Deciphering the early events in *Sw-5b* NLR-mediated resistance against *Tomato spotted wilt orthotospovirus*

By

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DISSERTATION

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Dissertation Abstract

This dissertation contains the first comprehensive study using RNA-seq to reveal early responses (12, 24, and 72 hours post-inoculation (HPI)) to *Sw-5b*-mediated resistance against *Tomato spotted wilt orthotospovirus* (TSWV) infection. TSWV is a significant challenge to successful tomato production and is spread in nature by a relatively small number of thrips species, especially western flower thrips (WFT), *Frankliniella occidentalis* (Pergande). Investigations to identify TSWV resistance in tomato have been almost entirely conducted using mechanical inoculation with sap prepared from infected tissue. The *Sw-5b* gene encodes a nucleotide-binding leucine-rich repeat (NLR) protein, the most commonly used resistance gene against TSWV in commercial tomato. The mechanism by which the NLR recognizes the TSWV NSm elicitor has been elucidated; however, the downstream cascade of events leading to *Sw-5b* NLR-mediated resistance are not known, nor are differences in downstream defense signaling that could be associated with different methods of inoculation.

In my dissertation, I investigated the early events in *Sw-5b*-mediated resistance, and the potential differences between thrips and mechanical inoculation, by examining the differential expression of genes at 12, 24, and 72 HPI with RNA-seq in the near isogenic lines, *Solanum lycopersicum* 'Santa Clara' (*Sw-5b*-, the TSWV-susceptible line), and *S. lycopersicum* 'CNP-LAM 147' (*Sw-5b*+, the TSWV- resistant line) following TSWV mechanical and thrips inoculation. I found that the *Sw-5b* NLR immune receptor induced earlier transcriptome responses to thrips inoculations (12 and 24 HPI), while transcriptome responses to mechanical inoculation were not detected until 72 HPI. Across the three-time points investigated, 80 and 95 differentially expressed genes (DEGs) were detected with thrips and mechanical inoculations, respectively, most of which were upregulated with both inoculation methods. The majority of

DEGs differed depending on the inoculation method (66 uniquely expressed in mechanically inoculated treatments, and 49 uniquely expressed in thrips inoculated treatments). Only 26 DEGs were shared between the inoculation methods. Examination of the functional categories in which DEGs of interest were classified showed that while thrips and mechanical inoculation resulted in the detection of different DEGs, many DEGs of interest placed into similar functional categories, e.g., pathogenesis-related proteins and cytochrome P450s. At the same time, there were marked differences in the functional categories into which the detected DEGs were placed. Unlike mechanical inoculation, thrips inoculation resulted in DEGs in the NLR and receptor-like kinases and calmodulin-binding protein functional categories. These findings suggest that the inoculation method plays a role in the downstream signaling elicited by activation of *Sw-5b*. These data are the first to show that the TSWV inoculation method results in differences in *Sw-5b*-mediated immune signaling and that the *Sw-5b* NLR-immune receptor induces earlier transcriptome responses to thrips inoculations. Identification of transcriptomic responses at these early time points and with different inoculation methods provides new insight into the components of *Sw-5b*-mediated plant defense pathways. Also, in nature, TSWV is primarily spread by thrips; however, most resistance studies focus their attention on mechanical inoculation. My results support the use of thrips inoculations in studying *Sw-5b* resistance, and suggest that when mechanical inoculation is the sole method used in resistance investigations, genes most likely of greatest importance in nature may be missed.

To examine the functional importance of some of the DEGs detected with RNA-seq, I established a preliminary functional analysis strategy using a *Tobacco rattle virus* based virus-induced gene silencing (VIGS) system, and a transgenic *Sw-5b Nicotiana benthamiana (Nb::Sw-5b).* I successfully used VIGS to silence *Sw-5b* and showed a loss of function resulting in a

phenotype with no HR production and systemic infection. I established that VIGS could be used as a preliminary genomic tool to perform functional assessment of genes that may play an integral role in *Sw-5b*-mediated resistance against TSWV. Based on intensity of fold change, and functional classifications, 15 DEGs were selected for preliminary functional analysis using VIGS. While the outcome of these experiments was not entirely conclusive, results from TRV-based VIGS revealed two DEGs of interest; ERF1, and a receptor-like kinase family protein. The results suggest these two genes are possibly required for *Sw-5b*-mediated resistance against TSWV and are worthy of further investigation. The variability and difficulty in replicating results I encountered using the TRV-based VIGS system, suggests further investigations should consider other methods for screening DEGs of interest, such as CRISPR. Finally, I used TRV-based VIGS to explore the function of SGT1 in *Sw-5b*-mediated resistance by silencing of SGT1. In these experiments, I found that silencing of SGT1 resulted in loss of HR, and systemic infection of plants. These findings show that SGT1 is required for *Sw-5b*-mediated immune signaling. In contrast, silencing of EDS1 resulted in no effect on HR production or resistance to TSWV infection, indicating that like many CC-NLRs, *Sw-5b* does not require EDS1 for immune signaling.

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Chapter 1

Introduction

INTRODUCTION

My dissertation examines early events in resistance against *Tomato spotted wilt orthotospovirus* (TSWV) mediated by the intracellular immune receptor *Sw-5b* in tomato. I have presented my work in three chapters: **Chapter One**: *Introduction*; **Chapter Two**: *The Sw-5b NLR immune receptor induces earlier transcriptome changes in response to thrips-mediated inoculation of Tomato spotted wilt orthotospovirus compared to mechanical inoculation*; and **Chapter Three**: *Preliminary functional analysis of differentially expressed genes during Sw-5b NLR-mediated resistance to Tomato spotted wilt orthotospovirus.*

My specific focus was on revealing and comparing - transcriptomic changes occurring during *Sw-5b*-mediated resistance in the first 72 hours following inoculation of TSWV with the insect vector of the virus, the western flower thrips (WFT), *Frankliniella occidentalis* (Pergande) and mechanical inoculation with sap prepared from TSWV-infected tissue. Upon identifying differentially expressed genes (DEGs) of interest, I focused on functional analysis of a subset of DEGs using VIGS, and *Sw-5b* transgenic *Nicotiana benthamiana.*

In this chapter, I will introduce my research objectives, the importance of my research, the model systems I deployed in my research, and provide a concise review of relevant plant immunity systems.

RESEARCH OBJECTIVES

The experiments in this dissertation were designed to decipher the early signaling events that contribute to *Sw-5b*-mediated resistance in tomato. My goal was to gain insight into the differential expression of genes at early times post-inoculation (12, 24 and 72 hours post inoculation) with sap prepared from TSWV-infected tissue (mechanical inoculation) and TSWV transmitted through thrips. The following were my specific objectives.

1. Optimization and synchronization of mechanical and thrips inoculations. This work was necessary to determine the age of plants to be used in my experiments and establish the best possible synchronicity of infection in the absence of an infectious clone of TSWV, which did not exist at the time of this work.
2. Identify genes that may be involved in the early signaling pathways associated with *Sw-5b*-mediated resistance to TSWV and investigate whether the inoculation method (mechanical vs. thrips) alters timing or type of gene expression. To achieve this objective, RNA-seq was used to compare differential expression of genes at 12, 24, and 72 hours post-TSWV inoculation of tomato *Sw-5b* near isogenic lines, *Solanum lycopersicum* 'Santa Clara' (*Sw-5b-,* the TSWV-susceptible line) and *S. lycopersicum* 'CNP-LAM 147' (*Sw-5b+*, the TSWV-resistant line) (Hallwass et al. 2014). The RNA-seq approach allowed me to decipher differential expression of genes when *Sw-5b* was present, and to elucidate changes in differential gene expression over time post-inoculation in response to the two inoculation methods (mechanical and thrips).
3. Investigate the possible function of DEGs of interest in *Sw-5b*-mediated resistance. A transgenic *Sw-5b N. benthamiana* line provided by Dr. Xiaorang Tao, (Nanjing Agricultural University) was used to perform functional analyses on 15 DEGs of interest (identified from the RNA-seq analysis in objective 2) using VIGS. The DEGs were selected for functional analysis based on their potential role in *Sw-5b*-mediated resistance against TSWV. Following silencing with VIGS, I challenged plants with TSWV MR-01 ($TSWV^{MR-01}$) and recorded phenotypic responses and infection status.

IMPORTANCE OF THE INVESTIGATION

Tomato is the second most important vegetable crop grown worldwide. In many growing regions, TSWV is a significant challenge to successful tomato production (Oliver and Whitfield 2016; Zhu et al. 2019). Resistance mediated by *Sw-5b* has been a critical component of many management programs seeking to limit losses due to TSWV epidemics; however, resistance-breaking isolates are challenging the durability of this important gene (Batuman et al. 2016; Kabaş et al. 2021; Batuman et al. 2020). Hence, the information gained in this dissertation will be important to future researchers seeking to preserve and/or enhance *Sw-5b* resistance and may guide those seeking new resistance mechanisms. My research will also expand our knowledge regarding the mechanisms underlying resistance mediated by the intracellular immune receptor *Sw-5b* in tomato.

Investigations of TSWV resistance and host defense genes in tomato have primarily relied on mechanical inoculation (Padmanabhan et al. 2019; Ramesh et al. 2017a), even though the primary means of spread of the virus in nature is by thrips (Bautista 1993; Oliver and Whitfield 2016). Furthermore, with regards to *Sw-5b*-mediated resistance against TSWV, there are no transcriptomic studies exploring gene expression during the first 72 hours post-inoculation. Not only is this project the first to attempt to decipher early responses to infection in *Sw-5b*-mediated resistance, but it is also the first to show that thrips inoculation results in expression of host defense genes earlier than mechanical inoculation. This is important for many reasons, especially because plants in the field are inoculated with TSWV by thrips.

RESEARCH SYSTEM

The pathosystem underlying the model system I utilized in my research includes $TSWV^{MR-01}$, its primary insect vector (WFT), and tomato *Sw-5b* near isogenic lines, *S.*

lycopersicum ‘Santa Clara’ (*Sw-5b-,* the TSWV-susceptible line) and *S. lycopersicum* ‘CNP-LAM 147’ (*Sw-5b+*, the TSWV-resistant line) (Hallwass et al. 2014). In the following, I describe each component of this pathosystem in more detail.

