primer: GTCGTTTTAGGTTGGTGCCTTC and reverse primer: CCCTCATAGTTAGCGTAACG).

Sequencing and analysis

To sequence ssDNA, 100ng of purified ssDNA from samples were sent to sequence at North Carolina State University Genomic Sciences Lab with a -96 primer contained in the Ph.D.™-C7C Phage Display Peptide Library Kit (NEB). Data files were aligned using SnapGene software to view the presence or absence of a randomized C7C insert sequence. Aligned sequences were compared to the M13KE sequence obtained from the NEB site (https://www.neb.com/tools-and-resources/interactive-tools/dna-sequences-and-maps-tool) with MUltiple Sequence Comparison by Log- Expectation (MUSCLE). To compare the obtained peptide sequence to bunyavirales, orthotospoviruses and TSWV proteins, BLASTP was used to identify similar sequences within those databases.

TSWV Acquisition-Inhibition Assay

Phage Feeding

To evaluate the effect of the peptide on thrips acquisition of TSWV, thrips were fed on different treatments of phage displaying peptides and viral abundance was compared. A cohort of 300-400, 16hr age-synchronized thrips were collected in microcentrifuge tubes and starved for 1hr. Thrips were then placed on 10^{-11} purified binding phage (10^{-11}) + 7% (w/v) sucrose and TBS solution in a microcentrifuge cap (total of 220µL) with parafilm for 3hrs. For controls, a random phage was isolated from the C7C-Phage display library stock (10^{-11}) and combined with TBS and 7% sucrose, and a positive control consisting of only TBS + 7% sucrose. Treatment solutions prepared and added to a microcentrifuge cap and secured with parafilm to feed the thrips in microcentrifuge

tubes. Phages were previously purified by collecting individual plaque forming units in LB media, and following growth, ssDNA and sequencing was performed to verify purity of each plaque.

TSWV inoculum

TSWV source material was maintained using alternating thrips and mechanical inoculation to obtain fresh viral inoculum for viral acquisition experiments. *Emilia sonchifolia* were maintained in a growth chamber at 24°C with a photoperiod of 14 hrs light and 10 hrs dark. The Hawaiian TSWV strain (MT2 isolate) was used to infect *E. sonchifolia* plants twice per week by mechanical inoculation from plants exposed to TSWV infected thrips. Mechanical inoculations of leaves was carried out by adding carborundum to the leaves and rubbed with cotton swabs with the inoculum buffer. To prepare the buffer, symptomatic *E. sonchifolia* leaves were collected from caged infected plants, maintained under the same conditions, and ground in a cold mortar with 10mL of 100mM Na_2SO_3 (sodium sulfate). Fourteen days post infection, symptomatic leaves were used for 8hr acquisition access period (AAP) by 1st instar larval thrips and later, adult thrips were placed for 4 days on healthy *E. sonchifolia* for inoculation access period (IAP).

TSWV Acquisition

Four hundred 16hr-old age-synchronized 1st instar larval thrips were fed on different treatments (binding phage, random phage and TBS + 7% sucrose) in microcentrifuge tubes. After 3hrs, thrips were transferred to a 16oz plastic deli container covered with thrips-proof screen containing one TSWV infected *Emilia sonchifolia* leaf in a bouquet using a centrifuge tube with water for 5hr AAP. After, leaves were removed, and green beans were added to each treatment cup for the thrips to feed on and incubated for ~2 days (until late 2nd instar).

RNA extraction

To test virus abundance in thrips, pooled samples composed of 30 late 2nd instar thrips were collected in triplicate using microcentrifuge tubes and ground up in 500 µL of TRIzol™ Reagent (Invitrogen™, Waltham, MA) using a pestle (FisherScientific™, Hampton, NH). Processing was done following the TRIzol™ Reagent user guide. After the TRIzol reagent, 100µL of chloroform was added and incubated for 2 min, followed with centrifuging for 15 min at 12,000 × g at 4°C. The aqueous phase was transferred into sterile microcentrifuge tubes and RNA was precipitated with 250µL of isopropanol for 10 min and centrifuged for 10 minutes at 12,000 × g at 4°C. The pellet was then washed in 500µL of 75% ethanol by vortex and centrifuged at 7500 × g for 5 min at 4°C. Finally, the pellet was resuspended in 50µL of RNase free water and left at 60°C for 15 min before determining RNA yield, to prepare cDNA using 200ng/µL of RNA.

To test TSWV acquisition rate (number of infected individuals by the number of individuals tested) and virus abundance in individual thrips, insects were processed using Chelex 100 Molecular Biology Grade Resin (Bio-Rad Laboratories, Inc., Hercules, CA) to isolate RNA (Boonham et al., 2002). One thrips per tube, total of 10 per treatment, was ground in 50µL of RNase free water, followed by addition of a 50% (w/v) Chelex slurry and vortexed, tubes were placed on a heat block set to 94°C for 5 min and centrifuged at 4°C for 5 min at 13,000 x g. From this, 12.5µL was used for cDNA synthesis.

RT-qPCR

cDNA from thrips was prepared after RNA extraction for individual and pooled samples, using the Verso cDNA synthesis kit (Thermo Scientific™, Waltham, MA) and set using the following settings on the thermocycler: cDNA synthesis at 40°C for 30 mins and Inactivation at 90°C for 5 min. 10µL reactions were prepared as follows: cDNA samples diluted 6-fold and 2µL added into

a 96-well plate, with 0.5µL of each primer for TSWV nucleocapsid (Forward primer: 5'-GCT TCC CAC CCT TTG ATT G-3' and Reverse primer: 5'-ATA GCC AAG ACA ACA CTG ATC-3') or Actin (reference gene, Forward primer: 5'-GGT ATC GTC CTG GAC TCT GGT G-3' and Reverse primer: 5'-GGG AAG GGC GTA AAC TTC A-3') and 5µL of SYBR® Green (Bio-Rad Laboratories, Inc, Hercules, CA) and 2µL nuclease free water. qPCR was performed as followed: 3-min initial heating step at 95°C followed by 39 amplification cycles of 10 sec at 95°C and 30 sec at 60°C, with an additional step of 5s at 65 and 5s at 95 to detect the melt curve using a CFX Connect Real-Time PCR System (Bio-Rad Laboratories, Inc, Hercules, CA). Normalized virus abundance was calculated using inverse Pfafll equation: E_ref Ct_ref / E_N Ct_N (Rotenberg et al., 2009).

Identifying peptide binding location in thrips

Immunolabeling

Thrips were fed on the binding phage obtained from biopanning to detect the possible attachment location of TSWV on the midgut. Three-day-old larval thrips were fed on three treatments (binding phage, random phage and TBS + 7% sucrose) during a 3hr period. Each treatment contained 10^{-12} phages and TBS with 7% sucrose and thrips were starved 1hr prior to placement on treatments. Dissection and immunolabeling were performed on 2nd instar larvae to increase the size, dissection, and labeling efficiency by following methods described in Montero-Astúa et al. (2016). Dissections of guts took place immediately after to ensure binding of phages. Guts were then fixed in 4% (w/v) paraformaldehyde and washed in PBS. Incubation with PBS + 1% (v/v) Triton x-100 was done overnight. The following day, guts were washed in PBS and incubated in blocking buffer for 30min. A primary mouse monoclonal antibody was applied at 50µg/mL and incubated to target

the M13 bacteriophage (GP8) major coat protein (Invitrogen™, Carlsbad, CA) for 2.5hrs, followed by a secondary rabbit anti-mouse conjugated with Alexa Fluor 488 (Invitrogen™, Carlsbad, CA) antibody at 10µg/mL for 2 hrs. Lastly, phalloidin conjugated with Alexa Fluor 594 (Invitrogen™, Carlsbad, CA) was applied to stain actin at 4 units/mL for 2hrs. Guts were placed on a slide with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen™, Carlsbad, CA) with a coverslip, cured for 24hrs at room temperature.

Confocal Fluorescence Microscopy

Fixed and immunolabelled thrips guts were observed using a Zeiss LSM 880 with Airyscan confocal microscope (The Cellular and Molecular Imaging Facility at North Carolina State University, Raleigh, NC) to determine attachment of the binding peptide. Guts were examined using LD C-Apochromat 40x/1.1 W Korr M27 objective with water, laser intensity set to 561nm: 3.5% for Phalloidin, 488nm: 2.4% for M13 phage coat protein and 405nm: 2.0% for DAPI and pinhole set to 40 µm for all three channels. Gain for the green channel was set to 820 µs, while blue and red channels were adjusted accordingly.

Fluorescence analysis

Confocal images were exported using Zeiss Zen Black version 2.3 SP1 and further analysis of fluorescence was done with ImageJ version 1.52a. Mean gray fluorescence, integrated density and area were determined using the free-hand tool to select the thrips midgut region 1 in images showing only the green channel for all treatments and additional background readings. Mean fluorescence intensity signal was calculated as follows: integrated density – (area measured x mean background fluorescence).

Statistical Analysis

A mixed model approach was used to compare acquisition as a continuous binomial variable (0 – no infection and 1 – infection) with constructed model effect as the different treatments (binding phage, random phage, TBS) and Tukey's honest significant test (α= 0.050) to evaluate difference between treatments and bio-replicates in JMP. One-way analysis of variance followed by a Tukey's honest significant test was performed to compare means of virus acquisition in R for four treatments (binding phage, random phage, TBS and non-treated thrips) for two assay replicates using the number of individuals with < 36.0 Ct values by the number of individuals tested. Kruskal-Wallis non-parametric was applied to normalized virus abundance in R for two assay replicates for individuals with Ct values. Mean fluorescence intensity signal was compared using Kruskal-Wallis and Pairwise Wilcoxon to determine difference in the signal intensity of the between treatments in thrips guts fed on the binding phage, random phage, and TBS.

Results

Biopanning

A C7C-phage display library was used to screen candidate peptides with the means to enrich for strong protein interactions. All plates contained blue colonies (plaque numbers shown in table 2.1a and 2.1b), which indicated positive phage infection on the ER2738 strain while bacterial colonies not containing any phage would produce white plaques. In the case of the first round of the first replicate, colorless colonies were observed, meaning that wild type M13 phages were present on the plate, though not observed later after amplification.

In the first replicate of biopanning, the number of plaques counted on eluted titer plates decreased from round one to round two (Table 2.1a), however the number of plaques increased after

amplification step for round two. For the second assay, similar observations were made on titer plates (Table 2.1b). For both eluted and amplified titer plates, blue plaques were well distinguished (unless titer was too low or high), which led to easy counting of the blue phage containing colonies from the rest of the white bacterial colonies. In the case of the third round for both replicates, no individual plaques were obtained, but instead, full blue covered plates resulted from having high phage concentration. Since no individual plaques were obtained from diluting the eluted phages up to 10^{-11} using top agar, ER2738 was infected with phages at different dilutions (10^{-1}-10^{-6}) and 100µL were streaked on a plate. This method resulted in easy isolation of blue plaques, from which individual plaques were visible and randomly collected for ssDNA extraction.

Sequencing of plaque forming units

After conducting three rounds of biopanning in the first replicate using thrips, 26 plaque forming units (PFU) were amplified and purified, and PCR was performed to confirm the presence of DNA in each sample. Bands were positive for M13 phage gene III for 25 samples, and only one did not have any bands for ssDNA. From all 25 samples, 16 samples were randomly selected, sequenced, and aligned using SnapGene software to the M13KE gene III sequence, which does not contain the randomized insert, leading to rapid and immediate visualization for the presence and sequence of each ssDNA sample. For the second replicate, 29 plaques were selected for amplification and ssDNA purification, from which only 9 resulted in detection of M13 phages by PCR. For the 16 samples from rep 1 and eight samples from rep 2, all peptide sequences obtained (shown in table 2.2) contained the C-NSTPGES-C; the remaining sample from rep 2 did not have a sequence data. The presence of the same sequence in 24 different samples supports the idea that this peptide had a high binding affinity to thrips gut proteins.

Two different NEB phage display libraries were tested due to technical problems with the first stock solution. From the initial stock, plaques obtained from round 3 of biopanning from eight independent replicates mostly resulted in M13 bacteriophages without any inserts in the Gene III. Table 2.2 (PI.6.03 and PI.8.04 clones) shows the only two inserts obtained from over 60 plaques sequenced, and when the library was plated, resulted in frame or insert-less bacteriophage ssDNA. The two sequences obtained from this were C-TYQEKLH-C and C-HAMHRHL-C. Because of this problem and the low number of plaques with inserts, no further research was done using this library stock.

BLASTP

The binding peptide sequence was aligned using the Basic Local Alignment Search Tool for proteins (BLASTP) to universal sequences and to all orthotospoviruses, TSWV, and all bunyavirales to compare to viral protein sequences. The peptide C-NSTPGES-C resulted in complete alignment of the 7 amino acids with zinc finger protein 37 from *Elysia marginata* (E-value 4471), CHAT domain-containing protein from Spirulina major (E-value 4480), NFkB protein isoform X1 from *Strongylocentrotus purpuratus* (E-value 4484), diaphanous homolog 2 from Aplysia californica (E-value 4492), ligand-gated channel protein from *Serratia proteamaculans* (E-value 4499), and TonB-dependent Receptor Plug domain protein from *Shigella flexneri* (E-value 4501). The peptide had 100% coverage to 5 amino acids of the nucleocapsid of groundnut bud necrosis virus (E-value 74) and while 4 amino acid coverage to the RdRp to soybean vein necrosis virus (E-value 632), bean necrotic mosaic virus (E-value 632), impatiens necrotic spot virus (E-value 632), Norway nairovirus 1 (E-value 632), Bole tick virus 1 (E-value 632), and others (Supplementary table S2.1). Meanwhile, 100% coverage to 4 and 5

amino acids of the glycoprotein precursor for TSWV (E-value 38) and different bunyaviruses such as Crimean-Congo hemorrhagic fever orthonairovirus (E-value 632), Keterah virus (E-value 632), Qalyub orthonairovirus (E-value 632), Kairi virus (E-values 632). When directly compared with TSWV's glycoprotein sequence, global alignment of amino acids resulted in SQTPG sequence at the 78-82aa position of G_N (NCBI: Q9IKB5.1), where the S in this sequence is similar to N (S1N) from the C-7-C peptide, with substitution of S for Q in the second position (Q2S). A local alignment with G_N (NCBI: APG79554.1) was identified at residues 253-256 with identity to PGES, where E makes important electrostatic interactions with other amino acids (personal communication with Moshe Dessau, Bahat et al., 2020). This is an interesting finding, since TSWV G_N is required for thrips transmission.