Tomato spotted wilt orthotospovirus

Tomato spotted wilt orthotospovirus (order Bunyavirales, family *Tospoviridae*, genus *Orthotospovirus*) causes one of the most damaging viral diseases affecting tomato production worldwide (Pappu et al. 2009b; Whalen 2005). In 1915, tomato spotted wilt disease was first described in Australia, and TSWV was described as the causal agent in 1930 (Samuel et al. 1930; Brittlebank 1919). Viruses in the family *Tospoviridae* are tripartite, single-stranded, negative-sense RNA viruses. Although TSWV is classified as a negative sense virus, two of the RNA segments are ambisense (de Haan et al. 1990; Kormelink et al. 1992a). TSWV virions are spherical, measure 80 -120 nm, and are thought to package the three RNA segments that are named: small, 2.9 kb (S RNA); medium, 4.8 kb (M RNA); and, large, 8.9 kb (L RNA). In addition, several RNA dependent RNA polymerase molecules (RdRp) are packaged within the envelope that is derived from host membranes (Oliver and Whitfield 2016). Each RNA segment is coated by nucleocapsid (N) protein molecules forming ribonucleoprotein (RNP) complexes. The L RNA encodes the RdRp in the viral complementary sense (De Haan et al. 1991). The S RNA and M RNA are ambisense, each with two open reading frames. The S RNA encodes a nonstructural protein (NSs) in the viral complementary sense, and the N protein in the viral sense (De Haan et al. 1991). The NSs protein likely has multiple functions, one being as a silencing suppressor (Takeda et al. 2002). The M RNA segment encodes a nonstructural movement protein (NSm) in the viral complementary sense, and the polyprotein precursor to two glycoproteins (G_n and G_c) in the viral sense (Kormelink et al. 1994; Kormelink et al. 1992a)). This polyprotein is

cleaved by a host factor, likely a signal sequence peptidase, generating the two mature glycoproteins, which are post-translationally glycosylated. These are named G_n and G_c because they correspond to the N- and C- termini of the polyprotein, respectively (Kormelink et al. 1992a; Adkins et al. 1996; Löber et al. 2001; Bergeron et al. 2007; Whitfield 2004). Notably, expression of viral genes is via sub-genomic messenger RNAs initiated via cap-snatching (Kormelink et al. 1992b; Kormelink et al. 2011).

The RNA segments have highly conserved 3' and inverted 5' ends that hybridize within the virion, creating a panhandle-like structure and giving the RNPs a circular appearance (Kellman J.F 2001). Numerous studies suggest that there are distinct similarities between insect and plant host cells with regards to particle morphogenesis and viral protein interactions (Snippe et al. 2005, 2007; Kikkert et al. 2001; Ullman et al. 1995a). Of particular importance to my work, the two nonstructural proteins, NSm and NSs are only present after the virus replicates

The TSWV isolate I used in my research ($TSWV^{MR-01}$), was collected for me in Monterey County, California in 2015 from radicchio, sometimes known as chicory, *Cichorium intybus,* by Farm Advisor Steve Koike, (University of California). I provided the isolate to colleagues, and the full genome was determined and a phylogenetic analysis was performed with other isolates from multiple geographic locations (Adegbola,R.O., Adkins,S.T. and Naidu,R.A., unpublished data). Complete sequences of the three RNAs can be found in NCBI (https://www.ncbi.nlm.nih.gov/nuccore) using these accession numbers: S RNA MG593199; M MG593198; and L RNA MG593197. The L-RNA is 8,920 nucleotides long, the M-RNA is 4,770 nucleotide and the S-RNA is 2,927 nucleotides long. Phylogenetic analysis showed that the S RNA and L RNA clustered with Asian type TSWV isolates, while the M RNA clustered with American type isolates (Adegbola,R.O., Adkins,S.T. and Naidu,R.A., unpublished data).

Western flower thrips, Frankliniella occidentalis

Tomato spotted wilt orthotospovirus is transmitted by the WFT, *F. occidentalis*, as well as at least nine additional thrips species (Montero-Astúa et al. 2016b). The WFT colony used in my investigations has been maintained in the Ullman Laboratory since 1995. It originated from the Kamilo Iki Valley on the Hawaiian island of O'ahu (Bautista 1993). This colony of WFT was also used to create the WFT lines used to produce the first Thysanopteran genome (Rotenberg et al. 2020), providing an important tool to better investigate specific interactions between this important insect, its plant hosts, and the viruses it transmits.

Western flower thrips earned the title *supervector* (Gilbertson et al. 2015) as the principal vector of multiple orthotospoviruses as well as other viruses, likely due to their wide distribution globally, high reproductive rates, very large plant host range, and propensity to develop resistance to pesticides. When WFT feed, they use their stylets to pierce plant cells, inject saliva, and suck out cytoplasm, thus having a significant interaction with the plant and plant defenses (Hunter and Ullman 1989, 1992; Hunter et al. 1994; Ullman et al. 1989; Ullman et al. 1992; Stafford et al. 2011; Stafford et al. 2012; Montero-Astúa et al. 2016a; Montero-Astúa et al. 2016b; Kumar et al. 1995). The WFT transmits orthotospoviruses in a circulative-propagative manner, and the viruses ultimately invade and replicate in the foregut, midgut, tubular, and principal salivary glands (TSG, PSG, respectively) of the vector, after which the insect injects the virus with its saliva during feeding (Ullman et al. 1993a; Ullman et al. 1993b; Ullman et al. 1995b; Montero-Astúa et al. 2016b; Rajarapu et al. 2021). Only larvae acquire virus; however, inoculation of a new host requires PSG infection that only occurs in the late second larval or adult stage (Rajarapu et al. 2021). Where as first instar larvae are key to virus epidemiology because they are most efficient in acquiring TSWV, infected adults are most significant in virus

spread because they are winged and mobile, and larvae are not (Montero-Astúa et al. 2016b). For this reason, my investigation focused on adult WFT. Male and female adult WFT are known to differ in their ability to inoculate plants, therefore, my inoculations included an equal number of each sex.

Pesticide use is expensive and can have significant detrimental impacts on environmental and human health. Crop growers apply over 500 million pounds of pesticide annually in the US, costing $842/acre in conventional tomato production (Fernandez-Cornejo et al. 2014). An estimated 20,000 pesticide poisonings occur yearly in the US and pesticide exposure is associated with increased risk and incidence of various cancers (Alavanja et al. 2004; Beane Freeman et al. 2005; Engel et al. 2005; Purdue et al. 2007). Many producers recognize that alternatives to pesticides are needed to protect the environment and human health, and that the most promising strategy is host plant resistance and integrated pest management (IPM). The gene that my investigations focus upon, *Sw-5b*, mediates the most effective known resistance against TSWV. However, prior to my investigations, the response of *Sw-5b* to thrips-mediated TSWV inoculation has not been investigated.

Plant System: TSWV Resistance Genes, and Tomato (S. lycopersicum) isogenic lines 'CNPH-LAM 147' (Sw-5b+) and 'Santa Clara' (Sw-5b-)

Several TSWV resistance genes (*Sw-1a, Sw-1b, Sw-2, Sw-3, Sw-4, Sw-5, Sw-6,* and *Sw-7*) have been described (Brommonschenkel et al. 2000; Spassova et al. 2001). *Sw-5*, a single dominant resistance gene locus, was introgressed from *Lycopersicum peruvianum* into a commercial tomato line named 'Stevens' (Stevens 1964) and mapped to the gene locus to the end of chromosome nine (Stevens et al. 1992). The *Sw-5* gene locus consists of five paralogs referred to as the *Sw-5* gene cluster (Spassova et al. 2001; de Oliveira et al. 2018). However, in

reference to resistance against TSWV, this cluster has been commonly been referred to simply as *Sw-5*. Several studies of *Sw-5a* and *Sw-5b* revealed that *Sw-5b* is the functional gene copy that mediates broad-spectrum resistance against all tested American-type orthotospoviruses (Spassova et al. 2001; Hallwass et al. 2014; de Oliveira et al. 2018; Zhu et al. 2017a). While *Sw-5a* does not mediate resistance against TSWV, a recent study revealed that it is involved in functional recognition of the AC4 effector mediating hypersensitive response and resistance against *Tomato leaf curl New Delhi virus* (Sharma et al. 2021). The *Sw-5*+ near isogenic lines used in my investigations, 'CNPH-LAM 147', has *Sw-5*a and *Sw-5b* gene copies; however, based on the findings just described, we can assume that *Sw-5b* is the gene copy mediating TSWV resistance. Throughout this dissertation, I will refer specifically to *Sw-5b*, when referring to my findings with 'CNPH-LAM 147', and when citing work prior to the discovery of the functionality of *Sw-5b.*

In my investigations, I used the near isogenic lines 'CNPH-LAM 147' *(Sw-5b+)* and 'Santa Clara' *(Sw-5b-),* with and without the *Sw-5b* gene. The *Sw-5b* near isogenic line used were obtained from Leonardo Boiteaux (Centro Nacional de Pesquisa de Hortaliças (CNPH)/EMBRAPA, CP 0218, 70359-970, Brasilia, DF, Brazil. These near-isogenic lines will be referred to as the susceptible (*S. lycopersicum* 'Santa Clara') or resistant line (*S. lycopersicum* 'CNPH-LAM 147'), respectively, throughout.

The *Sw-5b* gene encodes a coil-coil-(CC) domain-nucleotide binding oligomerization domain-like receptors (NLR) protein (Brommonschenkel, SH et al. 2000, Spassova et al. 2000). In addition, *Sw-5b* contains a N-terminal Solanaceae domain (SD) that is only present in some CC-NLRs from solanaceous species (Chen et al. 2016). Interestingly, *Sw-5b* is most similar to tomato gene *Mi*, *1.2*, which is a CC-NLR protein, that confers resistance to root-knot nematode,

psyllids, whiteflies, and aphids (Casteel et al. 2006; Vos et al. 1999; Milligan et al. 1998; Nombela et al. 2003). Other examples of this type of gene include the tomato *Prf* gene that confers resistance to bacterial pathogen and potato *Rx* that confers resistance to *Potato virus X* (Salmeron et al. 1996; Grant et al. 1995; Milligan et al. 1998; Rouppe van der Voort et al. 1999; Brommonschenkel et al. 2000).

Unlike most NLRs, *Sw-5b* confers broad-spectrum resistance to all tested American-type orthotospoviruses (Zhu et al. 2017b; De Oliveira et al. 2016). The *Sw-5b* NLR recognizes the movement protein, NSm, of TSWV and induces a localized cell death response called the hypersensitive response (HR) at the site of infection to limit virus spread (López et al. 2011; Peiró et al. 2014; Hallwass et al. 2014). Because NSm is a nonstructural protein, the virus must replicate before this protein is expressed. *Sw-5b* specifically recognizes a conserved 21 amino acid motif (NSm^{21}). *Sw-5b* uses a two-step recognition mechanism to induce a robust immune response against TSWV (Li et al. 2019). In addition to the LRR domain recognizing NSm and NSm^{21}, the N-terminal SD also directly interacts with NSm, and this interaction is critical for recognition. In the absence of TSWV NSm, the CC domain keeps the NB-LRR region of *Sw-5b* in an autoinhibited state to avoid induction of an autoimmune response (Chen et al. 2016). The recognition of NSm by SD releases the autoinhibition state leading to activation of the NB-LRR region, and the induction of HR cell death. The SD also facilitates recognition of low levels of NSm by NB-LRR to activate immunity. Therefore, *Sw-5b* uses two domains to perceive the viral effector NSm, and to activate a robust defense against TSWV (Li et al. 2019). While *Sw-5b* has provided effective resistance against TSWV in tomato for many years, resistance breaking isolates have been identified in many locations due to an amino acid substitution of either C118Y or T120N in the NSm protein (López et al. 2011; Batuman et al. 2016).

Despite the widespread use of *Sw-5b*, we know very little about signaling components and mechanisms underlying *Sw-5b*-mediated resistance. Furthermore, next-generation sequencing has not been used to explore the early events in *Sw-5b*-mediated resistance against orthotospoviruses. Aside from the work I present in this dissertation, the only other transcriptomic studies of TSWV resistance genes focus on *Sw-7*-mediated resistance against TSWV. These studies compared susceptible and resistant lines and identified differences in gene expression among host defense-related genes e.g., those encoding protein kinases, phytohormone signaling, transcription factors, and cell-wall related genes (Campos et al. 2021; Padmanabhan et al. 2019). Whereas a specific host resistance gene was not investigated, another transcriptomic analysis identified host genes targeted by TSWV-derived small interfering RNAs (Ramesh et al. 2017b), albeit not at early times following inoculation. Importantly, in all of the previously mentioned studies, the mode of inoculation was mechanical or rub inoculation with sap from infected plant tissue (Ramesh et al. 2017b; Padmanabhan et al. 2019). This investigation is the first transcriptomic study to focus on identifying the early events in *Sw-5b*-mediated resistance, and to compare differential expression of genes in response to mechanical and thrips inoculation of an orthotospovirus.

Functional Analyses with VIGS Systems

VIGS uses the natural defense mechanism that plants evolved to eliminate aberrant RNAs of transposable elements and viruses by post-transcriptional gene silencing (PTGS) (Baulcombe 1999; Robertson 2004; Becker and Lange 2010). VIGS has become an efficient genomic tool used to silence genes of interest in order to conduct rapid functional studies. This technique involves using a virus vector to express a double-stranded (ds)RNA portion of the gene of interest, which is then triggers silencing by the plant (Baulcombe 1999). Several viruses have

been utilized as vectors, such as *Tobacco mosaic virus* (TMV), *Potato virus X,* and *Tobacco rattle virus* (TRV*)* (Ratcliff et al. 2001; Liu et al. 2002; Robertson 2004; Lacomme and Chapman 2008). Functional assessment can be identified by either a loss of function or change in plant phenotype. As an example of how VIGS functions, TMV was one of the first VIGS virus vectors used to silence the *phytoene desaturase* (*PDS*) gene in *N. benthamiana* (Kumagai et al. 1995; Shi et al. 2021). *Nicotiana benthamiana* is often used in functional analyses as a model system because it is easily and quickly grown, and readily infected with viral vectors. When plants were infected with TMV engineered with a *PDS* sequence, the virus replicated and produced dsRNA. The dsRNA is digested by dicer-like proteins into small dsRNA targeting *PDS* mRNA for destruction. The silencing of *PDS* is easy to observe because a photobleached plant phenotype occurs (Zhang et al. 2014; Waterhouse et al. 2001). Due to the limited host range of some virus vectors, TRV has become a widely used vector for VIGS studies (reviewed in (Shi et al. 2021). The TRV-based VIGS system has been easily modified and suitable for silencing of many genes in tomato (Liu et al. 2002). My functional analyses of DEGs of interest were conducted using a TRV-based VIGS system developed by laboratory of Dinesh Kumar (Liu et al. 2002). Silencing of the *PDS* gene was used as a visual indication that silencing was successful in each VIGS experiment. My analyses were aimed at understanding the function of DEGs of interest in *Sw-5b*-mediated resistance. Since *N. benthamiana* can be quickly grown and is amenable to gene silencing with VIGS, the availability of transgenic *Sw-5b N. benthamiana* allowed us to perform rapid screening for the selected DEGs.

Tomato Genome

The tomato genome was first published in 2012 and is continuously updated. This genome is well annotated and readily accessible (Tomato Genome Consortium 2012). The

availability of the SL4.0 version and the International Tomato Genome Sequencing Project 4.0 annotation allowed me to explore the nature of the genes that were differentially expressed following mechanical and thrips inoculation of TSWV.

General Comments on Plant Immunity

Plant immunity is dependent on both membrane-localized pattern recognition receptors (PRRs) and intracellular nucleotide-binding oligomerization domain-like receptors (NLRs) (Dangl and Jones 2001a). The PRRs often include receptors, e.g., receptor kinases, that recognize pathogen-associated molecular patterns (PAMPs), e.g., flagellin, resulting in PAMP-immunity (PTI) that is often broad-spectrum and not particularly strong (Couto and Zipfel 2016; Wu et al. 2017). Many pathogens deliver effectors to induce disease and suppress defense responses. Plants use intracellular NLR receptors to recognize specific pathogen effectors and induce effector-triggered immunity (ETI) (Jones et al. 2016). Effector trigger immunity (ETI) is more robust than PTI and often leads to programmed cell death (PCD), and a hypersensitive response (HR) at the site of pathogen infection (Cui et al. 2015; Bendahmane et al. 1999).

Nucleotide-binding oligomerization domain-like receptors contain a centrally located nucleotide-binding domain (NB) and a leucine-rich repeat (LRR) domain at the C-terminus (Dangl and Jones, 2006). Generally, NLRs also possess a toll-interleukin-1 homology domain (TIR) or coil-coiled (CC) domain at the N-terminus. Direct recognition of the pathogens via the NLR occurs when the pathogen effector binds directly to the NLR (Dangl and Jones 2001b). Indirect recognition by NLRs is more common and can be described by the “guardee” or “decoy” models (Cesari et al. 2014). In the “guardee" model, the pathogen effector alters another plant protein, and the NLR recognizes this modification (Dangl and Jones 2001b). In the

"decoy" model, the pathogen effector interacts with a plant decoy protein and the NLR recognizes this interaction resulting in resistance (van der Hoorn and Kamoun 2008).

MANAGEMENT OF TSWV

Management of TSWV in food, forage, and horticultural crops is complicated for several reasons. First, the virus has an extensive host range exceeding 1,090 plant species (Pappu et al. 2009a; Parrella et al. 2003), as does its principal vector, the WFT, with a host range exceeding 1200 plant species (Lewis 1973). Because TSWV can replicate both in the plant and the insect vector, and the insects are ubiquitous, insecticides are widely used to manage TSWV spread. For example, plants have been drenched with neonicotinoids as a mode of protection for short-duration crops (Coutts and Jones 2005; Bielza 2008). Spinosad initially had high efficacy against WFT, although its widespread use led to thrips resistance (Bielza 2008; Bielza et al. 2007). Indeed, a major obstacle in controlling thrips populations is their propensity to rapidly develop insecticide resistance. This trait may have a basis in WFT physiology (Rotenberg et al. 2020), and also occur due to the WFT's short generation time, high fecundity, and haplodiploid reproductive system (Jensen 2000; Bielza 2008). Therefore, the management of WFT and its transmission of TSWV requires an integrated pest management (IPM) program. Results from a long-term epidemiology study tracking TSWV spread and thrips populations in Central California, resulted in implementing an IPM strategy to manage TSWV infections specific for processing tomatoes (Batuman et al. 2020). This IPM strategy recommends avoiding planting near bridge crops, controlling weeds and volunteers, reducing thrips inoculum sources, monitoring thrips and TSWV, and planting TSWV-resistant tomato varieties (Batuman et al. 2020). In addition, producers are recommended to use a field risk index and thrips projections from a degree day model to make decisions about when to spray for thrips (Batuman et al. 2013).

Several models to predict and make decisions about control of TSWV and thrips vectors have also been developed for peanut in the southeastern USA, in particular Georgia and North Carolina (Chappell et al. 2020; Srinivasan et al. 2017; Fulmer et al. 2019). Host plant resistance is also used when it is available; however, the only successful, widely used resistance gene in tomato is *Sw-5b* and resistance breaking isolates threaten the sustainability of this gene (Batuman et al. 2016)

Conclusions:

In conclusion, this dissertation describes my findings, which are the first to reveal differentially expressed genes during early responses (12, 24, and 72 hours post-inoculation) to *Sw-5b*-mediated resistance against TSWV infection. In addition to providing a comprehensive list of genes that are differentially expressed with different inoculation methods, I have shown that thrips inoculation results in earlier differential expression of genes than mechanical inoculation. Furthermore, the results revealed which DEGs occur in response to thrips and mechanical inoculations, and how inoculation methods differed with regard to the DEGs detected. My functional analysis of a subset of DEGs of interest with VIGs provided preliminary information about DEGs produced interesting phenotypes and should be pursued in the future with gene manipulation strategies

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A modified version of the following chapter will be submitted for publication with the target journal as Molecular Plant-Microbe Interactions

Chapter 2

The *Sw-5b* NLR immune receptor induces earlier transcriptome changes in response to thrips-mediated inoculation of *Tomato spotted wilt orthotospovirus* compared to mechanical inoculation

Abstract:

Tomato spotted wilt orthotospovirus (TSWV) is one of the most damaging plant viruses affecting tomato production worldwide. TSWV is spread in nature primarily by the western flower thrips (WFT), *Frankliniella occidentalis*, but investigations to identify TSWV resistance in tomato have been almost entirely conducted using mechanical inoculation with sap prepared from infected plant tissue. The *Sw-5b* gene is a single dominant resistance gene that encodes a nucleotide-binding leucine-rich repeat (NLR) protein. This is the most commonly used resistance gene against TSWV in commercial tomato cultivars. The *Sw-5b* NLR is known to recognize the TSWV movement protein (NSm); however, the downstream cascade of events leading to *Sw-5b* NLR-mediated resistance are not known, nor are differences in downstream defense signaling that could be associated with different methods of inoculation. In this study, we investigated the early events in *Sw-5b*-mediated resistance, as well as differences between thrips and mechanical inoculation, by examining the differential expression of genes at 12, 24, and 72 hours post-inoculation (HPI). This was accomplished with RNA-seq in the *Sw-5b* near isogenic lines, *Solanum lycopersicum* 'Santa Clara' (*Sw-5b*$^{-}$, the TSWV-susceptible line) and *S. lycopersicum* 'CNP-LAM 147' (*Sw-5b*$^{+}$, the TSWV- resistant line) following TSWV mechanical and thrips inoculation. We found that the *Sw-5b* NLR immune receptor induced earlier transcriptome responses to thrips inoculations (12 and 24 HPI), whereas transcriptome responses to mechanical inoculation did not appear until 72 HPI. Across the three-time points investigated, we detected 80 and 95 differentially expressed genes (DEGs) with thrips and mechanical inoculations, respectively; most of which were upregulated regardless of inoculation method. Among these DEGs, only 26 were detected with both inoculation methods, whereas the majority of DEGs were associated specifically with an inoculation method (66 DEGs were specific to

mechanically inoculated treatments, and 49 were specific to thrips inoculated treatments). Examination of the functional categories in which DEGs of interest were classified showed that while thrips and mechanical inoculation resulted in detection of different DEGs, many DEGs of interest were grouped in similar functional categories, e.g., pathogenesis-related proteins and cytochrome P450s. At the same time, there were marked differences between the inoculation methods with regards to the functional categories into which the detected DEGs were placed. Specifically, thrips inoculation resulted in upregulation of DEGs in the NLR and receptor-like kinases and calmodulin-binding protein functional categories, while none of the DEGs detected in mechanically inoculated treatments were within these functional categories. These findings suggest that inoculation method plays a role in the downstream signaling elicited by activation of *Sw-5b*. Our findings are the first to show that the TSWV inoculation method results in differences in *Sw-5b*-mediated immune signaling and that the *Sw-5b* NLR immune receptor induces earlier transcriptome responses to thrips-mediated TSWV inoculations. Our identification of transcriptomic responses at these early time points and with different inoculation methods provides new insight into the components of *Sw-5b*-mediated plant defense pathways.