The other two sequences obtain from biopanning, C-TYQEKLH-C and C-HAMHRHL-C were aligned, from which both had 100% coverage to four or five amino acids of bunyavirales glycoprotein to Tinaroo virus (E-value 150), Wenling crustacean virus 9 (E-value 150), Chinese mitten crab virus 1 (E-value 150), Dante Muikkunen virus 1 (E-value 36), and Haartman Institute snake virus 2 (E-value 36) and RdRp to Dipteran phenui-related virus OKIAV281 (E-value 12), Lepidopteran phenui-related virus OKIAV262 (E-value 13), Hymenopteran phenui-related virus OKIAV255 (E-value 150), Huangshi Humpbacked Fly Virus (E-value 3.1), and Dabieshan Tick Virus (E-value 74), while C-TYQEKLH-C also had 75% coverage to TSWV RdRp (E-value 89). Comparison with G_N and G_C of TSWV (NCBI: Q9IKB5.1) resulted in G_C alignment (residues 967-969) to C-TYQEKLH-C while C-HAMHRHL-C did not have any alignments.

Lastly, a random insert, obtained from the library plaques sequenced to reduce bias, resulted in in alignment to bunyavirales RdRp for species Punta Salinas virus (E-value 73), Aino virus (E-value 74) and Akebane virus (E-value 74) resulted in 100% coverage to five amino acids, and 83%

coverage to the glycoprotein precursor to Crimean-Congo hemorrhagic fever orthonairovirus (E-value 150), Hippeastrum chlorotic spot virus (E-value 150) and Hemipteran phasma-related virus OKIAV245 (E-value 150). The peptide sequence C-STHASLT-C (shown in table 2.2 and figure 2.2e) also had 100% identity to four amino acids in TSWV's: RdRp (E-value 89), NSs (E-value 43) and glycoproteins (E-value 43). When compared to TSWV's glycoprotein sequences (NCBI: Q9IKB5.1), G_C and the C-STHASLT-C peptide had four equal amino acids (STHA) positioned 682-685. The C-STHASLT-C peptide was used as a random peptide control in future testing to evaluate C-NSTPGES-C binding and interactions in thrips.

TSWV Inhibition Assay

Given that G_N is the TSWV attachment protein mediating binding to thrips(Whitfield et al., 2004; Sin et al., 2005), and our binding peptide had two alignments to two regions in G_N, the peptide could potentially bind to the same receptor and disrupt TSWV from attachment. We tested the effects of the binding peptide C-NSTPEGS-C, obtained from biopanning, on TSWV acquisition in thrips by feeding larvae on the peptide followed by a TSWV AAP. Individual thrips were collected for each treatment and processed for RT-qPCR in two biological replicates using positive (TBS), negative (no virus) TSWV controls and a random peptide (phage) control. Ct values showing NA or > 36.0 were considered TSWV negative, and the number of values were divided by the total number of individuals sampled to obtain the acquisition rate for each treatment (Figure 2.3). The binding phage resulted in 40% and 20% acquisition through the two bioreplicates, while the random phage resulted in similar percentages at 40% and 50%. TSWV positive control had 70% acquisition in both reps while negative control had 0%. A total average of 57% decrease of TSWV resulted from the C-NSTPEGS-C binding phage treatment when compared to the positive

control, and a 33% decrease in infection when compared to the random phage. Following an analysis of response in acquisition, where acquisition was treated as a continuous binomial variable (0 - no infection, 1 - infection), a mixed model approach with constructed model effects (rep, treatment, rep*treatment) was used to assess study effects. We found no significant difference between reps (acquisition inhibition assays). However, significant differences were found between treatments. A Tukey HSD (α= 0.050) test indicated that No Phage (TBS) treatment acquisitions were significantly different from Binding Phage treatments. The same test revealed that Random Phage treatments were not significantly different from No Phage (TBS) or Binding Phage Treatments. Results were interpreted by considering the response acquisition to be determined by using the average value between 0 and 1 as a threshold (0.5). This approach utilized the average value of the continuous binomial variable within treatments, where less than 0.5 constituted a net infection and greater than 0.5 constituted the opposite effect (no infection). ANOVA was applied to the mean for both bioreplicates and comparison p-value 0.0040, was significantly different. Tukey's honest significance test revealed a significant difference (p-value 0.0242), between the binding phage and positive control, while no difference was observed between the random phage and positive control (p-value 0.1075).

Average virus abundance (Figure 2.4a and 2.4b) was evaluated in individual thrips using Kruskal-Wallis, leading to a p-value = 0.4449. Pooled samples were collected in triplicate and averaged to determine viral titer change in each treatment (Figure 2.4a and 2.4c). Kruskal-Wallis was used to statistically evaluate treatments for pooled thrips, p-value = 0.334, in which no significance was detected, although a titer decrease in rep 1 binding phage was lower than random phage and positive control. Table 2.4 summarizes acquisition percentages and normalized abundance in

individual and pooled samples for each bioreplicate. Overall, no significance was identified in virus abundance in individual or pooled treatments.

Immunodetection of thrips binding peptide

Localization of the attachment site for the binding peptide was investigated in the anterior region of thrips guts, the interacting site of TSWV. Thrips were fed for 3hrs on three different treatment solutions: TBS, binding phages, and random phages; and guts were labeled with antibodies and observed through confocal microscopy to detect M13 phage coat protein (GP8). A total of 175 guts were imaged, from which 81 were binding phage, 48 random phage and 46 TBS. Figure 2.5 shows difference in treatments for thrips midgut region 1. In 29 guts, a clear fluorescent signal was observed under the green channel in midgut region 1 for thrips fed on the binding phage, while TBS and random phage did not show this signal. Images for binding phage 1 in figure 2.5 shows maximum fluorescent signal observed in guts, while images for binding phage 2 shows minimum signal, used to compare to TBS and random phage control. Images were analyzed using ImageJ to obtain the mean fluorescent intensity signal from midgut region 1 in all treatments and compared in Kruskal-Wallis. Resulting p-value 0.001235 established difference in mean fluorescence within treatments. Pairwise Wilcoxon test was applied to establish differences between treatment medians (Figure 2.6). Differences were significant between TBS and the binding phage (p-value 0.0012) and the binding phage and random phage (p-value 0.0288) medians, but not between the random phage and TBS (p-value 0.5277). Signal from the anterior midgut region 1 indicates possible interaction of the binding peptide with TSWV receptors.

Discussion

Phage display libraries are a revolutionary strategy used to screen and identify protein-protein interactions (Sera, 2017). We used a biopanning method to enrich and identify peptides that could interact with receptors in WFT. We identified a highly binding C7C-peptide (C-NSTPEGS-C) in WFT in two independent replicates of biopanning where thrips were fed TBS solutions containing phages from the library. To eliminate the possibility that the library was over-enriched for this thrips-binding peptide, we plated the phage library and then sequenced 10 plaques. These randomly selected plaques yielded unique sequences for each colony and the thrips-enriched peptide was not found in any of them. Having only one peptide out of all possible interactions could be due to a very high specificity in the gut receptors of thrips. We attempted to identify the possible peptide binding sites in thrips using antibody labeling. Preliminary findings support the hypothesis that the thrips enriched peptide does bind to thrips midguts. Our thrips-enriched peptide was compared through BLASTP to orthotospoviruses, TSWV, and bunyavirales. Table 2.4 shows similarity with TSWV glycoproteins and other bunyavirales. This is an interesting finding since the G_N glycoprotein from TSWV is involved in thrips acquisition (Whitfield et al., 2004 and 2008). This identity is based on a 4 amino acid alignment using short peptide, and having high E values, due to the chance of match in short peptide sequences being much higher. This identity, however, could lead to identifying required regions of G_N for thrips transmission of TSWV. Because of the small size of the peptide, there is also much higher possibility of interactions with multiple targets. To establish binding of this 7-mer, BiFC with this sequence could be used to evaluate the interactions with proteins in thrips that have been identified to interact with G_N (Badillo-Vargas et al., 2019).

Previous work involving phage display libraries has shown feeding the peptides with pathogens leads to decrease of infection in the vector (Ghosh et al., 2001; Liu et al., 2010; Sparks et al., 2011). We examined our C7C-peptide through competition feeding assays with TSWV to test whether the peptide could decrease virus infection in larval thrips. In total, TSWV acquisition was decreased by 57% in thrips that fed on our C7C-peptide (C-NSTPEGS-C), while only 36% decrease from the random peptide (C-STHASLT-C). This decrease in virus acquisition indicates that the binding peptide may be targeting the receptor for TSWV. Reduction in the random phage could potentially indicate the interactions of other phage proteins with thrips receptors, leading to the need of a control for the phage itself without random peptides. However, additional experimentation is required to substantiate this preliminary finding that hints at competitive binding between the peptide and TSWV particles.

To further evaluate the possible binding of C7C- phage on TSWV receptors, we identified the attachment sites of the phages in thrips guts. TSWV has previously been shown to adhere to the anterior region of the midgut (Nagata et al., 1999). Fluorescent signal and statistical difference in fluorescence from the binding peptide does in fact indicate attachment in the same binding region as TSWV; meanwhile, little to no fluorescent signal was observed on the anterior region of the midgut when fed on the random peptide. Although we did not answer if it binds to the same receptor, it does indicate this possibility. In future work, we will try to identify the thrips attachment receptor and compare this to TSWV's. This could help uncover important regions in TSWV's attachment protein required for entry.

The phage library technique takes into consideration 1.28×10^9 different peptides and decreases the candidates by enriching the strongest interactions, leading to high affinity binding. Sparks et al. (2011) found seventeen different peptides bound to some receptor of tobacco budworm,

Heliothis virescens (HV). These peptides were compared to occlusion-derived virus (ODV), leading to identifying two similar sequences, HV1 and HV2, to the envelope protein of five different ODV. Using *Autographa californiaca* multiple nucleopolyhedrovirus (AcMNPV), competition assays were performed with the peptides, leading to an increase in insect survival when HV1 bound to the midgut of the tobacco budworm (Sparks et al., 2011). The receptor that AcMNPV uses seems to be blocked by HV1, leading to a decrease in the ability of virus to infect.

Studies involving the use of peptide to deliver toxins continue to identify new targets for crop pest (Bonning and Chougule, 2014). Chogule et al. (2013) for example, used *Bacillus thuringiensis* Bt-toxin fused to a 12-aa peptide that could bind to the aphid gut, resulting in increase of toxin delivery. Future directions for this project include evaluating thrips transmission to plants after feeding on our C7C-peptide followed by TSWV AAP. We also plan to test binding efficiency on larval and adult stages for WFT and other vector species of thrips. It is possible to target more than one vector if it can transmit a common virus. TSWV is transmitted by nine different species of thrips, meaning that decrease in virus acquisition in WFT should be similar to other vectors of TSWV.

With an increase in pesticide resistance, there is an urgent need for alternative control methods. We also plan to evaluate thrips mortality in response to different substances and develop fusion peptides to deliver candidate toxins into thrips. Bonning et al. (2014) developed a toxin fusion protein that targets aphids. They used a region of the CP of the Pea enation mosaic virus (PEMV) in which had previously been shown to be required for aphid transmission of PEMV (Liu et al., 2009; Doumayrou et al., 2016). When fused to GFP, the CP protein region was observed to enter the hemolymph. Later, fusion of the CP to *Hadronyche versuta* derived spider toxin increased

mortality in aphids. Further research on our C7C-peptide could lead to insights on the possibility of targeting thrips species.

Lastly, we could adapt the approach from Mishra et al. (2021), where they applied Next Generation Sequencing (NGS) to all eluted phages from each round of biopanning from different arthropods. This approach can result in identifying similarities in gut binding peptides between different TSWV vectors. Further comparison between thrips and beneficial insects binding peptides can decrease the possibility of targeting valuable arthropods, such as pollinators. Using NGS analysis and comparing peptides to identify common features from other thrips species could help with developing a multi-targeting thrips biopesticide.

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Tables and Figures

Figure 2.1. Biopanning flow chart demonstrating the process by which thrips are fed on a solution with 1.28 x 109 possible peptide sequences that can target and interact with proteins in the insect. After three rounds of biopanning, peptides are enriched for the strongest interactions by only eluting and amplifying those in solution after removing the non-targeting peptides. Once biopanning is completed, purification and sequencing of plaques is done to determine the binding sequences.

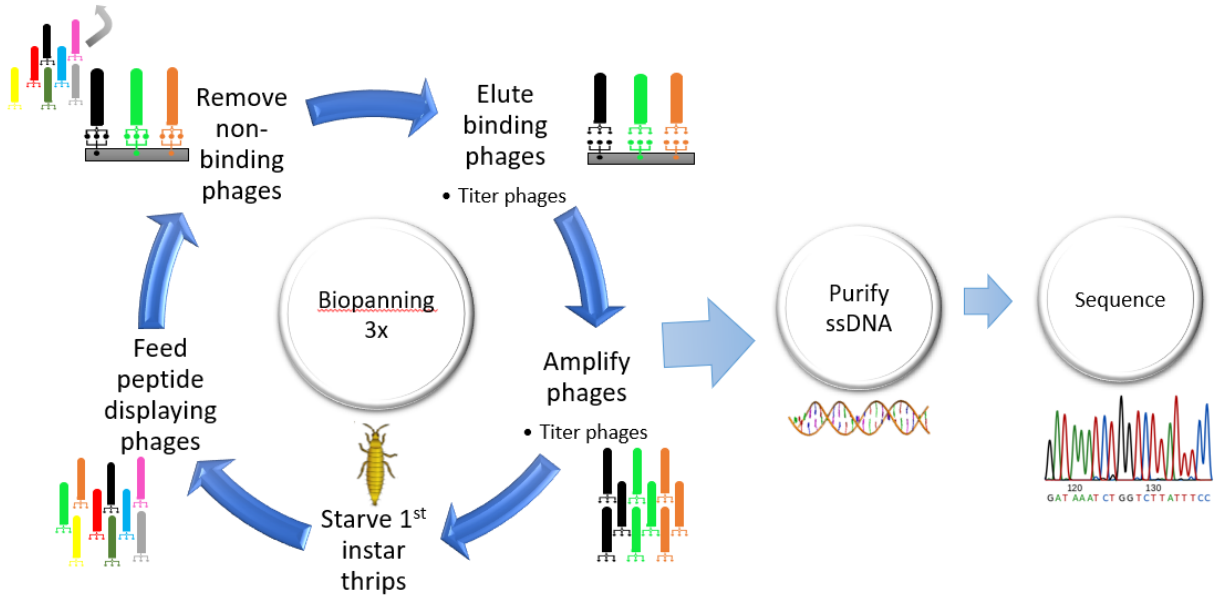


Table 2.1 a and b. Biopanning plaque count for each titer dilution plate. Table a) refers to the number of plaques in trial 1 and table b) contains plaques from trial 2. At both trials, the third round had a large number of plaques from which individual plaques were collected and ssDNA isolated for sequencing of peptides.