Introduction

Tomato spotted wilt orthotospovirus (TSWV) is one of the most economically important plant viruses affecting tomato production worldwide (Zhu et al. 2019; Oliver and Whitfield 2016). TSWV is the type member of the genus *Orthotospovirus*, family *Tospoviridae*, order *Bunyavirales*, and is characterized by a single-stranded negative-sense RNA genome composed of three RNA segments enclosed in a host-derived virion envelope with two embedded glycoproteins (Oliver and Whitfield 2016). TSWV can be transmitted mechanically with infected sap (Rotenberg et al. 2015); however, in nature, the western flower thrips (WFT), *Frankliniella*

occidentalis (Pergande) primarily transmits orthotospoviruses. Transmission by the insect is in a circulative-propagative manner, and the viruses ultimately invade and replicate in the foregut, midgut, tubular, and principal salivary glands of the vector, from which they are injected into plants during feeding (Ullman et al. 1993a; Ullman et al. 1993b; Ullman et al. 1995; Montero-Astúa et al. 2016; Whitfield et al. 2005). An essential component of any integrated pest management strategy is using host plant resistance when it is available; however, *Sw-5b*-mediated resistance is virtually the only effective source of resistance against TSWV in tomato and is widely deployed globally (López et al. 2011; Oliver and Whitfield 2016; Pappu et al. 2009). Unfortunately, *Sw-5b* resistance-breaking orthotospovirus isolates are arising in many locations, challenging the durability of this important resistance gene (Batuman et al. 2016; Ciuffo et al. 2005; López et al. 2011).

Several TSWV resistance genes (*Sw-1a, Sw-1b, Sw-2, Sw-3, Sw-4, Sw-5, Sw-6,* and *Sw-7*) have been described (Brommonschenkel et al. 2000; Spassova et al. 2001). With regards to orthotospovirus resistance, the most efficacious among them is *Sw-5*, a single dominant resistance gene locus that was introgressed from *Lycopersicum peruvianum* to a commercial tomato line 'Stevens' (Stevens 1964). The gene was mapped to the end of chromosome nine (Brommonschenkel et al. 2000; Abe et al. 2011; de Oliveira et al. 2018). Within the *Sw-5* gene cluster, *Sw-5a* has been shown to be nonfunctional against TSWV (De Oliveira et al. 2016; Spassova et al. 2001; Hallwass et al. 2014), but was recently shown to provide resistance against the geminivirus, *Tomato leaf curl New Delhi virus*, by recognizing an AC4 protein (Sharma et al. 2021). *Sw-5b* has been shown to mediate resistance against TSWV, and several other orthotospoviruses (De Oliveira et al. 2016; de Oliveira et al. 2018). The *Sw-5b* gene encodes a coiled-coil nucleotide-binding leucine-rich repeat receptor (CC-NLR) class of immune receptor

(Brommonschenkel, Frary et al. 2000). In addition, *Sw-5b* contains an extended N-terminal Solanaceae domain (SD) that is only present in some CC-NLRs from solanaceous species (Chen et al. 2016).

The *Sw-5b* NLR recognizes the movement protein, NSm, of TSWV and induces a localized cell death response called the hypersensitive response (HR) at the site of infection to limit virus spread (López et al. 2011; Peiró et al. 2014; Hallwass et al. 2014). The *Sw-5b* NLR specifically recognizes a conserved 21 amino acid motif (NSm^{21}), and confers resistance against most of the American-type orthotospoviruses (Zhu et al. 2017). Interestingly, *Sw-5b* adopts a unique two-step recognition mechanism to induce a robust immune response against TSWV (Li et al. 2019a). In addition to the LRR domain recognizing NSm and NSm^{21}, the N-terminal SD also directly interacts with NSm, and this interaction is critical for recognition. In the absence of TSWV NSm, the CC domain keeps the NB-LRR region of *Sw-5b* in an autoinhibited state to avoid induction of an autoimmune response (Chen et al. 2016). The recognition of NSm by SD releases the autoinhibition state leading to activation of the NB-LRR region, and induction of HR cell death. The SD also facilitates recognition of low levels of NSm by NB-LRR to activate immunity. Therefore, *Sw-5b* uses two domains to perceive the viral effector NSm, and to activate a robust defense against TSWV (Li et al. 2019a).

Although the recent studies cited in the previous paragraph provided some insights on how *Sw-5b* NLR recognizes viral effector NSm, we know very little about players involved immediately downstream of effector recognition, especially the transcriptional changes that activate immune responses leading to cell death and containment of virus to the infection site. There is no report of gene expression changes that occur in *Sw-5b* plants in response to TSWV infection. Although gene expression changes that occur in *Sw-7* containing tomato line have been

reported in response to mechanical inoculation of TSWV, the differentially regulated genes were identified from newly emerged leaves at 4 to 35 days post virus inoculation (Padmanabhan et al. 2019). Because in resistant *Sw-7* lines, TSWV is limited to the infection site in the inoculated leaves, the response that occurs systemically is due to a later response by the resistant plant or possibly systemic acquired resistance (SAR) (Qi et al. 2021). Furthermore, there are no reports of *Sw-5b* or *Sw-7*-mediated transcriptional responses when TSWV is delivered via thrips inoculation. This is an important gap in our knowledge considering thrips-mediated transmission is how the virus spreads in nature (Oliver and Whitfield 2016; Gilbertson et al. 2015; Ben-Mahmoud et al. 2019).

Therefore, we investigated the transcriptional changes that occurred early, within the first 72 hours post inoculation, during *Sw-5b* NLR recognition of TSWV in tomato when infected with TSWV via thrips and compared it with TSWV infection through mechanical inoculation. We found that the *Sw-5b* NLR immune receptor induces earlier transcriptome changes in response to thrips-mediated inoculation of TSWV compared to mechanical inoculation and identified genes that are differentially regulated at these early time points with both methods of inoculation. Our findings provide new insight into candidate genes that could play a role in *Sw-5b*-mediated resistance to TSWV and highlight the importance of understanding the *Sw-5b*-mediated signaling pathway when activated by thrips inoculation.

Results and Discussion

Optimization of TSWV inoculation and tissue collection for RNA-seq experiments

We used the near-isogenic tomato lines, 'Santa Clara' (*Sw-5b*$^{-}$, the TSWV-susceptible line) and 'CNPH-LAM 147' with *Sw-5* (*Sw-5b*$^{+}$, the TSWV-resistant line) (Hallwass et al. 2014) in experiments described herein. In order to investigate the *Sw-5b* response to TSWV infection, it

is important to have a robust infection. Therefore, we first optimized the developmental stage (DS) of plants that would best support a robust virus infection using susceptible 'Celebrity' tomato plants, and the MR-01 strain of TSWV ($TSWV^{MR-01}$). To achieve this, we rub inoculated a known amount of sap prepared from $TSWV^{MR-01}$ infected tissue (hereafter referred to as mechanical inoculation) onto true leaves of tomato plants at the 2, 3, 4, 5, 6, and 7 leaf stage (DS2 to DS7) (Figure 2.1A). Our results showed that nearly 100% of DS2 – DS4 stage susceptible tomato 'Celebrity' plants were systemically infected as measured by enzyme linked-immunosorbent assay (ELISA) and developed symptoms by 12 days post-inoculation (Figures 2.2A, 2.2B). We observed that beyond DS 4, symptoms of $TSWV^{MR-01}$ infection decreased as the developmental stage increased (Figure 2.2A, 2.2B).

Because of the robust infections we observed and the ideal size of the plants for our cages (Figure 2.1B) and growth chamber, we used DS2 stage 'Santa Clara' and 'CNPH-LAM 147' plants for thrips and mechanical inoculations with $TSWV^{MR-01}$ using our optimized conditions (see methods section for details). We collected the two inoculated true leaves from each plant at 0, 12, 24 and 72-hours post-inoculation from three biological replicates (Figure 2.1C). Total RNA was extracted, and RNA-seq libraries were prepared. Illumina sequencing was performed as described in the methods section.

Plant developmental stage and symptom expression are correlated

As stated earlier, we based our decision to use developmental stage (DS) 2 stage plants in our RNA-seq experiments on the robust infections and size of plants at this stage of development. We were also interested in whether plant developmental stage affected symptom expression in infected plants. We observed that symptoms of $TSWV^{MR-01}$ infection decreased as the developmental stage inoculated increased (Figure 2.2B), and regression analysis showed a

positive and statistically significant (p=0.0004) correlation between DS and symptom expression (Figure 2.2B). While symptom expression typically revealed the infection status of DS 2-5 plants; notably, symptom expression did not always reflect the infection status of DS 6 and 7 plants. We observed that 47% and 83% of the plants at these DS6 and 7 respectively, were non-symptomatic, but infected (Figure 2.2B).

Overview of differentially expressed genes in *Sw-5b* plants in response to TSWV infection

A total of 1.1 billion 50 bp reads were generated using the Illumina sequencing platform. The number of reads per sample ranged from 5 – 30 million. After quality control and filtering low quality reads, 84% of the reads (843,811,642) were mapped to the reference tomato cDNA sequences (version ITAG4.1; https://solgenomics.net/). The mapped reads ranged from 4.5 – 25 million reads per sample. The general linear model of the edgeR package in R was used to identify the differentially expressed genes (DEGs) in the resistant compared to the susceptible genotype and for each inoculation method and time point. Mock inoculation with sap prepared from non-infected plants or non-infected thrips were included as experimental controls. These controls were used to observe base gene expression and identify whether any DEGs from TSWV inoculated treatments were being expressed in the mock inoculations with sap prepared from non-infected plants or inoculation with non-infected thrips. These comparisons allowed removal of any DEGs activated by mechanical wounding and non-infected thrips feeding damage from our analysis. Among these experimental controls, we found no differences between resistant and susceptible lines using the general linear model. Consequently, the differences in gene expression we detected in the resistant line are specifically due to the interaction between TSWV and *Sw-5b*. Genes with more than two-fold log expression difference (false discovery rate < 0.05) were considered significantly different.

Our RNA-seq studies revealed early transcriptome differences between the inoculation methods. We identified a total of 80, and 95 DEGs in the resistant line across three different time points post- thrips and mechanical inoculation, respectively (Figure 2.3). Of the 175 DEGs identified, 140 were upregulated, and 35 were downregulated. We observed distinctive differences between the inoculation methods, with thrips inoculations resulting in detection of DEGs as early as 12 hours post thrips inoculation (HPTI), with a single downregulated gene. At 24 HPTI, we observed 12 upregulated and three downregulated genes. Among the 64 DEGs identified at 72 HPTI, 62 were upregulated, and two were downregulated. In contrast, for the mechanically inoculated treatment, DEGs were not detected until 72 hours post mechanical inoculation (HPMI). Among the 95 detected, 66 were upregulated, and 29 were downregulated.

The *Sw-5b* NLR immune receptor induces earlier transcriptome changes to thrips-mediated inoculation of TSWV.

Earlier transcriptomic changes were detected in the resistant line, 'CNPH LAM-147' in response to thrips inoculations of $TSWV^{MR-01}$ than in mechanical inoculations. DEGs were detected at the earliest time points in the experiment at 12 and 24 HPTI (Figure 2.3, Supplementary Table 2.1), while transcriptome responses were not observed until 72 hours HPMI (Figure 2.3, Supplementary Table 2.2). The majority of DEGs (74) detected in response to thrips inoculation were upregulated, ranging from 1.00 to 9.12 fold, whereas only six were downregulated, ranging from -1.15 to -8.19 fold (Figure 2.3, Supplementary Table 2.1).

At 12 hours post inoculation, we detected a single DEG in thrips inoculated samples (Table 2.1, Supplementary Table 2.1). The DEG (Solyc09g082340) is predicted to encode a vicilin-like protein that was downregulated, -8.18 fold at 12 HPTI. Vicilin was not differentially expressed at later time points in thrips inoculated treatments nor in mechanically inoculated

treatments, suggesting this gene is not only a thrips-specific response, but of potential importance in early *Sw-5b*-mediated resistance to thrips inoculation of TSWV^{MR-01}. Vicilins are plant-specific proteins and structurally belong to the cupin superfamily of proteins (Dunwell et al. 2001; Chen et al. 2013). Interestingly, structure-guided biochemical analysis of vicilin from *Capsicum annuum* (pepper) and tomato indicate that vicilin may function as superoxide dismutase (SOD) to regulate oxidative stress (Shikhi et al. 2018; Shikhi et al. 2020). Furthermore, the C-terminal cupid fold of pepper and tomato vicilin in the crystal structure is bound by the defense hormone salicylic acid (SA), suggesting that SA could modulate the SOD activity of vicilin (Shikhi et al. 2020; Shikhi et al. 2018). Given that reactive oxygen species (ROS), such as superoxide, are known to play a role in defense (Kawano 2003; Qi et al. 2017), *Sw-5b*-mediated recognition of TSWV^{MR-01} could downregulate vicilin to suppress SOD activity. In *Arabidopsis*, *PAP85* encodes a vicilin-like protein that is upregulated early during *Tobacco mosaic virus* (TMV) infection, and virus accumulation was reduced in *AtPAP85* RNAi plants (Chen et al. 2013). Since TMV replication was reduced in protoplasts prepared from RNAi plants, the author concluded that *AtPAP85* might be involved in virus replication by facilitating endoplasmic reticulum (ER) membrane transition that is important for TMV replication. Interestingly, the NSm movement protein of TSWV has been shown to physically interact with the ER membrane, and disruption of this interaction inhibits the cell-to-cell movement property of NSm (Feng et al. 2016). Furthermore, in a *rdh3* mutant in which the ER network is altered, intracellular trafficking of NSm and systemic movement of TSWV were significantly affected (Feng et al. 2016). Therefore, we hypothesize that TSWV may use the vicilin-like protein for membrane interaction or reorganization to support virus replication and movement. Hence, *Sw-5b* activation could downregulate vicilin early during the defense

response to limit TSWV to the infection site. Future studies should provide insights into the potential role of vicilin in *Sw-5b*-mediated resistance to TSWV.

At 24 HPTI, 15 DEGs specific to the resistant line were identified (Figure 2.3). Twelve of these DEGs were upregulated (ranging from 2.24 – to 8.59 fold), and three were downregulated (ranging from 1.22 – to 3.01 fold) (Figure 2.3, Supplementary Table 2.1). Among the 15 DEGs detected at 24 HPTI, five were uniquely detected in response to thrips inoculations at this time point and were not detected at 72 HPTI, nor in any mechanically inoculated treatments (Figure 2.4, Supplementary Table 2.1). These five unique DEGs included a class II heat shock protein - HSP17, pleiotropic drug resistance protein, NOD26-like intrinsic protein 2.1,WEB family protein (similar to At4g27595), and a rhamnogalacturonate lyase 6 (Supplementary Table 2.1). These five DEGS are associated with the plant response to thrips inoculation of $TSWV^{MR-01}$ and initiation of a defense response. A function of small class II heat shock proteins is to act as a chaperone for protein folding and prevention of aggregation of proteins (Upadhyay et al. 2020; Waters 2012). Pleiotropic drug resistance protein and the NOD26-like intrinsic protein 2.1 are known to function as transporters facilitating the movement of diverse molecules across membranes as a response to stimuli and stresses (Nuruzzaman et al. 2014a; Xie et al. 2021; Wallace et al. 2006). In tobacco, pleiotropic drug resistance proteins have been shown to be regulated by defense hormones jasmonic acid (JA) and salicylic acid (SA), which indicates that pleiotropic drug resistance proteins might have a regulatory role in plant defense (Xie et al. 2021). Our data showing that genes encoding pleiotropic drug resistance proteins are differentially expressed in response to thrips inoculation of $TSWV^{MR-01}$ show that with thrips inoculation, the plant mounted a defense response against $TSWV^{MR-01}$ within 24 HPTI. These

DEGs are of special interest in an early *Sw-5b*-mediated response, especially because they were specifically seen only in the thrips inoculated treatments.

Among the 64 DEGs detected at 72 HPTI, 44 were detected only in response to thrips inoculation and were not detected at 24 HPTI (Figure 2.4 and Supplementary Table 2.1). Functional classification of DEGs of particular interest can be found in Table 2.1 and is further described below.

The *Sw-5b* NLR immune receptor induces delayed transcriptome changes to mechanical inoculation of TSWV

Mechanical inoculation of the resistant near-isogenic line with $TSWV^{MR-01}$ resulted in a delayed transcriptome response to *Sw-5b*-mediated resistance. DEGs were not detected at 12 or 24 HPMI in the resistant line, but at 72 HPMI, we observed 95 DEGs in the resistant line (Figure 2.3). Among these, 66 genes were upregulated (ranging from 1.91 – to 10.51 fold), and 29 DEGs were downregulated (ranging from 1.03 – to 5.65 fold) (Figure 2.3, Supplementary Table 2.2). Sixty-nine of the 95 DEGs detected at 72 HPMI were detected only in mechanically inoculated treatments (Figure 2.4, Supplementary Table 2.2). Functional classifications of DEGs of particular interest to plant defense can be found in Table 2.2 and are further described below.

Specific transcriptome responses important to early plant defense responses were shared among thrips and mechanical inoculations

Figure 2.4 shows that some of the DEGs detected at 24 HPTI were also detected in the 72-hour post inoculation treatments (72 HPTI and 72 HPMI), and Table 2.3 shows the identity and changes in their expression. Among the 15 DEGs identified at 24 HPTI, four also were detected at 72 HPTI, and also detected at 72 HPMI. These DEGs included three pathogenesis-related proteins and beta (1,3) glucanase, all of which were upregulated (Table 2.3), suggesting

an important role in early and ongoing aspects of *Sw-5b*-mediated resistance. The upregulation of these DEGs indicates that by 24 HPTI, the *Sw-5b* NLR had initiated the defense response as expected (Takken and Tameling 2009; van Wersch et al. 2020). It is notable that at 72 HPTI, the expression of three of four genes decreased, suggesting that as *Sw-5b*-mediated resistance proceeds, these genes may no longer be needed to modulate resistance. Taken together, these data suggest that thrips inoculations allowed for faster recognition of NSm by the *Sw-5b.* The detection of these same genes in the 72 HPMI treatment supports their importance in the *Sw-5b* resistance response, independent of the inoculation method (Table 2.3). Furthermore, these four DEGs had higher-fold expression at 72 HPMI than they did in either of the thrips inoculated treatments (24 HPTI, 72 HPTI). These data suggest that although *Sw-5b*-mediated transcriptome responses were delayed in mechanically inoculated treatments, the plant defense response was heightened once it was initiated.

Six DEGs detected at 24 HPTI were no longer detected at 72 HPTI but were detected at 72 HPMI. Among these were four upregulated enzymes known to be of importance in resistance responses: terpene synthase, pyruvate decarboxylase (PCD), phenylalanine ammonia lyase (PAL), and FAD-binding berberine family protein (Figure 2.4 and Table 2.3). All of these have been shown to play a role in immunity in many plant systems (Kim and Hwang 2014; Vanitha et al. 2009; Benedetti et al. 2018; Daniel et al. 2017; Chen et al. 2018; Köllner et al. 2008; Tadege et al. 1998; Rojas et al. 2014; Kim et al. 2009). In maize, overexpression of terpene synthase has been shown to enhance resistance to a fungal pathogen (Chen et al. 2018). The C2 protein from begomoviruses has also been shown to compromise terpene synthase resulting in a decrease in resistance to virus infection (Li et al. 2019b). Our findings of upregulation of terpene synthase could explain the enhanced defense mediated by *Sw-5b* NLR against thrips inoculation of

TSWV^{MR-01}. The upregulation of PCD is known to induce pathogenesis proteins that will ultimately produce HR (Tadege et al. 1998; Rojas et al. 2014). PAL has been demonstrated to be a key enzyme in several defense-related secondary compounds that increase resistance against bacterial pathogens (Kim et al. 2009). Studies in lettuce and sunflowers have shown that FAD-binding berberine family proteins and BBE-like enzymes are upregulated after infection (Daniel et al. 2017; Benedetti et al. 2018). The Class III heat shock protein is the only one among the six DEGs detected at 24 HPTI and 72 HPMI that was downregulated with thrips and mechanical inoculation methods. Small heat shock proteins are known to respond to heat stresses and sequester unfolded proteins to be degraded (Ohnesorge and Bejarano 2009).