Table 2.1a	Number of plaques (pfu)	pfu/ml	Number of plaques (pfu)	pfu/ml	Number of plaques (pfu)	pfu/ml
Dilution factor	**Round 1**		**Round 2**		**Round 3**	
-1	TMTC[1]	TMTC	TMTC	TMTC	TMTC	TMTC
-2	306	2.0E+02	84	56	TMTC	TMTC
-3	312	2.1E+01	19	1.2667	TMTC	TMTC
-4	162	1.1E+00	0	0	TMTC	TMTC
-5	183	1.2E-01	0	0	TMTC	TMTC
-6	84	5.6E-03	TMTC	TMTC	TMTC	TMTC
-7	57	3.8E-04	TMTC	TMTC	TMTC	TMTC
-8	80	5.3E-05	351	0.0002	TMTC	TMTC
-9	43	2.9E-06	29	1.933E-06	TMTC	TMTC
-10	101	6.7E-07	1	6.667E-09	TMTC	TMTC
-11	108	7.2E-08	1	6.667E-10	TMTC	TMTC

Table 2.1b	Number of plaques (pfu)	pfu/ml	Number of plaques (pfu)	pfu/ml	Number of plaques (pfu)	pfu/ml
Dilution factor	**Round 1**		**Round 2**		**Round 3**	
-1	121	80.6667	320	TMTC	TMTC	TMTC
-2	32	2.1E+01	101	67.3333	TMTC	TMTC
-3	13	8.7E-01	3	0.2	TMTC	TMTC
-4	0	0.0E+00	0	0	TMTC	TMTC
-5	1	6.7E-04	0	0	TMTC	TMTC
-6	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC
-7	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC
-8	TMTC	TMTC	344	0.0002	TMTC	TMTC
-9	768	5.1E-05	148	9.9E-06	TMTC	TMTC
-10	403	2.7E-06	36	2.4E-07	TMTC	TMTC
-11	227	1.5E-07	17	1.1E-08	TMTC	TMTC

[1] TMTC: Too many to count.

Figure 2.2. ssDNA sequence from biopanning plaques. Each sequence is as follows: a) peptide insert site (blue labeled bases) on M13KE vector without an insert, b) PI.6.03, c) PI.8.04 and d) PII.2.11 e) PDLII.8. Figures b), c), d) and e) highlighted sequences show the peptide insertion site with the random 7-mers located between the two Cysteines. Clones and peptide sequence are shown in table 2.3.

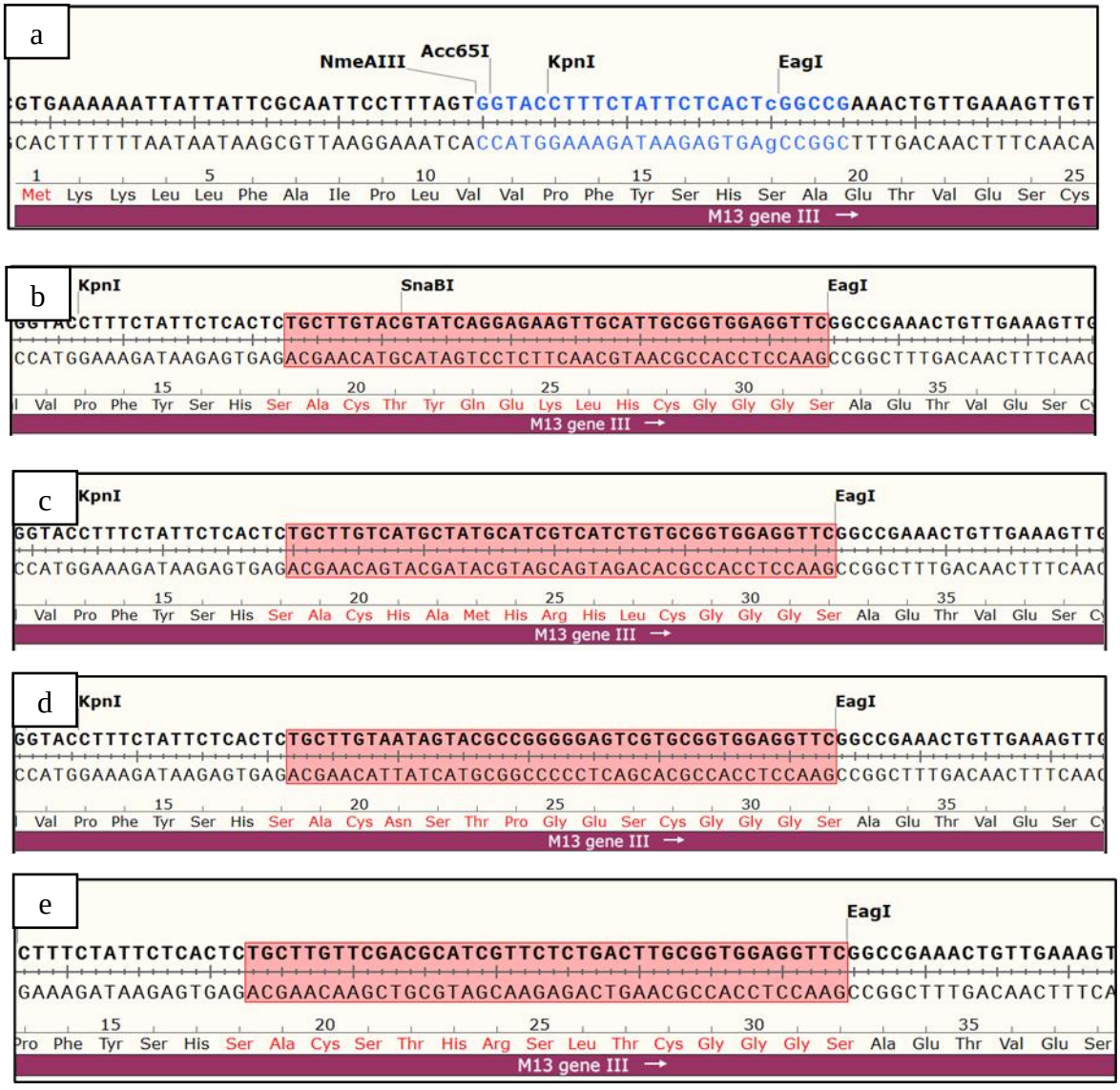


Table 2.2. Peptide sequences from randomly isolated plaques.

Experiment	Name	Phage clone	Sequence	Frequency	Total number of pfu[2] sequenced
2	Binding phage	PII.2.11	C-NSTPGES-C	24	24
2	Random phage	PDLII.8	C-STHASLT-C	1	1
1	NA	PI.6.03	C-HAMHRHL-C	1	2
1	NA	PI.8.04	C-TYQEKLH-C	1	13

NOTE: For clone PII.2.11 sequence was also identified in 23 more isolated plaques using a second library stock (experiment 2). The PDLII.8 clone was sequenced from a plate obtained from plating the library. Clones PI.6.03 and PI.8.04 were only observed once in two different independent replicates from a library stock (experiment 1) that resulted in mostly empty M13 phages (containing no insert in gene III, which displays the C7C-peptide).

[2] Pfu: plaque forming units

Table 2.3. Summary for C7C-peptide inhibition assay of TSWV. Table displays TSWV acquisition percentages for each treatment obtained from Cq values, and average virus abundance in individual thrips and pooled thrips samples. A decrease of 57% TSWV acquisition was observed for thrips fed on binding phages before TSWV acquisition, while only 36% decrease from the random phage.

Bioreplicate	Treatment	Percent acquisition[1]	Combined acquisition[2]	Mean TSWV abundance in individual thrips[3]	Mean TSWV abundance in pooled thrips[4]
1	Untreated[5]	0%	0%	0.00	0.00
2		0%			
1	Binding phage	40%	30%	3.296 ± 4.451	0.641 ± 0.600
2		20%			
1	Random phage	40%	45%	1.599 ± 2.227	0.437 ± 0.0002
2		50%			
1	TBS	70%	70%	0.585 ± 0.0091	1.951 ± 1.070
2		70%			

[1]Percent acquisition was obtained from the number of Ct scores < 36.0 or non-NA divided by the number of samples.
[2]Combined acquisition is the mean acquisition from both bioreplicates.
[3,4]Individual or pooled thrips N-gene Ct values were normalized to Actin-gene values using Inverse Pfaffl to calculate the average (Avg) and standard deviation (SD) for virus abundance for both bioreplicates.
[5]Untreated thrips were used as a negative control. Insects were not exposed to phages or TSWV infected tissue

Figure 2. 3. Tomato spotted wilt virus acquisition rate in thrips. Thrips were fed on binding phage, random phage and TBS followed by a 5hr AAP of TSWV to determine virus acquisition qPCR in two bioreplicates. Ct values of NA and > 36.0 were determined as no acquisition and mean acquisition was determined between both bioreplicates. Means were compared in ANOVA (p-value = 0.003974) and Tukey's honest significant difference test, which determined a 95% level difference between the positive control (buffer-TBS) and binding phage (p-value = 0.0241991), while no significance was observed between the random phage (p-value = 0.3552124) or negative control (p-value = 0.0624388) with the binding phage. Overall, the binding phage resulted in a decrease of 57% acquisition of TSWV.

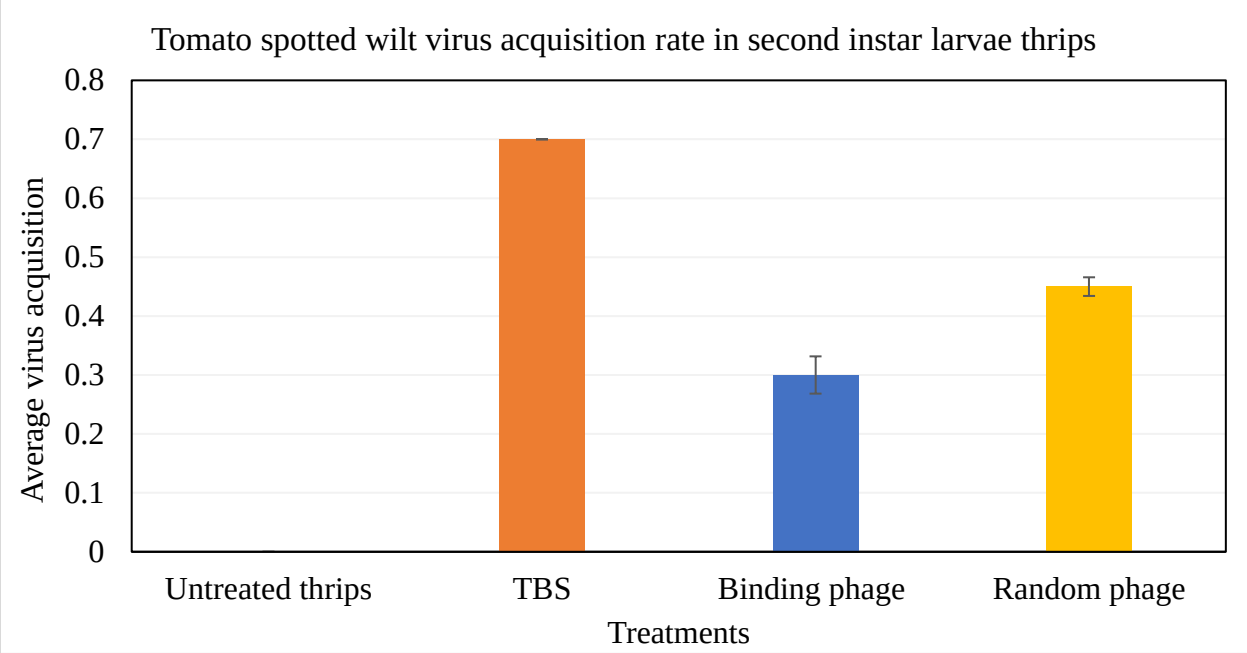


Figure 2.4 a, b and c. Tomato spotted wilt virus normalized abundance in Western flower thrips. Thrips Ct values for TSWV-N gene were normalized to actin gene using the Inverse Pfaffl equation. Averages for each treatment were compared by Kruskal-Wallis test, where no difference was identified for either individual (p-value 0.4449) and pooled (p-value 0.334) samples. Figure 2.4 shows a bar graph with the average for each treatment and standard error for individual and pooled thrips. Figures b and c are stripcharts constructed with the normalized values for each treatment in individual and pooled samples respectively.