Sixteen DEGs were detected at 72 hours with both inoculation methods, all of which were upregulated regardless of inoculation method; however, nine (pathogenesis-related protein 1, pathogen-related protein, major allergen Pru ar 1-like, polyphenol oxidase-chloroplastic, glucan endo-1,3-beta-glucosidase,1-aminocyclopropane-1-carboxylic acid, nucleoside hydrolase family protein, arogenate dehydrogenase 2,,1-aminocyclopropane-1-carboxylate) had a greater fold change at 72 HPTI (Table 2.3, Supplementary Table 2.6). Most of these DEGs are indicators of activation of plant defense responses (Chandra-Shekara et al. 2004; Park et al. 2004; Sinha et al. 2014; Stintzi et al. 1993; Linthorst and Van Loon 1991). Pathogen-related proteins were among the DEGs shared at 72 hours post-inoculation; eight out of 16 belong to PR-1, PR-2, PR-3, PR-4 and PR-5 (Figure 2.4, Table 2.3). Interestingly, we also observed upregulation of the two enzymes required for ethylene production via aminocyclopropane-1-carboxylate oxidase 1 (Houben and Van de Poel 2019). Infection with TSWV has been previously shown to increase ethylene production and was associated with a difference in expression of PR-5 in *Sw-7* resistance at 21 days post-inoculation (Padmanabhan et al. 2019).

We also observed upregulation of lipoxygenases, which is usually associated with responses to wounding from the mechanical damage or thrips feeding damage resulting in the synthesis of jasmonic acid (Yan et al. 2013). However, our experimental controls included mock inoculations with sap prepared from non-infected plant, and inoculation with non-infected thrips, which allowed us to remove responses due to mechanical wounding and thrips feeding damage from our analysis. Hence, upregulation of the lipoxygenases may be specific to the interaction of TSWV and *Sw-5b*-mediated resistance. We also observed an upregulation of arogenate dehydrogenase and polyphenol oxidase, both known to play an integral part in coordinating reactive oxygen species that are critical early events leading to HR, which is the response seen at 72-hours post-inoculation on the *Sw-5b* resistant line. As observed in other studies (Padmanabhan et al. 2019; Schaff et al. 2007; Zheng et al. 2013; Yang et al. 2016), upregulation of genes tends to occur during pathogen replication as the plant responds to pathogen invasion and replication; hence, we were not surprised that the majority of the DEGs we detected were upregulated. We were surprised by the overall small number of DEGs identified in response to *Sw-5b* NLR-mediated resistance. The relatively smaller number of DEGs we observed may be due to our use of a highly isogenic line, an aspect that was not available to previous transcriptomic studies (Padmanabhan et al. 2019). The near-isogenic lines used in our investigation shared the same background, with only known difference being presence or absence of *Sw-5b*; hence, any transcriptomic changes identified are likely a very specific response to *Sw-5b*-mediated resistance against TSWV.

Functional classes of DEGs varied with inoculation method

DEGs detected only in thrips inoculations. Table 2.1 shows functional categories for DEGs of interest detected only in thrips inoculated treatments at 72 HPTI. Of the 64 DEGs, 44

were detected specifically to thrips inoculation and were not found in mechanically inoculated treatments (Figure 2.4). Thirty of these DEGs were of interest relative to plant defense, and these were found in several functional classifications: NLR and receptor-like-kinases, transporters, transcription factors, pathogenesis-related proteins, cytochrome p450s, and calmodulin-binding proteins (Table 2.1). As expected for plant immune responses to pathogens (Tsuda and Somssich 2015; van Wersch et al. 2020; Cook et al. 2015), we observed upregulation of pathogenesis-related proteins and transcription factors. These findings indicate the plant's defense response was activated by the thrips inoculations within 24 hours. Thrips inoculation also induced five NLR or receptor-like kinases that were not found in any mechanically inoculated plants. These DEGs are of particular interest due to their known role in resistance responses (Wiermer et al. 2005; van Wersch et al. 2020; Zhao et al. 2018; Zeng et al. 2012). For example, overexpression of G-type lectin S receptor-like kinases (G-type lec-RLK) has been shown to increase plant defense to biotrophic pathogens (Zhao et al. 2018). G-type Lec-RLKs in *Nicotiana attenuate* have been demonstrated to be involved in recognition of insect feeding, and required to induce a full defense response against *Manduca sexta* (Bonaventure 2011; Gilardoni et al. 2011). The three transporters identified at 24 and 72 HPTI fall into the ABC transporter family, which has been implicated in modulating phytohormones, secondary metabolites, and signal molecules, such as salicylic acid and jasmonic acid (Nuruzzaman et al. 2014b). We detected downstream calmodulin-binding proteins at 72 HPTI that were upregulated. This finding is of interest because these proteins play a role in the biosynthesis of signaling molecules altering plant defenses against various pathogens (Lv et al. 2019; Cheval et al. 2013). At 72 HPTI, we also observed an upregulation of three cytochrome P450s which have been implicated in several metabolic pathways that result in defense mechanisms against pathogens and vectors

(Xu et al. 2015; Mizutani 2012; Jensen 2000). Interestingly, thrips inoculated treatments included DEGs from functional classifications that we did not find in mechanical inoculations, particularly the NLR and receptor-like kinases and calmodulin-binding proteins. These results support findings in *Sw-7*-mediated resistance against mechanically inoculated TSWV, where NRL and receptor-like kinases were only shown to be downregulated at 14, 21, and 35 days post-inoculation (Padmanabhan et al. 2019). Padmanabhan et al 2019, did not observe any difference in gene expression at the earliest time points tested, 4- and 7-days post-inoculation. Our observations showing differences in expression of NLR and receptor-like kinases suggest the importance of these genes at earlier time points during the *Sw-5b* NLR-mediated resistance.

DEGs detected only in mechanical inoculations. At 72 HPTI, 69 of the DEGs detected were specific to mechanical inoculation and were not found in thrips inoculated treatments (Figure 2.4, Supplementary Table 2.2). Thirty-nine of these DEGs were of special interest to plant defense, and these were classified in several functional classes, including pathogenesis-related proteins, transcription regulators, degradation proteins, cytochrome P450's, transporters, as well as a diversity of "other" categories (Table 2.2). As would be expected for the early phases of a plant defense response, mechanical inoculation induced many pathogenesis-related proteins and transcription regulators (Miller et al. 2017; Tsuda and Somssich 2015). The up and downregulation of genes related to protein degradation, e.g. RING-Type E3 ubiquitin ligase (upregulated), and F-box phloem protein2-like proteins (downregulated) is also not surprising, as protein degradation is known to be part of an early defense response (Dinant et al. 2003). Surprisingly, DEGs involved in protein degradation were not found in the thrips inoculated treatments (Table 2.1). We also observed 4 differentially expressed cytochromes; three of them were upregulated while one was downregulated. There were other cytochrome P450s that were

also differentially expressed in the HPMI treatment. However, mechanical inoculation resulted in the upregulation of a cytochrome p450 gene with a fold change of 8.92, indicating that a strong response was mounted against TSWV^{MR-01}. Cytochrome P450s play an integral part as secondary metabolites in defense responses (Mizutani 2012; Jensen 2000; Xu et al. 2015) which may explain their detection in thrips and mechanically inoculated treatments. Among the DEGs in the "other" category, fatty acid desaturase (FAD) was one of the most highly upregulated DEGs in our analysis (fold change of ~10.51) and was only differentially expressed with mechanical inoculation (Table 2.2). FADs have been identified as key to producing metabolites used in defense signaling pathways (Daniel et al. 2017; Avila et al. 2013; Berestovoy et al. 2020). Our observation of differences in FAD expression solely with mechanical inoculation could indicate that thrips and mechanical inoculations fundamentally differ in their induction of defense pathways.

Differences in *Sw-5b* transcriptome changes in response to thrips and mechanical inoculation of TSWV is not due to differences in virus titer

We hypothesized that the earlier induction of defense genes we observed with thrips inoculations could be explained by faster or more efficient viral replication in thrips inoculated leaves. To test this hypothesis, we tested the plant materials used to prepare our RNA-seq libraries with RT-qPCR. Figure 2.5A shows that low TSWV^{MR-01} titers were detected in thrips and mechanically inoculated leaves from susceptible and resistant lines as early as 12 hours post-inoculation, with only slight, non-significant increases occurring at 24 hours post-inoculation. Both inoculation methods resulted in a significant increase in virus titer in the susceptible line at 72 hours post-inoculation, but virus titers remained low in all time points in the resistant line with both inoculation methods. Although the abundance of TSWV N gene transcripts in thrips

inoculated resistant plants was higher compared to mechanical inoculation, the difference was not significant. These findings suggest that differences we observe in gene expression profiles relative to the inoculation method are not related to differential rates of virus increase during the first 72 hours post inoculation.

Thrips inoculation of TSWV^{MR-01} results in earlier and higher rates of systemic infection of susceptible plants

Following the removal of inoculated leaves for RNA-seq studies, plants were maintained in the same growth chamber. At 12 days post-inoculation, newly emerged leaves were tested with ELISA for systemic infection. These measurements showed that thrips inoculations resulted in systemic infection of the susceptible line earlier than mechanical inoculations, reaching a mean of 89% infection in the 12 hours post-inoculation treatment, and a mean of 94% in the 24 hours post-inoculation treatment, compared to means of 22% and 15%, respectively, in the mechanical inoculations at the time points (Figure 2.5B, Supplemental Table 2.3, 2.4). Surprisingly, in the resistant line, thrips inoculations led to 26% of the plants having systemic infections as early as 12 hours post-inoculation, whereas none of the mechanically inoculated plants were systemically infected at this time point. 24 HPTI also resulted in significantly higher systemic infection than mechanical inoculations, 26% compared to 5%, respectively. Although mechanical inoculation was less likely to cause a systemic infection (26% compared to 36% in thrips inoculations), the 72 hour post-inoculation treatments in the resistant line were not significantly different relative to the inoculation method. Virus titer in the inoculated leaves at the time of collection for RNA-seq did not vary significantly with the inoculation method (Figure 5A); hence, another mechanism underlies the faster, higher rates of systemic infection in susceptible plants (Figure 2.5B). Most studies use mechanical inoculations to identify new

sources of resistance (Dianese et al. 2011; Kabaş et al. 2021); however, our findings highlight the importance of using thrips inoculation when testing new cultivars for resistance against TSWV and when identifying new *Sw-5b* resistance breaking isolates.

Controls support the efficiency of virus inoculations and lack of cross-contamination in biological experiments.

Biological experiments were done in the same growth chamber to avoid variation between experiments, and plants were held in individual cages, as shown in Figure 2.1B. A series of positive controls were inoculated with $TSWV^{MR-01}$ mechanically, and with thrips at the same time as the experimental treatments to validate the infectivity of the $TSWV^{MR-01}$ isolate. ELISA confirmed that the susceptible line positive controls in thrips and mechanically inoculated treatments were 100% infected in all post-inoculation treatments. In contrast, resistant positive controls were only infected when inoculated by thrips (33, 50 and 33% at 12, 24, and 72 hours post-inoculation, respectively) (Supplementary Table 2.3). Unlike our experimental treatments, mechanical inoculation did not result in infection at any time (Supplement Table 2.4). Negative controls to test against cross-contamination with $TSWV^{MR-01}$ included plants mechanically "inoculated" with sap from non-infected plants, exposed to non-infected thrips, or given no treatment. These plants were held in the same growth chamber as treatments in each experiment. ELISA confirmed that none of the negative controls were infected with $TSWV^{MR-01}$ (Supplementary Table 2.3, 2.4), indicating that there was no cross contamination of plants occurring in our experiments.