Figure 2.4a. Normalized Tomato spotted wilt virus abundance in Western Flower thrips

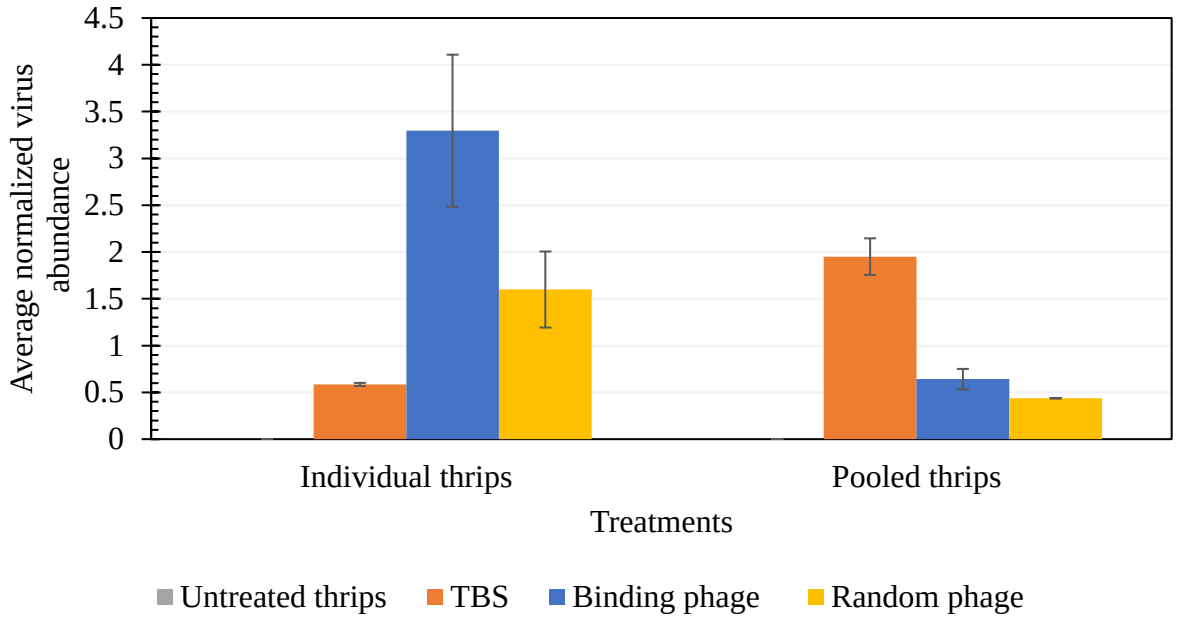


Figure 2.4b. Normalized virus abundance in individual thrips

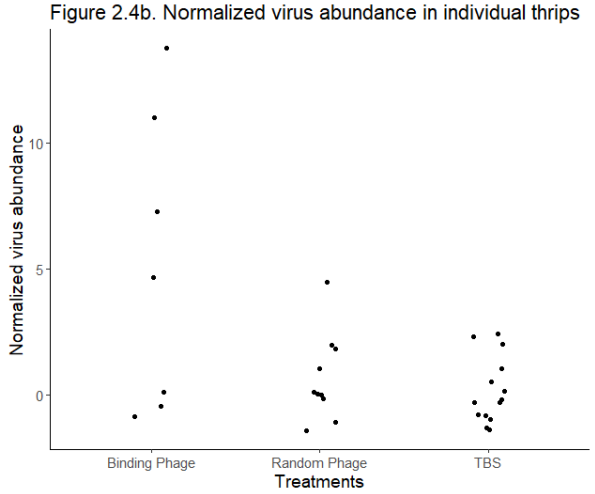


Figure 2.4c. Normalized virus abundance in pooled thrips

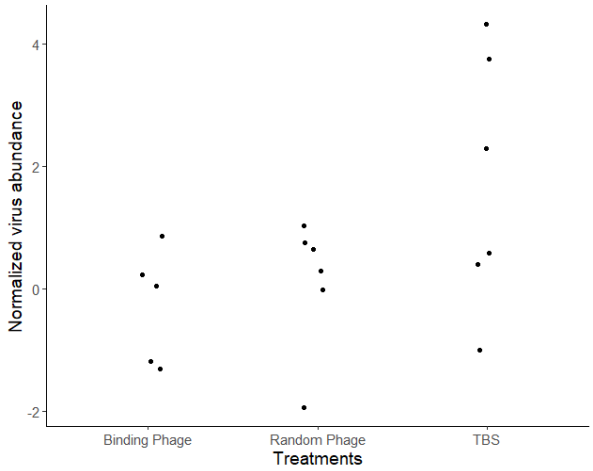


Figure 2.5. Localization of C7C-peptides through immunofluorescence. Larval thrips were fed on treatments (binding phage, random phage and TBS) and guts dissected to incubate with antibodies that target M13 phage coat protein (GP8), actin (Phalloidin) and DAPI. Images show thrips midgut, arrows indicate the anterior region of the midgut for Alexa 488 and scale bars indicate 100µm. Both random phage and TBS did not display any fluorescence from this region, however, when guts were exposed to the binding phage and labeled, signal was observed in the anterior region of midgut 1. This is also the initial site of TSWV infection in thrips vectors.

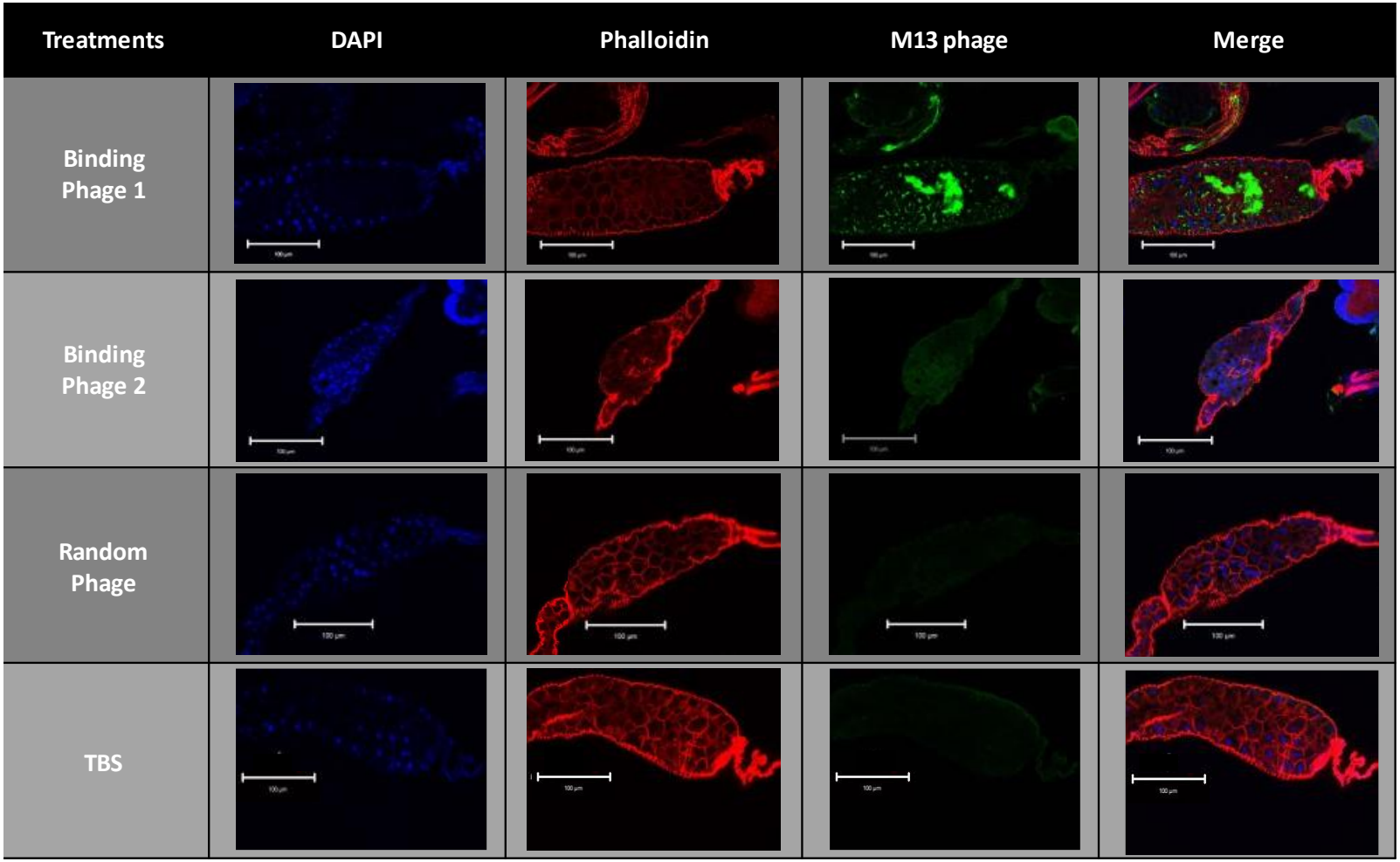


Figure 2.6. Thrips guts immunofluorescence signal intensity. Mean fluorescence intensity signal was calculated for immunolabeled thrips guts images showing green fluorescence for M13 phage coat protein. Mean fluorescence was compared in Kruskal-Wallis (p-value = 0.001235) which indicated mean difference, and Wilcoxon pairwise comparison to identify difference in TBS and binding phage medians (p-value = 0.0012) but no difference between the other treatments.

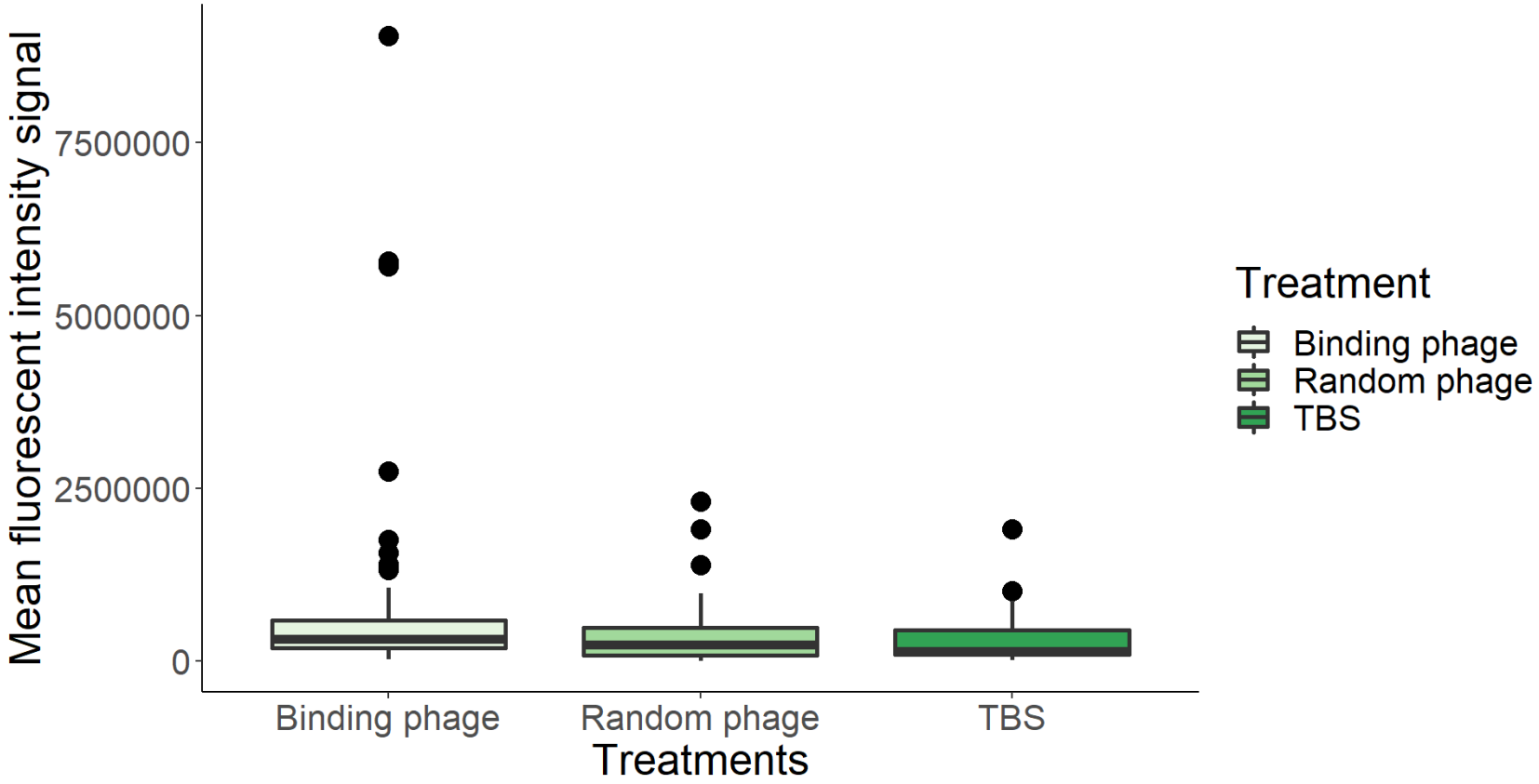
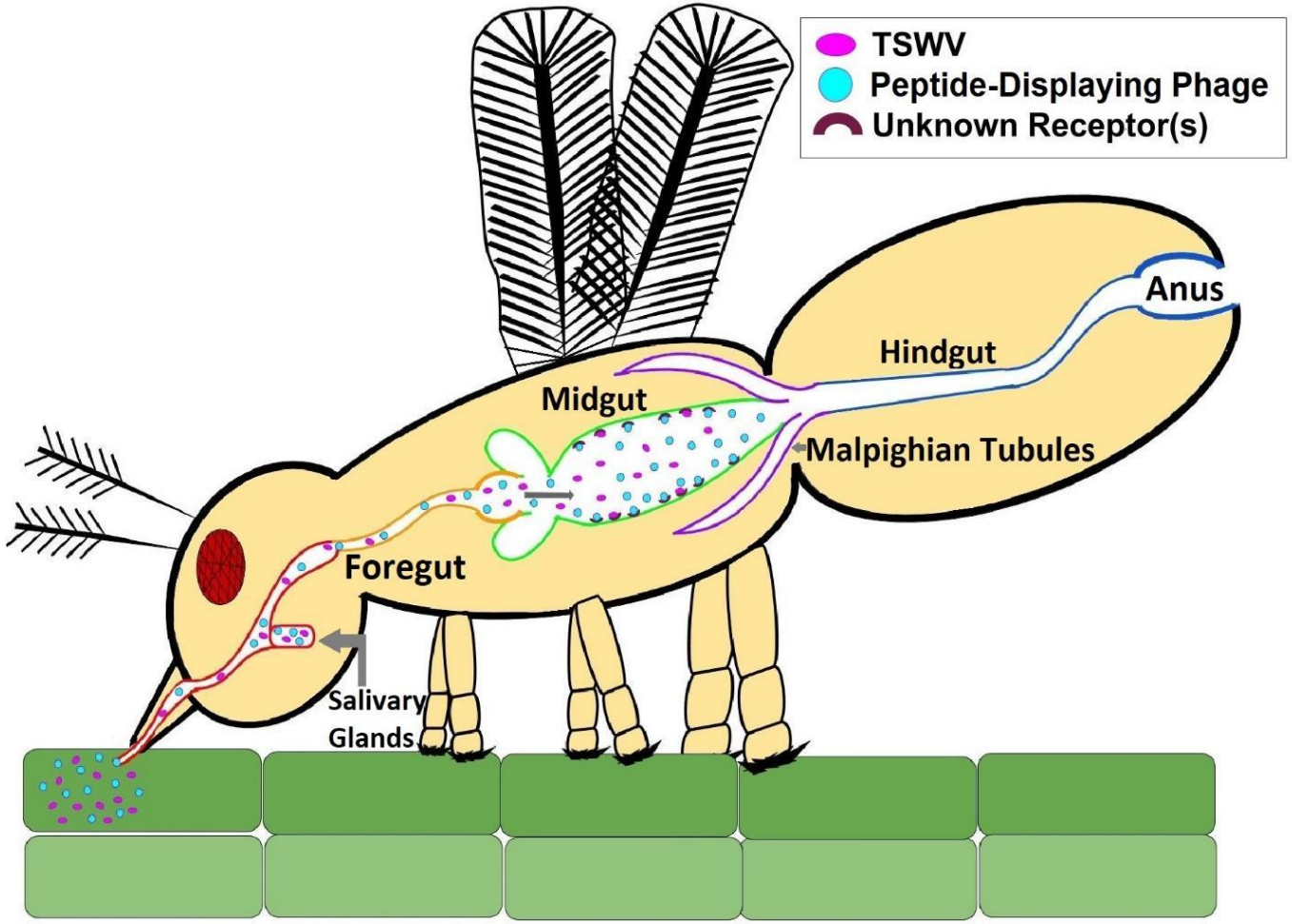


Figure 2.7. Model of C7C-binding phage inhibition of TSWV receptors. Thrips fed on the binding peptides resulted in a decrease of 57% TSWV acquisition. Based on this information, we hypothesize that the peptide is utilizing the same receptor as TSWV G_N glycoprotein, resulting in entry inhibition to thrips midgut epithelial cells.



CHAPTER 3: Transgenic tomato lines that exhibit resistance to field isolates of tomato spotted wilt virus (TSWV) show promise against a Sw-5 resistance-breaking strain.

Abstract

Tomato spotted wilt virus (TSWV) is among the most damaging disease-causing plant viruses worldwide. Management of TSWV is challenging and genetic resistance and vector control are two of the primary control options for tomato production systems. In 2016, the first report of resistance breaking strains of TSWV were reported in California tomatoes, resulting in loss of the effectiveness of the resistance gene, *Sw-5*.. These vector-borne viruses rely on thrips to move from host to host, resistant-breaking (RB) TSWV variants may reach other regions in the United States. Previously, transgenic tomato plants expressing hairpins of conserved regions from the open reading frame sequences of all five orthotospovirus genes were constructed and tested against a Hawaiian isolate of TSWV (MT-2) and tomato chlorotic spot virus (TCSV), resulting in resistance at the T1 and T2 generations. To further explore the resistance phenotype, I tested these transgenic plants against two field isolates of TSWV: a fresh market tomato isolate from North Carolina and a resistance-breaking strain from processing tomatoes in California. Eight different T2 generation plant lines (T3 progeny) representing four different hairpin constructs were confirmed for the transgene and selected for screening by leaf rub-inoculation in greenhouse trials. Four lines showed high-level resistance, and stunting and common tomato spotted wilt disease symptoms consistently developed on two lines, while two lines exhibited intermediate resistance. These transgenic plants show promise of broad-spectrum resistance for orthotospovirus control in the US, including *Sw-5* resistance-breaking strains of TSWV.

Introduction

Tomato spotted wilt virus (TSWV) is a persistent threat to food production worldwide, infecting over 1,000 different plant species. The virus is transmitted by nine species of thrips in a persistent circulative propagative manner (reviewed in Rotenberg et al., 2015). This pathogen is extremely difficult to control due to insecticide resistance in the vector populations, as well as the limited number of methods for viral control (Zhao et al., 2020). Current control measures rely on resistance genes such as *Sw-5* for management of TSWV and other orthotospoviruses in field and greenhouse production of tomatoes.

In 1930, a source of resistance to TSWV resistance gene Sw-5b was first identified in *Solanum peruvianum*, a wild relative of tomato that occurs naturally in Peru (de Oliveira et al., 2018). Later investigations revealed a resistance gene cluster (locus) containing five paralogs (named the Sw-5 gene cluster, Spassova et al., 2001) located on chromosome 9 (Chagué et al., 1996; Stevens et al., 1995). One of these genes, Sw-5b, was functionally characterized to be responsible for resistance to orthotospoviruses (Spassova et al., 2001; Hallwass et al., 2014; Leastro et al. 2017). The Sw-5b protein has an N-terminal extension known as the "Solanaceae" domain which interacts with TSWV-NSm protein (Li et al., 2019; de Oliveira et al., 2016), and it contains nucleotide binding and leucine rich repeat domains (Spassova et al., 2001), common motifs found in plant resistance proteins. The Sw-5b gene provides tomato with broad-spectrum resistance against TSWV, groundnut ringspot virus (GRSV), tomato chlorotic spot virus (TCSV) and other closely related (TSWV clade) orthotospoviruses.

Genetic resistance has been used extensively for TSWV control since the first release of cultivars containing *Sw-5*b gene (herein referred to as *Sw-5* gene) in 1988 (Thompson and van Zijl, 1996). As with deployment with other single dominant resistance genes, researchers had expectations for

occurrence of variant strains able to overcome resistance. Resistance breaking (RB) strains of TSWV developed five years after the first introduction of resistant plants into the field, where infections were identified in South Africa (Thompson and van Zijl, 1996).

The concern of losing the effectiveness of *Sw-5* resistance materialized with the increase in the occurrence of RB-TSWV strains. In Spain, (Ciuffo et al., 2005) the control of TSWV was lost around 2005, when symptoms were observed on *Sw-5* tomato plants in different regions of the country. Meanwhile, in California U.S., Batuman et al. (2017) reported TSWV infection of *Sw-5* plants from production fields that had history of TSWV infection since 2016. Genotypic analysis of RB-TSWV revealed that the resistance breaking virus phenotype is associated with a single amino acid change in the NSm protein (de Oliveira et al., 2018; Ciuffo et al., 2005; Batuman et al. 2017; López et al., 2011). Two different changes have been linked to these RB strains: C118Y and T120N, with the C118Y mutation as the most frequently observed mutation occurring in TSWV populations associated with decline in effectiveness of *Sw-5* plants in different geographic regions (Peiro et al., 2014; López et al., 2011).

TSWV, once acquired by thrips vectors, replicates, resulting in transmission throughout the adult life span. Because TSWV infection in the thrips vector leads to increase in viral titer, the risks of RB strains reaching new hosts and reservoir plants increases, and the likelihood of spreading RB strains to other countries increases through thrips contamination of plant material shipped globally (Morse and Hoddle, 2006). As RB mutations have occurred independently from each other (López et al., 2011), there is also the chance of eventually completely losing resistance as a method of control for TSWV in regions where RB strains have not been reported. These possibilities are only a matter of time, and scientists are diligently working on breeding programs and alternative methods to increase resistance, but these take extensive time to develop and release commercially.

We propose to use transgenic tomato plants that express short hairpin RNA sequences targeting TSWV and other orthotospoviruses by RNA interference as an alternative to *Sw-5* mediated resistance. Hairpins were previously created and inserted into plants to work as a defense strategy by targeting all five orthotospovirus genes (Oliver et al., unpublished). RNAi in our tomato system is an innovative method, however, it has been used in other plant-virus systems currently in the commercial use (Zhao et al., 2019; Ferreira et al., 2002; Fuchs et al., 1998). The mechanism of resistance is through generation of small-interfering RNAs (siRNAs) that target viral RNAs (reviewed in Brosnan and Voinnet, 2011). siRNA can travel to distant tissues and activating the host defenses (Brosnan et al., 2007, Palauqui et al., 1997). Infection of our transgenic plants by mechanical rub-inoculation with Hawaiian isolate (MT-2) of TSWV (HI-TSWV) and TCSV resulted in resistance of T2 plants in greenhouse trials (Oliver et al., unpublished). These hairpin-expressing plants have also previously been shown to have effective resistance to when challenged with viruliferous thrips infected with HI-TSWV (Oliver et al., unpublished). We challenged the 3rd generation (T3) hairpin transgenic plants containing one of four different transgene constructs (A, B, E or F) against a TSWV isolate obtained from fresh-market tomatoes in Western North Carolina and a *Sw-5* resistance-breaking (RB) TSWV strain collected from processing tomatoes in California. Our long-term goal is to identify transgenic lines that will provide breeding material for introgression into a desirable tomato breeding line to develop resistant varieties to serve as a durable and broad-spectrum solution for orthotopovirus control.

Materials and Methods

Transgenic tomato line selection and growth conditions

Hairpin lines showing resistance to TSWV and TCSV in previous experiments (Oliver et al., unpublished) were selected for testing against both NC and RB-TSWV. For NC-TSWV isolate, we tested seven plant lines from T2 generation seeds (T3 lines) available (A1.3.8, A3.2.3, B4.4.1, E4.2.1, E8.1.9, F2.5.3 and F4.2.10), while for the RB-TSWV, an additional 8th line (E2.4.3) was included. Plants were grown in MetroMix 900 potting soil (Sun Gro®, Agawam, MA, USA) and tested under greenhouse conditions in cages with 26^{o}C day and 24^{o}C night with a 12 hr photoperiod. Plants were fertilized weekly with Tomato Plant Food from Miracle-Gro® from one week after germinating and continuously through experiment.

TSWV isolate maintenance

TSWV isolates maintained for experiments included Hawaiian (MT-2), used as a positive control for molecular testing, a field-collected isolate from fresh market tomatoes in Western North Carolina (NC), and lastly, a *Sw-5* RB-TSWV isolate from processing tomatoes in California. All isolates were maintained under the same greenhouse conditions in cages at 26°C day and 24°C night with a 12 hr photoperiod in insect proof cages on 'Money Maker' tomato and *Emilia sonchifolia*, and RB-TSWV was also inoculated on 'Mountain Merit' (untreated F1 tomato lines, Harris Seeds, Rochester, NY, USA) to ensure that the *Sw-5* resistance-breaking mutation was maintained. All isolates were inoculated bi-weekly onto new plants to continue an infection cycle and high titer infectious inoculum for testing.

Sequencing of the RB-TSWV NSm gene

Testing of RB mutations in the TSWV NSm gene was performed prior to experiments to verify mutations associated with the RB phenotype. RB infected plant tissue was collected and processed for RNA extraction using Mini Total RNA Kit (plants) (IBI Scientific©, Dubuque, IA) and cDNA synthesized (Verso cDNA synthesis kit, from Thermo Scientific™). Primer sequences from di Rienzo et al. (Table 3, 2018) with the same PCR protocol were used to amplify the NSm gene. PCR products were sequenced at North Carolina State University Genomic Sciences Lab using the reverse primer from Rienzo et al. and verification of the RB mutation with SnapGene.

Identification of transgenic plants

T3 plants were sampled for the presence of the hairpin to confirm that the plants tested were transgenic (i.e. presence of the transgene construct, one of five possible constructs). Seed of selected lines were sewn and at the 2-leaf stage verified by DNA extraction and PCR for the transgene before inoculation with TSWV. Using the Genomic DNA Mini Kit (Plant) (IBI Scientific©, Dubuque, IA), 100 mg of tissue was collected per plant, frozen in liquid nitrogen and finely ground with a TissueLyserII (Quiagen, Germantown, MD) and Daisy Precision Max BB's (0.177 Caliber BB Zinc Plated Steel, Walmart) for 2 minutes at 30.0 1/s frequency and processed following the kit protocol. Hairpin presence was verified in 50 ng of the isolated DNA by PCR for using primers from Table 3.1 (Oliver et al., unpublished) with the following program: 2-min initial heating step at 95°C followed by 30 cycles of 30 sec melting at 95°C, 30 sec annealing at 55°C, and 50 sec elongation at 72°C with a final extension of 5 min at 72°C. PCR products from plants demonstrating hairpin presence were transplanted into 4-inch pots.

Leaf-rub inoculation of TSWV

To evaluate the resistance phenotype, T3 plants showing transgene presence were inoculated with the isolate or strain of TSWV (NC or RB, respectively). For leaf-rub inoculations, plants were dusted with carborundum to create small wounds for virus entry on plant leaves and sap/inoculum was prepared by grinding 4-5 infected leaves in a cold mortar and pestle with 10mM sodium sulfite buffer and applied to all leaves using two cotton swabs to cover in sap and fresh buffer prepared for every cage.

Experimental design and sampling strategy

Representatives of each transgenic line were placed into five 2x2 ft cages which served as blocks in a randomized complete block design, with three types of controls and mock-inoculated plants placed in each cage. Controls included: i) a hairpin negative control, shGFP, which contains a short hairpin RNA of GFP that confers no resistance to TSWV, ii) non-transgenic Money Maker (MM) tomato (the genetic background of the transgenic plants), and iii) Mountain Glory F1 tomato (Harris Seeds, Rochester, NY, USA), a tomato variety that contains the Sw-5 gene. Two plants representing each line (seven (NC) or eight (RB) transgenic and three controls) were placed into five blocks for NC-TSWV and eight blocks for RB-TSWV, one treated as mock and the second as virus inoculated. All plants were monitored over a 2-week period, at 14 days post inoculation (dpi) measurements were recorded and samples were collected for enzyme-linked immunosorbent assay (ELISA). Within each block, one representative virus-inoculated and one mock-inoculated plant were placed at random with three controls (virus and mock inoculated). For the RB-TSWV isolate, a second set of samples were collected at 24dpi for ELISA and RT-qPCR for infected plants. The experiment was conducted in November 2020 for NC-TSWV with ten plants per line (five virus-

inoculated and five were mock-inoculated), and twice for the RB-TSWV strain (Rep 1 = July 2021; Rep 2 = October 2021, with ten virus-inoculated and ten were mock-inoculated per replicate).

Plant measurements

Plants were measured at 14dpi using a yardstick in centimeters for height and width. Height was taken from soil to top of the last new incoming growth, while width was taken using the longest leaves from newer growth.

Evaluation of virus resistance

TSWV infection was determined serologically with the use of a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). For each plant, 100mg of samples for the newest emerged leaf tissue was collected in 2mL tubes (at 14 dpi and additionally at 24 dpi for RB-TSWV) and flash frozen using liquid nitrogen and pulverized using a TissueLyserII (Qiagen, Germantown, MD) and Daisy Precision Max BB's (0.177 Caliber BB Zinc Plated Steel, Walmart), and general extraction buffer was added into each tube. We used the Agdia TSWV DAS-ELISA (Agdia, INC, Elkhart, IN) kit for detection following the manufacturer's protocol. Collected samples included seven lines (A1.3.8, A3.2.3, B4.4.1, E4.2.1, E8.1.9, F2.5.3 and F4.2.10) challenged with the NC-TSWV isolate, and an additional line (E2.4.3) used for testing the RB-TSWV. Absorbance values of samples read from the ELISA plates were determined at a wavelength of 405 nm wavelength the Cytation™ 5 imaging reader (Biotek, Winooski, VT, USA). Mock-inoculated plants served to calculate the average background absorbance values for non-infected, healthy tissue for each plant line. OD values were determined positive if they were less than three times the average absorbance of healthy tissue.