Validation of RNA-seq results

RNA-seq data was verified by RT-qPCR to calculate the expression of 6 randomly selected DEGs (Supplementary Table 2.5). The RT-qPCR fold change of gene expression at 12

HPTI, 72 HPTI and 72 HPMI was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). We observed a positive correlation between the fold change of RNA-seq and RT-qPCR results ($r^2 = 0.74$).

Conclusion

This transcriptomic analysis showed that the *Sw-5b* NLR immune receptor induced earlier transcriptome responses to thrips inoculations (12 and 24 HPI), compared to mechanical inoculation, where differential expression of genes was not detected until 72 HPI. Our results indicate that thrips inoculations provide a mechanism allowing for faster induction of a defense response mediated by *Sw-5b* NLR resistance against TSWV. While mechanical inoculations resulted in a delayed *Sw-5b* resistance response, once initiated, this method of inoculation led to a more robust defense response with a greater number of DEGs identified. As recognition and activation of the *Sw-5b*-mediated defense requires TSWV NSm (Chen et al. 2016; Zhu et al. 2017), and this non-structural protein is only present following virus replication (Zhu et al. 2019; Storms et al. 1995), we hypothesized that a possible mechanism explaining earlier detection of DEGs in thrips inoculated treatments was that TSWV replicated more quickly when inoculated by thrips, making NSm available earlier. When we tested this hypothesis with RT-qPCR testing of the biological materials used for library preparation, we determined that virus titers were not significantly different between the inoculation methods (Figure 2.5A). Thus, some other mechanism, as yet unidentified, associated with thrips inoculation of TSWV is involved. One possibility is that thrips feeding, which involves salivation into the intracellular space of plant epidermal, parenchymal, mesophyll cells (Stafford-Banks et al. 2014) may result in immediate TSWV replication in the proximity of the *Sw-5b* NLR, enhancing the probability of

earlier recognition by the receptor. Future experiments should focus on this interesting finding, and those investigating *Sw-5b*-mediated resistance may want to consider using thrips inoculation.

While a small number of the DEGs we detected were shared across the inoculation methods, the higher number of DEGs found only in mechanical (69) or thrips (49) inoculation (Figure 2.4) suggests that the *Sw-5b* NLR uses different signaling pathways to provide resistance against TSWV depending on the method of inoculation. This hypothesis is supported by our finding that when thrips inoculate plants, DEGs classified as NLR and receptor-like kinases are present; however, they are not present in plants that were mechanically challenged. Similarly, among mechanically inoculated treatments, we observed DEGs classified under protein degradation that were not identified to be differentially expressed with thrips inoculations. Given that most investigations around *Sw-5b*-mediated resistance focus only on mechanical inoculations, these findings suggest important components of the defense signaling pathways activated by *Sw-5b* and leading to resistance are being missed.

Not only is this transcriptomic analysis the first to compare thrips inoculations and mechanical inoculations methods, but we also are the first to reveal the early post inoculation responses (12, 24, and 72 HPI) in *Sw-5b* NLR-mediated resistance against TSWV. Both the unique and shared DEGs all have possible roles in *Sw-5b* NLR-mediated resistance against TSWV. Our findings provide new insight into the genes that are differentially regulated early in *Sw-5b*-mediated resistance and provide a foundation for future functional analyses to determine the roles these genes may play in *Sw-5b*-mediated immune signaling.

Material and Methods

Plant Material

Transcriptomic experiments were performed on the near-isogenic lines, TSWV-susceptible line, *Solanum lycopersicum* 'Santa Clara' and a TSWV- resistant line, *S. lycopersicum* 'CNP-LAM 147' (Hallwass et al. 2014) (Figure 2.1C). These near-isogenic lines will be referred to as the susceptible line and resistant line, respectively, throughout this paper. Development of these near-isogenic lines by Hallwass et al. (2014) involved crossing *S. lycopersicum* cultivar 'Santa Clara' (highly susceptible to *Orthotospovirus* species) with the resistant cultivar 'Viradoro,' a germplasm source of the *Sw*-5 locus derived from the tomato cultivar 'Stevens'. Ultimately, a single dominant homozygous resistant (*Sw*-5/*Sw*-5) plant of the line 'CNPH LAM 147' and a single recessive homozygous susceptible (*Sw-5/Sw*-5) 'Santa Clara' plant was selected, and the seed was increased by Leonardo Boiteaux (Centro Nacional de Pesquisa de Hortaliças (CNPH)/EMBRAPA, CP 0218, 70359-970, Brasilia, DF, Brazil). Seeds from these increases were used in all plantings for these experiments. Optimization and standardization of planting and watering times, and the use of a single environment-controlled growth chamber prevented biases in transcriptomic data due to these aspects of plant treatment. Seeds from the near-isogenic lines were planted in individual plastic pots with SunGro professional mix (Agawam, MA.) in an environment-controlled growth chamber (light intensity, 300 µEinsteins; 26°C with 16h daylight/ 8h dark and 50% relative humidity) in the Controlled Environment Facility, University of California, Davis, California (Department of Plant Biology, One Shields Avenue, Davis, California 95616).

TSWV Isolate and maintenance

TSWV $^{MR-01}$ is a TSWV isolate collected originally from infected radicchio in Monterey County, CA, flash frozen and stored at -80°C. Complete sequences of the three RNAs can be found in NCBI (https://www.ncbi.nlm.nih.gov/nuccore) using these accession numbers: S RNA MG593199; M RNA MG593198; and, L RNA MG593197. Phylogenetic analysis showed that the S RNA and L RNA clustered with Asian type TSWV isolates, while the M RNA clustered with American type isolates (Adegbola et al. unpublished data).*Datura stramonium* for mechanical inoculations was grown in individual pots with SunGro Professional mix (Agawam, MA) in an controlled environment growth chamber (light intensity 300 µEinsteins; 30°C with 16h daylight/ 8h dark and 50% relative humidity) were mechanically inoculated with TSWV $^{MR-01}$ inoculum was prepared by grinding 1 gram of infected tissue with 30ml of ice-cold inoculation buffer (0.1M potassium phosphate, 1 mM sulphite, 1% celite, pH 7) on a chilled mortar and pestle. The inoculum was kept on ice while rub inoculations were performed using a pestle. Ten minutes after the inoculations, the inoculated leaves were rinsed with distilled water and maintained under the growing conditions (see above). The presence of TSWV^{MR-01} was confirmed by enzyme-linked-immunosorbent assay (ELISA) and maintained in an environment-controlled growth chamber maintained at 30°C with 16h daylight and 8h dark. Systemic infection was tested for the presence of TSWV^{MR-01} using ELISA following the manufacturer's protocol (Adgia, Elkhart, IN).

Thrips colony and maintenance

Frankliniella occidentalis from a lab colony originally collected from the Kamilo Iki valley on the Hawaiian island of O'ahu was reared on green pods (*Phaseolus vulgaris.* L) as previously described (Ullman et al. 1992; Bautista 1993). Thrips inoculations were standardized

and optimized by collecting thrips six hours post-hatching and placed on either TSWV^{MR-01} infected *D. stramonium* or non-infected *D. stramonium* for a 24-hour acquisition access period (AAP). After the AAP, thrips were reared on green bean pods and at 24-hr post adult eclosion.

Mechanical inoculation of TSWV on Near Isogenic Lines

Mechanical inoculations were conducted on seedlings of the near-isogenic lines when they reached the two true leaf stage. TSWV^{MR-01} inoculum was prepared by grinding one gram of infected TSWV^{MR-01} *D. stramonium* leaf tissue in 15 ml of inoculation buffer (0.1M potassium phosphate, 1 mM sulphite, 1% celite, pH 7) with a mortar and pestle on ice. The mechanical inoculation procedure was followed as described above with the only difference being that their conditions were as follows: 26°C with 16-hour daylight and 8-hour dark cycle.

Thrips inoculation of TSWV

Thrips inoculations were performed on seedlings of the near-isogenic lines when they reached the two true leaf stage. Each seedling was placed in individual tomato cages composed of two-32 ounce plastic cups (Lancaster, PA) with the bottom cut out and covered with no-thrips insect screens (BioQuip, Rancho Dominguez, CA) (Figure 2.1B). When the plant and thrips were placed inside, the two deli cups were joined by Parafilm. The pot was also covered using parafilm to prevent thrips' movement into the soil. Ten thrips (5 female and 5 males) were placed on leaf 1 and leaf 2 on each plant, and the cage closed. Thrips remained on the plants until samples were taken for RNA-seq.

Experimental conditions and tissue collection for RNA sequencing and confirmation of virus status

Samples were collected at 0, 12, 24 and 72 hours post inoculations. At each collection time, the two inoculated leaves from each plant (n=10) were collected from each treatment:

viruliferous thrips (VT), non-viruliferous thrips (NVT) (mock), mechanical inoculation with sap from virus-infected plants (VS), mechanical inoculation with sap from non-virus infected plants (NVS) (Figure 2.1C). Susceptible and resistant lines were included in our positive, negative, and no thrips controls. Verification of TSWV infection of the positive controls that were inoculated with VT and VS were used to verify inoculation success using ELISA. Negative controls were inoculated with NVT and NVS. The no thrips control was used to test for cross-contamination during the biological experiments. The collected samples were flash-frozen in liquid nitrogen and stored at-80° C. Additional confirmation of TSWV presence, and concentration was assayed using RT-qPCR with specific primers for the N gene of TSWV F-GCTTCCCACCCTTTGATTC and R-ATAGCCAAGACAACACTGC [4]. RT-qPCR was performed for three biological replicates, and six technical replicates/biological replicates were run on Bio-Rad CFX96 machine using the following conditions: 95 ◦C for 30 sec, followed by 39 cycles of 95 ◦C for 10 sec, 55 ◦C for 10 sec, and 60 ◦C for 20 sec.

RNA extraction and RNA-seq library preparation

Tissue samples of the near-isogenic lines were collected as described above and then pooled in each treatment and collection time (0, 12, 24, 76 hours post-inoculation). Samples were flash-frozen using liquid nitrogen and stored at -80° C. RNA-seq libraries were constructed using the protocol described in (Nagalakshmi et al. 2010). Briefly, mRNA was isolated using oligo (dT) magnetic beads, treated with DNase, first and second-strand cDNA synthesis, followed by fragmentation and addition of barcoded adaptors. A total of 96 barcoded libraries were created and pooled for sequencing using the Illumina HiSeq4000 platform at the Genomic Facility at the University of California, Berkeley.