RNA extraction & RT-qPCR

For RB-TSWV, plant samples were processed to determine mean virus abundance (titer) for each line. Newly emerged leaf samples of infected plants were collected at 14dpi and flash frozen with liquid nitrogen. Samples were kept at -80◦C, and pulverized in batches in the TissueLyserII (Qiagen, Germantown, MD) using Daisy Precision Max BB's (0.177 Caliber BB Zinc Plated Steel, Walmart) for 2 minutes at 30.0 1/s frequency for RNA extraction with the Mini Total RNA Kit (Plants) (IBI Scientific©, Dubuque, IA), by protocol. Samples were quantified on a NanoDrop (Thermo Fisher Scientific™,Waltham, MA) and 150ng were immediately added into the cDNA mix (Verso cDNA synthesis kit, from Thermo Scientific™) and into the thermocycler with the following program: cDNA synthesis at 40◦C for 30 mins and Inactivation at 90◦C for 5 min. cDNA was used to test viral presence and titer in each plant sample. For qPCR testing, 10µL reactions were prepared as follows: cDNA samples diluted 5-fold and 2µL added into a 96-well plate, with 0.5µL of each primer for TSWV nucleocapsid (Forward primer: 5'-GCT TCC CAC CCT TTG ATT G-3' and Reverse primer: 5'-ATA GCC AAG ACA ACA CTG ATC-3') or Elongation Factor 1 (reference gene, Forward primer: 5'-GAT TGG TGG TAT TGG AAC TGT C-3' and Reverse primer: 5'-AGC TTC GTG GTG CAT CTC-3') and 5µL of SYBR® Green (Bio-Rad Laboratories, Inc) and 2µL nuclease free water, with the following program: 3-min initial heating step at 95°C followed by 39 amplification cycles of 10s at 95°C and 30s at 60°C, with an additional step of 5s at 65◦C and 5s at 95◦C to detect the melt curve using a CFX Connect Real-Time PCR System (Bio-Rad Laboratories, Inc). Virus abundance (titer) was normalized using the Pfafll inverse equation: $E\_ref^{Ct\_ref}/E\_N^{Ct\_N}$ (Rotenberg et al., 2009).

Statistical Analysis

An 10x2 contingency table (for RB-TSWV 11x2 table) was constructed with disease incidence (infected and not infected) to compare by Fisher's exact test and pairwise comparison resistance of each transgenic line by evaluating difference in p-values of each line to shGFP in R-studio. Kruskal-Wallis non-parametric and Pairwise Wilcoxon tests were applied to compare plant height and width measurement values between mock- and virus-inoculated plants for both NC- and RB-TSWV, while also applied to the average virus titer from RB-TSWV infected plants at 14 dpi in R-studio.

Results

RB-TSWV NSm sequence

Previous studies (de Oliveira et al., 2018; Ciuffo et al., 2005; Batuman et al. 2017; López et al., 2011) have reported that *Sw-5* plants may become infected with TSWV, if the virus contains mutations in the NSm protein at C118Y or T120N. Prior to experiments, we sequenced the RB-TSWV strain NSm gene to verify that it had the mutation that causes *Sw-5* plants to be infected. Sequencing of the NSm gene revealed a C118Y mutation, indicating that our isolated strain was identical to many RB-TSWV strains documented.

Tomato seed emergence

Emergence rates were determined to evaluate the number of seeds that sprouted. Overall, emergence rates for the lines ranged from 68% - 90%. Two different soils were tested as well to determine which worked best for germination. The first was only MetroMix 900 and the second was 33% MetroMix 900 and 66% pea gravel (results on *table 3.2*). In the mix with gravel, all lines

had much higher emergence rates of 82% - 94%, while with only Metro mix, emergence rates ranged from 49% - 87%. Germination in the 33% Metro mix + 66% gravel resulted in the best soil for these transgenic plants.

Hairpin transgene detection

The transgenic plants are expected to contain one of four transgene inserts (A, B, E, or F). Prior to virus inoculation, PCR of genomic DNA was performed to confirm the presence of the hairpin-expression transgene. Table 3.2 reports the percent of plants that tested positive for the transgene for each line tested for all three trials combined. Lines A1.3.8, E2.4.3 and E8.1.9 had a low number (57%, 58% and 73% respectively) of plants containing a transgene, more than 75% of the plants tested from the other lines contained the transgene. Lines were not tested for homozygosity, but most lines did have more than half of the tested plants (progeny of these plants = T3) inheriting the transgene.

Testing T2 generation lines resistance to TSWV

Five of the seven lines challenged with NC-TSWV showed varying levels of resistance to the virus *(Figure 3.1* and *Table 3.3)*. All the negative control plants (Money Maker and shGFP) tested positive for TSWV and displayed common TSWV symptoms such as stunting, brown and yellow ringspots, and crinkling of leaves, while the positive controls for TSWV resistance (Sw-5 variety) did not express symptoms, and OD values indicated virus-negative plants (0% infection). To evaluate infected plant lines that were challenged with NC-TSWV, the contingency table was compared by Fisher's exact test for lines A1.3.8, A3.2.3, B4.4.1, E4.2.1, E8.1.9, F2.5.3 and F4.2.10 and the three control plants. Overall, the Fisher exact test (two-tail, overall p-value =

0.0000002644) indicated differences across lines and controls. Transgenic lines A1.3.8, A3.2.3, B4.4.1, E4.2.1and F2.5.3, did not have any visual symptoms and DAS-ELISA values indicated 0% infection indicating a strong and reproducible resistance to an isolate of TSWV found in tomato production systems. On the other hand, lines E8.1.9 and F4.2.10, did in fact have TSWV symptoms and resulted in 80% and 40% infection respectively.

For the first trial with RB-TSWV, at 14 dpi, all replicate plants of lines A3.2.3, E4.2.1, F2.5.3 and F4.2.10 tested negative for TSWV, while A1.3.8, B4.4.1, E2.4.3 and E8.1.9 lines exhibited moderate to low rates of infection. By 24 dpi, infection incidence remained stable for five of the eight lines. However, lines E8.1.9 (to 60%) and F4.2.10 (10%) exhibited an increase in the number of infected plants, while plants of line B4.4.1 that had detectable virus at 14 dpi, tested negative at 24 dpi (*figure 3.1* and *table 3.3*). Fisher's exact test compared lines and controls at 14 and 24 dpi where in both cases there was an overall significant difference in infection rates (p-values = 0.0004998 and < 2.2e-16, respectively) across the lines and controls, with four lines exhibiting a high level of resistance to the RB strain.

In replicate two of the screen (trial 2) for resistance to RB-TSWV, we observed similar trends to replicate one. Overall, there was a lower infection rate across the experiment, possibly due to temperature, light intensity and day length associated with seasonal changes between trials, which consequently affected plant growth. Nonetheless, trends were similar between the two trials, with a few exceptions. The best performing plant lines (A3.2.3, B4.4.1, E4.2.1 and F2.5.3) showed complete resistance - testing negative for virus and exhibiting no visible symptoms. Plants of lines A1.3.8 and E2.4.3, which had exhibited some level of infection in rep 1, resulted in 0% infection, 24 dpi in rep 2. Lines E8.1.9 and F4.2.10 both had a 25% infection rate at both sampling time points, a pattern consistent with rep 1. Low infection efficiency in the controls (Money Maker,

shGFP and Mountain Glory) suggested that lower temperatures and shorter day lengths could have impacted plant growth, virus accumulation and/or symptom development. There was an overall significant difference in infection rates (% plant testing positive) across lines and controls at both time points (14dpi p-value = 0.001935 and 24dpi p-value = 2.538e-05), with six lines representing four transgene constructs exhibiting 0% infection (tested negative by ELISA) regardless of the environmental conditions experienced by plants in second trial.

Virus abundance in plants infected with RB-TSWV

Mean virus abundance is presented in figure 3.2 for RB-TSWV-infected transgenic lines and controls for the two replicates. The non-transgenic 'Money Maker' control resulted in high mean virus accumulation (140 in rep 1 and >15 in rep 2) across the two trials, while Mountain Glory' (the Sw-5 resistance line) and 'shGFP' (hairpin transgene negative control in 'Money Maker' background) both had similar means and much lower titer (<40). Through all controls, similar number of plants resulted infected from ELISA. Both 'Mountain Glory' and shGFP were similar to E8.1.9 virus accumulation in both trials, but this line had lower number of infected plants. Other lines resulting in 1-3 infected plants were A1.3.8, E2.4.3 and F4.2.10, though the titer was below 1, leading to very low abundance detected. Kruskal-Walis in both replicates did not result in significant difference (rep 1 p-value = 0.4405 and rep 2 p-value = 0.06107). This data does inform us on how abundant the virus is within each line of the infected transgenic plants which express the hairpin, leading very low TSWV accumulation in some lines.

Plant Measurements

One primary, observable plant host symptom associated with systemic infection by TSWV is stunting. To assess the effect of NC-TSWV and RB-TSWV infection across lines and controls, plant height and stem width measurements were recorded. Comparisons were made between mock-inoculated and virus-inoculated plants for each line and control treatment (Table 3.4). For the NC-TSWV, Kruskal-Wallis p-values were 0.003289 for height and 0.0162 for width. The RB-TSVW strain Kruskal-Wallis test was employed to infect plants height for both replicates resulting in p-values of 1.73e-06 and 0.01802 respectively, and width for rep 1 with p-value 0.01089 (Pairwise Wilcoxon p-values located on supplementary table S3.3). For both NC and RB, significance was observed from statistical comparisons of infected and healthy plants. Transgenic plants inoculated with either virus resulted in low variation to the mock-inoculated, while for the control lines, virus infections demonstrate susceptibility and stunting phenotype (figures 3.3 and 3.4).

Discussion

The hairpin constructs were designed to contain one of two conserved regions from sequences of all five genes of tospoviruses inserted via agroinfiltration. These regions were carefully chosen within the open reading frame (ORF) of the *Orthotospovirus*, to promote recognition and targeting of a large number of these viruses. Plants having a hairpin that can target not one, but multiple regions from a viral genus increases resistance to multiple viruses while decreasing the chances of viral suppressors of RNA that could act on the complete hairpin products. We selected seven and eight T2 lines to test two different TSWV strains, NC and RB respectively. These lines represent

four different hairpin constructs (A, B, E and F), from which each T0 line, transgenes were inserted into plants independently, and eventually T2 lines were produced.

Our results indicate that all constructs are suitable for resistance. Still, there are T2 independent lines under a hairpin construction, which can result in low or no resistance, as we observed for E8.1.9. However, we also observed lines (A1.3.8, E2.4.3 and F4.2.10) whose infection was less than 30%. When interpreting the mean virus abundance, the low values for the three lines (A1.3.8, E2.4.3 and F4.2.10) in comparison with E8. 1.9 or the three controls (Money Maker, Mountain Glory and shGFP), we could ask ourselves, is the virus still transmissible by thrips? Variations from one line to another containing the same construct could suggest that the transgene insertion location (or site) could play a crucial role in how this defense mechanism acts in the plants.

We found four lines (A3.2.3, B4.4.1, E4.2.1 and F2.5.3) resulting in high levels of resistance against two species of orthotospoviruses. We directly compared *Sw-5* plants against both NC and RB strains of TSWV. Not only did the transgenic hairpin-expressing plants show better results against both viruses after 14 dpi, but they also displayed a healthy growth and appearance, exhibiting more leaves and vertical growth in our greenhouse trials than infected plants. The RB isolate can overcome the *Sw-5* resistance after 14 dpi while the four resistant hairpin lines show no symptoms after 24 dpi. We found that all infected plants were stunted and had brown ringspots for both viruses. These symptoms were observed in controls: Money Maker, shGFP and *Sw-5* plants, as well as transgenic plants that were infected (A1.3.8, E2.4.3, E8.1.9 and F4.2.10).

Plants in replicate 1 for RB-TSWV grew at optimal temperatures and light exposure. During replicate 2, we had fall weather and shorter days, which could have affected plant growth. Previously, we have noticed effects on symptom development and infection length during colder

months on non-resistant Money Maker plants, which could explain the overall lower infection percentages. Although this could have affected the experiment, transgenic plants still resulted in similar trend to that of replicate 1, in which four lines had no infections and two lines were susceptible in both experimental replicates.

RNA interference (RNAi) is an efficient method to control different pathogens. Plants naturally use an RNA mediated silencing pathway to control post-transcriptional gene silencing (PTGS) processes for protein regulation and other processes (Zhu et al., 2011). An RNAi based resistance focuses on the use of naturally occurring proteins in plants (siRNA and miRNA) which cleave to foreign dsRNA and provide a guide for sequence-specific degradation of pathogen RNA (Nahid et al., 2011). This resistance starts when plant RNAi-Induced Silencing Complex (better known as RISC) proteins target dsRNA, which then is used as a complementary strand to find and degrade matching sequences. RNAi can help in the activation of immune responses by sending siRNA to distant tissues (Buchon and Vaury, 2005). Because of this, the production of genes that contain pathogen information to form dsRNA in plants is an efficient tool to control plant pathogens, especially viruses, since some form dsRNA during replication or transcription (Zamore, 2004; Buchon and Vaury, 2005).

Previous attempts to develop transgenic plants expressing TSWV's N-gene have been made (Kim et al., 1994). The main problem was the expression of the N-gene RNA to produce resistance only delayed or reduced the infection and did not actually confer immunity to the plant. Expression of only one gene for this virus was not sufficient to overcome infection in tomato or tobacco plants. They did evaluate N-gene expression and saw that lines with less expression had higher TSWV incidence. We've shown that using multiple gene targets can help increase resistance, though we still need to assess if hairpin expression correlates to resistance.

Through the use of RNAi, resistance to plant viruses has been reported in cases such as Rice dwarf virus (RDV), where no natural resistance has been obtained against this virus (Shimizu et al., 2009). Meanwhile, cases like Citrus tristeza virus (CTV), where resistant cultivars of *Poncirus trifoliata* are used as rootstocks to grow citrus in New Zealand, but CTV has been able to overcome it (Harper et al., 2010) and new methods using RNAi have aroused in plants with use of genome sequences to target CTV (López et al., 2011). RNAi has been the new incoming method to control pathogens in a fast and efficient way by targeting one or multiple viruses or viral products with one transgene.

Commercial lines of transgenic plants carrying a gene to target viruses, such as those for Papaya ringspot viruses (PRSV), have been on the market in the US (Ferreira et al., 2002) since 1988. These plants in particular, were developed to target the coat protein (CP) of PRSV, providing high levels of resistance in Hawaii, but varying resistance in Florida (Davis and Ying, 2004). These genes were good at targeting viruses isolated in Hawaii, but not that functional in a large region like China (Wei et al., 2007). Because of the low resistance, scientists opted to design and develop plants expressing RNAi against conserved domains from variable isolates from China in the hopes to target a broader number of PRSV isolates (Jia et al., 2017). In the case of cassava brown streak disease (CBSD), there is also a limit to actual resistant cultivars available against the two possible viruses that can cause this disease, Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) in West Africa. Beyene et al. (2017) focused on utilizing an RNAi method to target the CP from two viruses using one cassette expressing sequences for both viruses and obtaining plants that confer resistance in the cassava cultivar preferred by farmers.

The use of multiple targets within one construct could decrease the ability of a plant to become infected. Zhang et al. (2011) used a different method to target three different plant pathogenic

viruses (Alfalfa mosaic virus, Bean pod mottle virus and Soybean mosaic virus) by using only small section (~100bp) of highly conserved regions of viral replicase genes to target via RNAi. Their method of construct is interesting due to the resistance of all three viruses from different families, even when all three are present together on a soybean plant, and the fact that using a small fragment of inverted repeat sequences for each virus was able to produce resistance. Although this is a good example of a broader targeted range, the use of small sequences can easily lead to viral suppressors or changes in the genome that can evade complementation.

Using other genes, such as those involved in replication may have a bigger advantage when targeting closely related viruses. Other research, interestingly, has focused on targeting viral genes involved in replication as Pns12 in RDV (Shimizu et al., 2009) a dsRNA virus and AC1 in Bean golden mosaic virus (Bonfim et al., 2007) a ssDNA virus. Pns12 is involved in the formation of the viroplasm, while AC1 initiates replication. AC1 was also targeted for two other Geminiviruses, Cotton leaf curl virus and Tomato yellow leaf curl virus, making this RNAi strategy also functional for three different ssDNA viruses (Asad et al., 2003; Yang et al., 2004).

All these previously mentioned examples are arthropod vectored viral diseases, which are difficult to manage, because many of the important insect vectors have developed resistance to insecticides and effective practices to reduce insect populations on plants are limited (Reitz et al., 2020; Umina et al., 2019). With our hairpin delivery strategy, plant health and production can increase without TSWV infection. This approach provides a good source of resistance by targeting all orthotospovirus genes via short hairpin RNA. We have tested and documented different constructs and independent lines resulting in plants able to confer resistance to RB-TSWV strains using RNAi. We plan to map the insertion site of these candidates to breed into grower-preferred tomato

cultivars in the US. Using these plants, we can test against other species of othotospoviruses to develop suitable plants with broad-spectrum solution control.

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Tables and Figures

Table 3.1. Primers used for transgene detection of transgenic orthotospovirus resistant tomato plant lines (Oliver et al., unpublished). For each construct (A, B, E or F) the TSWV_-R (reverse) primer was combined with the GUSF1 (forward) primer for PCR. The GUSF1 represents the hairpin linker which is common in all constructs, while each construct has a different primer for the arm.

Name	Purpose	Sequence
JEO-GUSF1	Sequencing of constructs in pANDA35HK	CACGCAAGTCCGCATCTTCA
TSWVA-R	Amplification of TSWV-A hairpin arm	GCCGTCGACAAGTTGAATTCCCC
TSWVB-R	Amplification of TSWV-B hairpin arm	GCCGTCGACATACTTAGTTCCAGC
TSWVE-R	Amplification of TSWV-E hairpin arm	GCCGTCGACAGAATGACTTGC
TSWVF-R	Amplification of TSWV-F hairpin arm	ATGAGGCCGTCGACTTATTCTGG

Table 3.2. Emergence rates and transgene-positive plants of T3 transgenic 'Money Maker' tomato. Transgenic tomato percent of germination in different potting soils for T2 generation seeds and percent of transgene presence. Two different soils were tested to determine which served best for germination purposes and together used to calculate the rate of germination for each line. For each experiment, Genomic DNA was extracted, and extraction and endpoint PCR was performed used to confirm presence of hairpin transgenes in plants prior to entering plants into the virus screen. Values represent data collected from all trials to evaluate phenotypic response to RB-TSWV (2 trials) and NC-TSWV (1 trial). RB = Sw-5 resistance-breaking isolate from California tomato field; NC = 'wildtype' TSWV isolate from North Carolina tomato field. to test for each virus variant and results were used to determine the transgene rate for which is present in each line.

Line	Overall Germination Rate	Germination Rate in Metro Mix	Germination Rate in 33% Metro mix + 66% gravel soil	Number of transgenic plants	Number of plants tested	Percent of plant containing the transgene
A1.3.8	0.6825	0.49	0.875	38	67	57%
A3.2.3	0.7175	0.55	0.885	55	71	77%
B4.4.1	0.87	0.81	0.93	57	70	81%
E2.4.3	0.79	0.7	0.82	23	40	58%
E4.2.1	0.78	0.85	0.94	39	50	78%
E8.1.9	0.825	0.545	0.865	37	51	73%
F2.5.3	0.895	0.865	0.91	48	50	96%
F4.2.10	0.89	0.85	0.92	42	51	82%

Table 3.3. Transgenic lines exhibit varying levels of resistance to NC-TSWV and RB-TSWV in greenhouse experiments. Combined = overall line performance against both TSWV strains (wildtype NC-TSWV and Sw-5 RB-TSWV). Infection incidence data was analyzed statistically using Fisher exact test of independence followed by pairwise comparisons between each transgenic line (or non-transgenic variety) vs. the transgene hairpin control (shGFP). Pairwise comparisons with p-values less than 0.05 indicate significant differences within pairs. NT = not tested; *Rep 2 produced a lower infection rate in the controls overall and there were fewer replicates per line in the screen, resulting in a loss of statistical power to resolve statistically supported differences. Nonetheless, the trend was robust in the transgenic lines in Rep 2 (0% vs 50% in hpGFP control) that exhibited strong resistance to NC-TSWV and the first replicate of the RB-TSWV.

Combined results			**NC-TSWV**				**RB-TSWV Rep 1**				**RB-TSWV Rep 2***			
Line	Percent infection	p-value	Number of infected plants	Number of uninfected plants	Percent infection	p-value	Number of infected plants	Number of uninfected plants	Percent infection	p-value	Number of infected plants	Number of uninfected plants	Percent infection	p-value
A1.3.8	20%	7E-05	0	5	0%	0.03	3	7	30%	0.010	0	8	0%	0.064
A3.2.3	0%	2E-07	0	5	0%	0.03	0	11	0%	0.000	0	8	0%	0.064
B4.4.1	0%	2E-07	0	5	0%	0.03	0	10	0%	0.000	0	8	0%	0.064
E2.4.3	11%	1E-04	NA	NA	NA	NA	1	8	11%	0.001	0	8	0%	0.064
E4.2.1	0%	3E-07	0	5	0%	0.03	0	9	0%	0.000	0	8	0%	0.064
E8.1.9	67%	8E-02	4	1	80%	1	6	4	60%	0.162	2	6	25%	0.881
F2.5.3	0%	2E-07	0	5	0%	0.03	0	10	0%	0.000	0	8	0%	0.064
F4.2.10	20%	7E-05	2	3	40%	0.37	1	9	10%	0.001	2	6	25%	0.518
Money Maker	100%	1E+00	5	0	100%	1	8	0	100%	1	4	4	75%	0.881
shGFP	100%	--	5	0	100%	--	8	0	100%	--	6	2	50%	--
Mount Glory	64%	7E-02	0	5	0%	0.03	9	0	100%	1	3	5	50%	0.881

Figure 3.1. Resistance in transgenic tomato infected with NC-TSWV and RB-TSWV by mechanical inoculation**.** Transgenic T3 generation plants (progeny from T2 lines) expressing *orthotospovirus* hairpins (detected by PCR) were challenged against NC-TSWV isolate and the RB-TSWV strain by mechanical inoculation. Virus was detected in plant samples at 14 (NC-TSWV) and 24 (RB-TSWV) days post-inoculation using DAS-ELISA. Bars indicate the percent of infected plants per line for one trial infected with either TSWV variant. Control plants Money Maker (MM), shGFP and Mountain Glory (MG) were included and used to compare ELISA results by Fisher's exact test. P-value for NC-TSWV 0.00000002644 and RB-TSWV 2.2e-16, both resulted in high significance. The star symbol over bars, indicates a (★) 95%, (★★) 99% and (★★★) 99.9% statistical difference confidence from negative control shGFP with a P values on *Table 3.3* by Fisher Pairwise comparison.

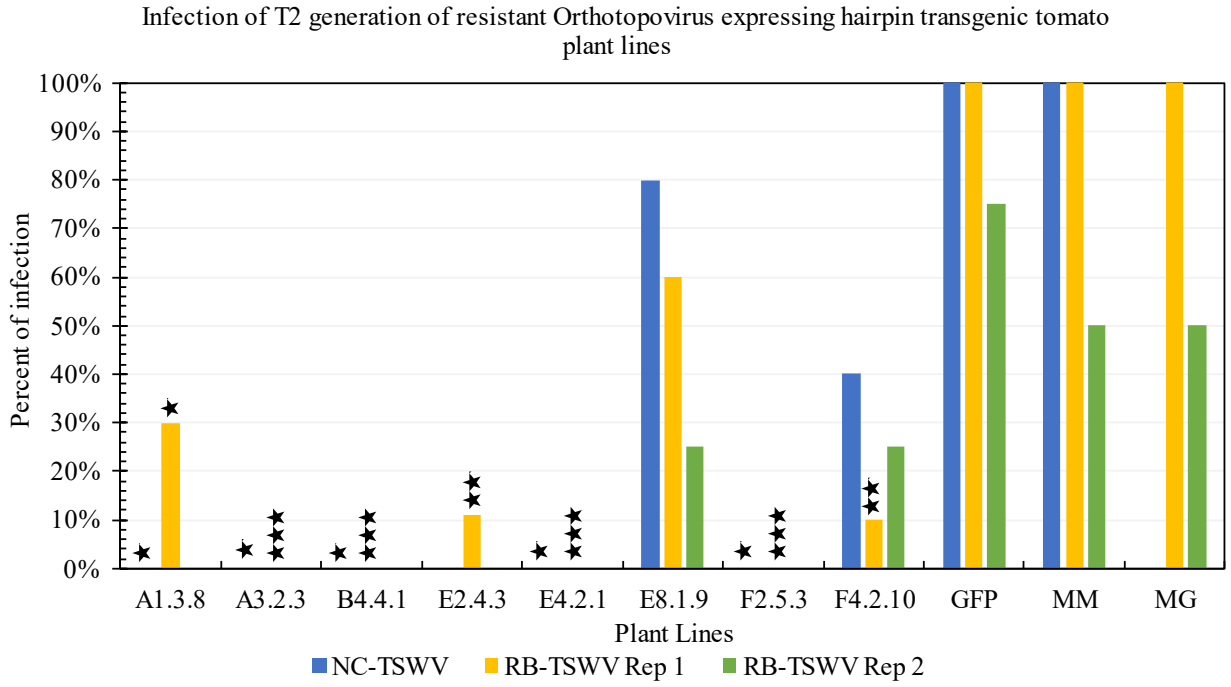


Figure 3.2. RB-TSWV virus abundance from infected T2 lines. Plant samples were collected from RB-TSWV infected plants for RT-qPCR and compared for plants resulting positive at 24 dpi (*figure 3.1*). Bars show normalized virus abundance for plants that resulted positive for virus in DAS-ELISA, calculated using reverse Pfafll with the *Elongation factor 1* reference gene and TSWV-N gene. Kruskal-Wallis test p-value 0.4405 for rep 1 and 0.06107 for rep 2 did not show difference in means.

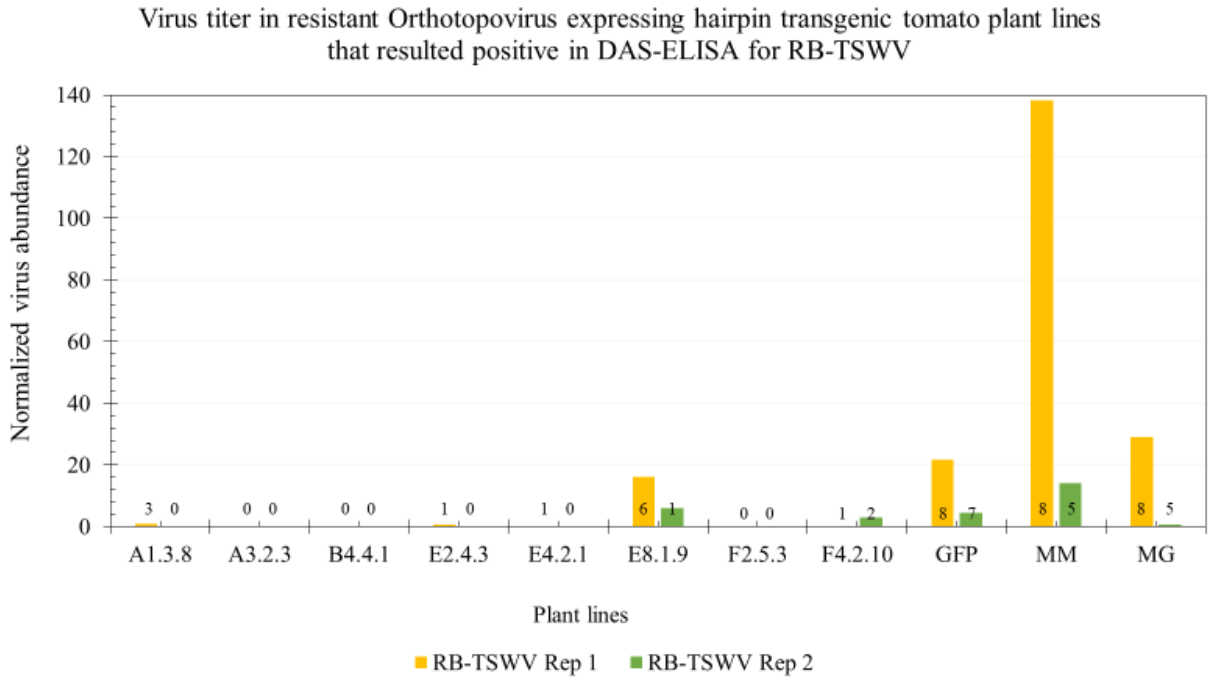


Figure 3.3. NC-TSWV and RB-TSWV infected plant heights. Bars for average height for all lines (A1.3.8, A3.2.3, B4.4.1, E2.4.3, E4.2.1, E8.1.9, F2.5.3, and F4.2.10) were plotted to show variation between NC-TSWV or RB-TSWV and mock inoculated plants after 14 days post inoculation. Kruskal-Wallis non-parametric p values 0.003289 and 1.73e-06 for NC and RB TSWV respectively (Pairwise Wilcoxon p-values located on supplementary table S3.3). Significance in both cases indicates a 99% level difference. Stunting from infected plants can be observed in negative controls Money Maker (MM), and shGFP (GFP), as well as the Sw-5 resistant control Mountain Glory (MG). All bars for T2 transgenic lines show low variation between mock and NC-TSWV infected plant height.

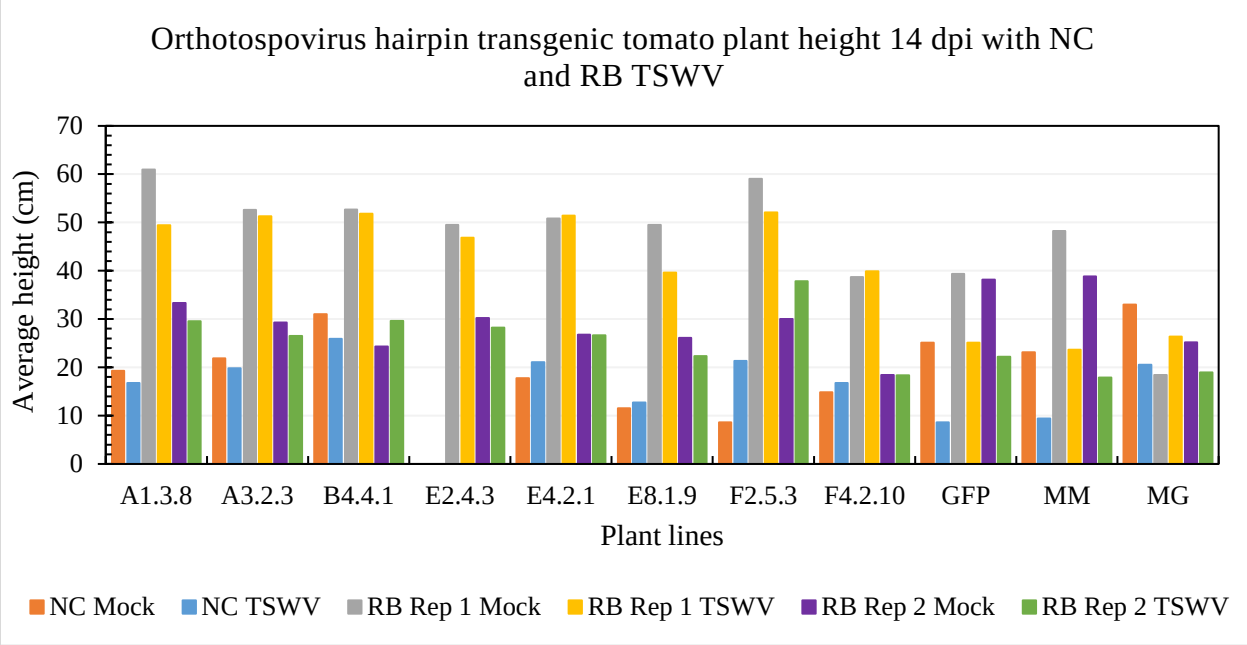
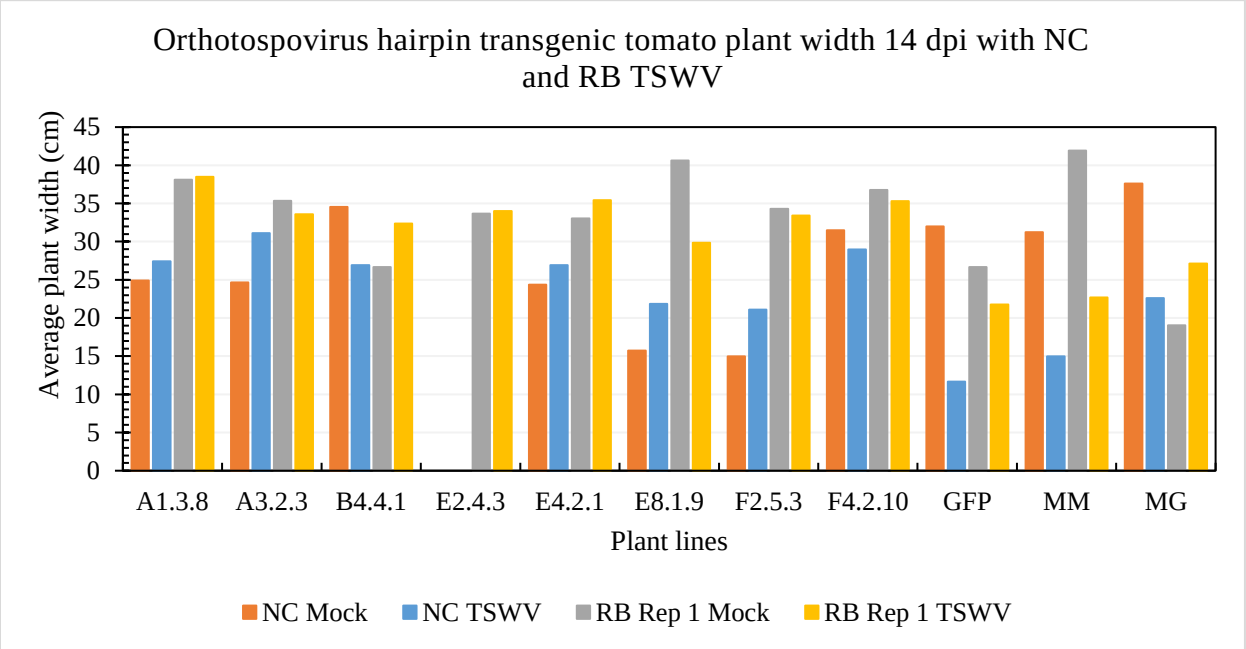


Figure 3.4. NC-TSWV and RB-TSWV infected plant widths. Bar graph was plotted using width measurement averages for all transgenic T2 lines (A1.3.8, A3.2.3, B4.4.1, E2.4.3, E4.2.1, E8.1.9, F2.5.3, and F4.2.10) to show variation between NC-TSWV or RB-TSWV and mock inoculated plants after 14 days post inoculation. Kruskal-Wallis p values 0.0162 and 0.01089 for NC and RB TSWV respectively, indicates significant difference for both with 95% level. Bars show high variation for Money Maker (MM) mock vs TSWV inoculated, while low variation for shGFP (GFP) or Mountain Glory (MG). For the T2 transgenic lines, low to no variation in plant width was observed between inoculated and mock plants.



APPENDICES

Supplementary Material Chapter 2

Supplementary Table S2.1. C7C-peptide alignment to BLASTP sequences for TSWV, orthotospoviruses and *Bunyavirales.*[3]

Accession no.	Peptide sequence	BLASTP Annotation	Viral Genome segment	E-Value[2]	Species	Blast search under
C7C phage	NSTPGES	Atachment	Gene III	--	M13 Phage Gene III insert	--
QHU78834.1	NSTPG	Nucleocapsid protein	S	6.3	Groundnut bud necrosis virus (GBNV)	Tospoviridae
AAW80651.1	DSTPGSS	Nucleocapsid protein	S	29	Peanut bud necrosis virus (PBNV)	Tospoviridae
QNT38479.1	NSTP	RdRp	L	58	Soybean vein necrosis virus (SVNV)	Tospoviridae
YP_006468898.1	NSTP	L protein	L	58	Bean necrotic mosaic virus (BeNMV)	Tospoviridae
AJT59689.1	TPGE	RdRp	L	58	Groundnut chlorotic fan-spot virus (GCFSV)	Tospoviridae
AJT59689.1	STPG	RdRp	L	121	Groundnut chlorotic fan-spot virus (GCFSV)	Tospoviridae
AWK77935.1	NSTP	RdRp	L	58	Impatiens necrotic spot orthotospovirus (INSV)	Tospoviridae
CBX23841.1	NSTP	NSm	M	26	Tomato spotted wilt virus (TSWV)	TSWV
QED87911.1	PGES	RdRp	L	38	Tomato spotted wilt virus (TSWV)	TSWV
APG79554.1	PGES	glycoprotein precursor	M	38	Tomato spotted wilt virus (TSWV)	TSWV

[3]High E values are the result of short alignments since the chance of these sequences are much higher.

Supplementary Table S2.1. (Continued)

Q9IKB5.1	SQTPG	glycoprotein	M	--	Tomato spotted wilt virus (TSWV)	Direct comparison to GN
AIE16132.1	PGES	glycoprotein	M	548	Crimean-Congo hemorrhagic fever orthonairovirus	Bunyavirales
YP_009361833.1	NSTP	glycoprotein	M	548	Keterrah virus	Bunyavirales
AKC89320.1	TPGE	glycoprotein	M	548	Qalyub orthonairovirus	Bunyavirales
ABV68909.1	NSTP	polyprotein	M	548	Kairi virus	Bunyavirales
YP_009666117.1	STPG	glycoprotein	M	548	Burana virus	Bunyavirales
YP_009666117.1	NSNPG	glycoprotein	M	548	Burana virus	Bunyavirales
ABV68909.1	TPGE	polyprotein	M	548	Kairi virus	Bunyavirales
WP_204103968.1	NSTPGES	CHAT domain-containing protein	--	4480	Spirulina major	--
GFR75741.1	NSTPGES	zinc finger protein 37	--	4471	Elysia marginata	--
XP_030834883.1	NSTPGES	NFkB protein isoform X1	--	4484	Strongylocentrotus purpuratus	--
XP_012943896.1	NSTPGES	diaphanous homolog 2	--	4492	Aplysia californica	--
WP_174353804.1	NSTPGES	ligand-gated channel protein	--	4499	Serratia proteamaculans	--
EIQ74234.1	NSTPGES	tonB-dependent Receptor Plug domain protein	--	4501	Shigella flexneri 1235-66	--

Supplemental Material Chapter 3

Supplementary Table S3.1. Table with significance for RT-qPCR of infected plants compared to sh-GFP (contains a non-resistant hairpin expressing construct).

Plant Line	RB-TSWV Rep 1			RB- TSWV Rep 2		
	Average virus abundance	Number of infected plants	Significance	Average virus abundance	Number of infected plants	Significance
A1.3.8	0.921	3	0.67	0.000	0	NA
A3.2.3	0.000	0	NA	0.000	0	NA
B4.4.1	0.000	0	NA	0.000	0	NA
E2.4.3	0.627	1	1	0.000	0	NA
E4.2.1	0.000	0	NA	0.000	0	NA
E8.1.9	16.109	6	0.75	6.124	1	0.889
F2.5.3	0.000	0	NA	0.000	0	NA
F4.2.10	0.162	1	0.67	2.959	2	0.889
shGFP	21.776	8	--	4.571	7	-
MM	138.214	8	0.67	13.994	5	0.082
MG	29.038	8	0.67	0.586	5	0.889

Supplementary Table S3.2. T2 tested lines with the NC-TSWV and RB-TSWV results from DAS-ELISA showing number of infected and uninfected plants, percent of infection and p-values compared to shGFP from Fisher's exact test pairwise comparison. The percent of infection and significance values are plotted on *figure 3.1*.

Measurement	Height (cm)						Width (cm)			
Plant Line	NC Mock	NC TSWV	RB Mock Rep 1	RB TSWV Rep 1	RB Mock Rep 2	RB TSWV Rep 2	NC Mock	NC TSWV	RB Mock Rep 1	RB TSWV Rep 1
A1.3.8	19.3	16.8	61.0	49.4	33.3	29.5	24.9	27.4	38.1	38.5
A3.2.3	21.8	19.8	52.6	51.3	29.3	26.5	24.6	31.1	35.3	33.6
B4.4.1	31.0	25.9	52.7	51.8	24.3	29.6	34.5	26.9	26.7	32.4
E2.4.3	NA	NA	49.5	46.8	30.2	28.3	NA	NA	33.7	34.0
E4.2.1	17.8	21.1	50.8	51.4	26.7	26.7	24.4	26.9	33.0	35.4
E8.1.9	11.6	12.7	49.5	39.6	26.1	22.3	15.7	21.8	40.6	29.8
F2.5.3	8.6	21.3	59.1	52.1	30.0	37.9	15.0	21.1	34.3	33.4
F4.2.10	14.9	16.8	38.7	39.9	18.4	18.3	31.5	29.0	36.8	35.3
shGFP	25.1	8.6	39.4	25.1	38.2	22.2	32.0	11.7	26.7	21.7
MM	23.1	9.4	48.3	23.7	38.8	17.9	31.2	15.0	41.9	22.7
MG	33.0	20.6	18.4	26.4	25.2	19.0	37.6	22.6	19.1	27.1

Supplementary Table S3.3. Table with significant differences for plant measurements compared to sh-GFP (contains a non-resistant hairpin expressing construct).

Measurement	Height P-values						Width P-values			
Plant Line	NC Mock to TSWV	NC TSWV to TSWV	RB Mock to TSWV Rep 1	RB TSWV to TSWV Rep 1	RB Mock to TSWV Rep 2	RB TSWV to TSWV Rep 2	NC Mock to TSWV	NC TSWV to TSWV	RB Mock Rep 1	RB TSWV to TSWV Rep 2
A1.3.8	0.790	0.380	0.778	0.035	0.92	0.6	1.000	0.300	1.000	0.140
A3.2.3	0.960	0.150	1.000	0.017	0.57	0.85	0.930	0.300	1.000	0.180
B4.4.1	1.000	0.260	2.000	0.017	0.4	0.57	0.910	0.300	1.000	0.180
E2.4.3	NA	NA	0.974	0.088	0.78	0.6	NA	NA	1.000	0.380
E4.2.1	0.710	0.150	1.000	0.020	0.49	0.59	1.000	0.300	1.000	0.140
E8.1.9	0.960	0.420	1.000	0.392	0.43	1	0.540	0.300	1.000	0.370
F2.5.3	0.310	0.200	0.654	0.014	0.66	0.27	0.710	0.360	1.000	0.140
F4.2.10	1.000	0.710	1.000	0.107	0.22	0.81	1.000	0.300	1.000	0.140
shGFP	0.150	--	0.654	--	--	--	0.300	--	1.000	--
MM	0.170	0.850	0.778	1.000	1	0.6	0.300	0.550	0.900	0.980
MG	0.710	0.310	1.000	1.000	0.43	0.9	0.920	0.300	1.000	1.000