Bioinformatic analysis

The sequencing adaptors and low-quality bases were trimmed from the raw reads using Trimmomatic version 0.36 (Bolger et al. 2014). The high quality reads were mapped against the tomato cDNA sequences (version 4.1) using Salmon version 0.8.1 (Patro et al. 2017). Differential gene expression analysis was performed using the generalized linear model (glm) functionality of the edgeR package (Robinson et al. 2010). Tomato genes with at least two-fold expression difference between the susceptible and resistant genotypes and False Discovery Rate (FDR) < 0.05 were considered differentially expressed. GO enrichment analysis was performed using PANTHER version 11 with conservative Bonferroni correction for multiple testing (Mi et al. 2017).

Validation of selected DEGs by reverse transcription quantitative PCR.

Six DEGs were selected and validated with RT-qPCR. Gene-specific primers were designed using Primer3Plus online for amplification of 93-180 bp fragments from each target gene (Supplementary Table 2.5). The F-box gene was used as the housekeeping gene to normalize the data (Liu et al. 2012). Total RNA was extracted from frozen leaf material using TRIzol® (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA was then treated with Ambion® DNase I (Invitrogen, Carlsbad, CA) followed by cDNA synthesis using Verso cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA). Three biological replicated were run on Bio-Rad CFX96 machine using the following conditions: 95 ◦C for 30 sec, followed by 39 cycles of 95 ◦C for 10 sec, 55 ◦C for 10 sec and 60 ◦C for 20 sec. The gene expression fold change was calculated using the $2(-\Delta\Delta CT)$ method (Livak and Schmittgen 2001).

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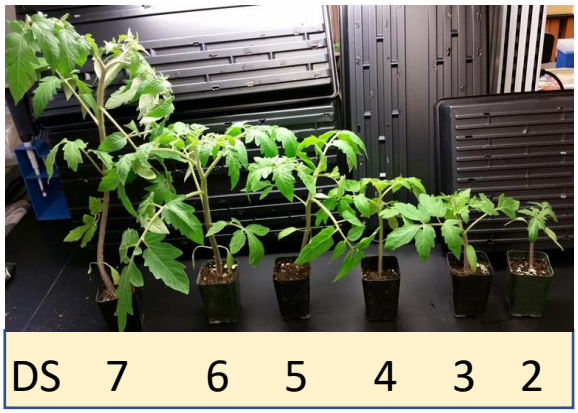
A



B

C

Inoculation method	Tomato cultivar	Inoculation Source	Sample time points
Mechanical	Santa Clara (-Sw-5)	Virus-infected plants (VS)	0,12, 24, and 72 Hours post inoculation
		Non-virus infected plants (NVS)	
	CNPH-LAM 147 (+Sw-5)	Virus-infected plants (VS)	
		Non-virus infected plants (NVS)	
Thrips	Santa Clara (-Sw-5)	Viruliferous thrips (VT)	
		Non- viruliferous thrips (NVT, mock)	
	CNPH-LAM 147 (+Sw-5)	Viruliferous thrips (VT)	
		Non- viruliferous thrips (NVT, mock)	

Figure 2.1. (A) Top photo depicts the six developmental stages (DS) used for mechanical inoculation of 'Celebrity' with *Tomato spotted wilt orthotospovirus* (TSWV). (B) Photo shows the cage used for thrips inoculation of tomato in biological experiments. (C) Table showing biological treatments for RNA-seq experiments at 12, 24 and 72 hours post inoculation (HPI) of the near *Sw-5b* isogenic lines *Solanum lycopersicum* ('Santa Clara, - *Sw5-b,* and 'CNPH-LAM 147', *+Sw5-b)* with TSWV using mechanical inoculations (virus-infected plants (VS) and non-virus infected plants (NVS)) western flower thrips (WFT), *Frankliniella occidentalis* inoculation (Viruliferous thrips (VT) and Non-viruliferous thrips (NVT)).

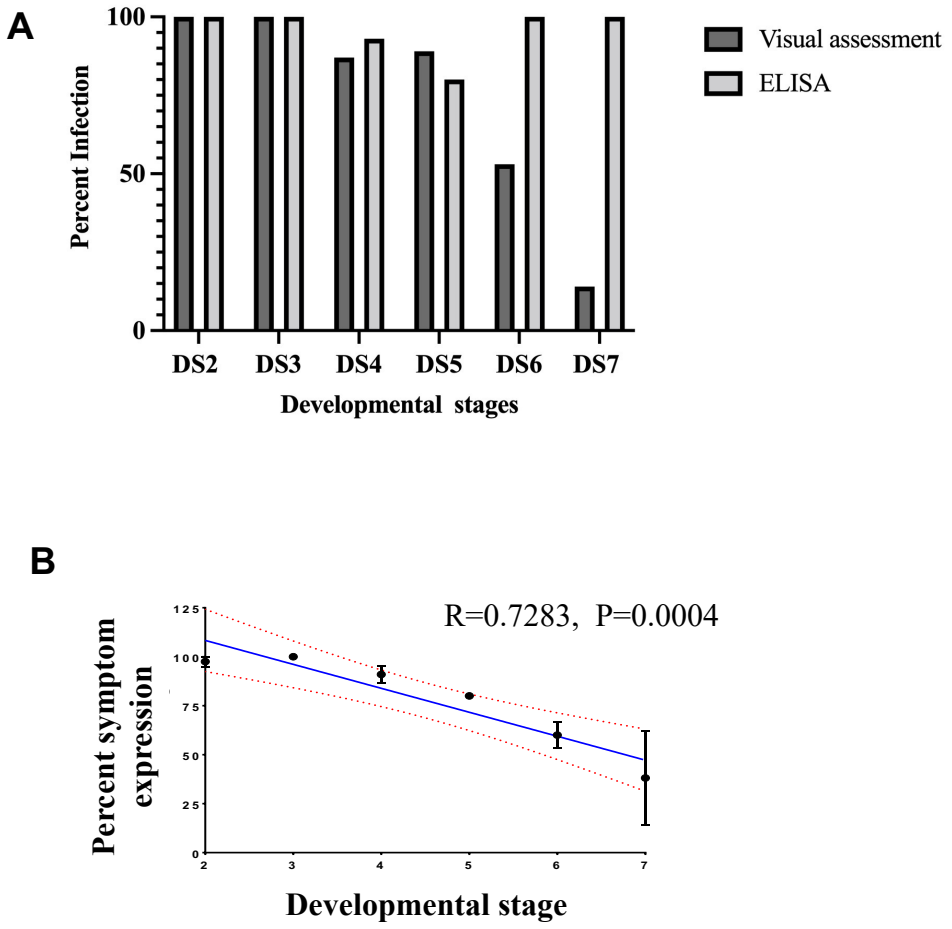


Figure 2.2. Relationship between tomato developmental stage, systemic infection by mechanical inoculation of *Tomato spotted wilt orthotospovirus* (TSWV) and symptom expression. (A) Comparison of mean percent infection based on visual assessment to enzyme linked-immunosorbent assay of 'Celebrity' tomato at six developmental stages (DS) (as defined by number of true leaves), at 12 days post mechanical inoculation (DPI) with TSWV-infected sap. (B) Correlation between DS and symptom expression at 12 DPI (regression analysis, mean plants with symptoms). (A and B) (2 separate experimental replicates, rep 1, n = 20-21 plants/ds) (rep 2, n = 5 to 15 plants/ds). S = Susceptible, 'Santa Clara' R = Resistant 'CNPH-LAM147', VT = Viruliferous thrips, VS = Infected Sap

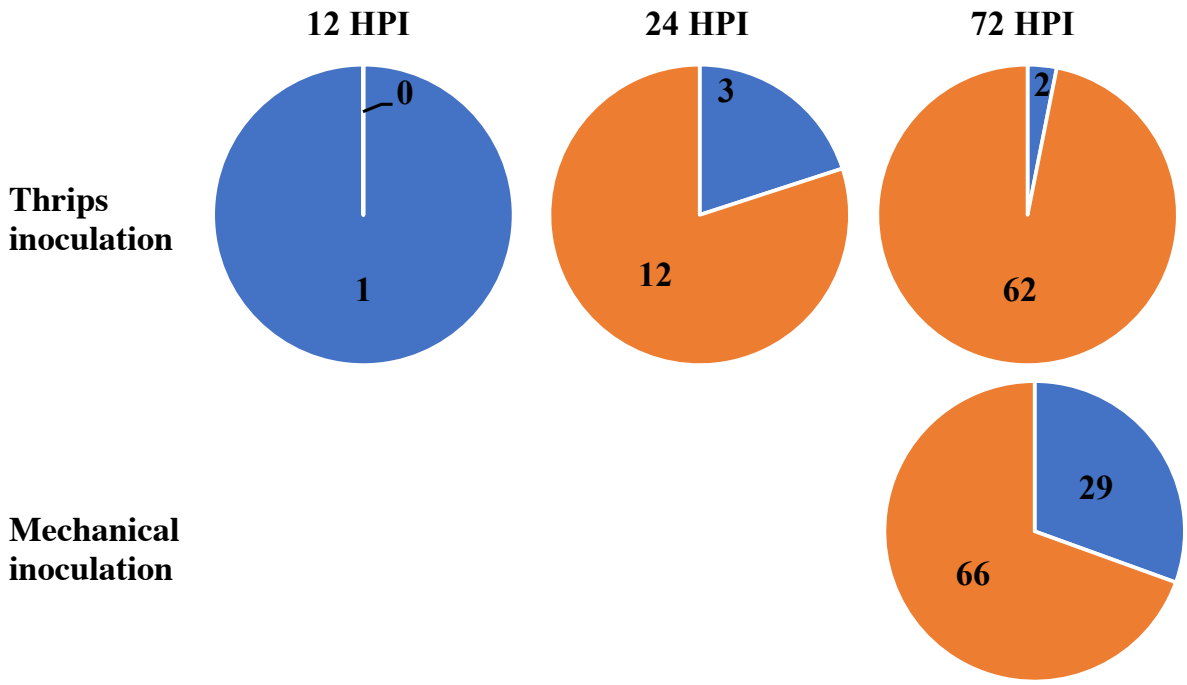


Figure 2.3. Venn diagrams showing the number of differentially expressed genes (DEGs) detected with RNA-seq at 12, 24 and 72 hours post inoculation (HPI) in the resistant *Solanum lycopersicum* 'CNPH-LAM 147', +*Sw5-b* line with *Tomato spotted wilt orthotospovirus* (TSWV) using sap prepared from TSWV^{MR-01} infected tissue (mechanical) or TSWV infected western flower thrips, *Frankliniella occidentalis*. Thrips inoculation induced differences in gene expression at 12, 24 and 72 HPI. Mechanical inoculation resulted in differences in gene expression only at 72 HPI. Orange = upregulated DEGs, and blue = down-regulated DEGS.

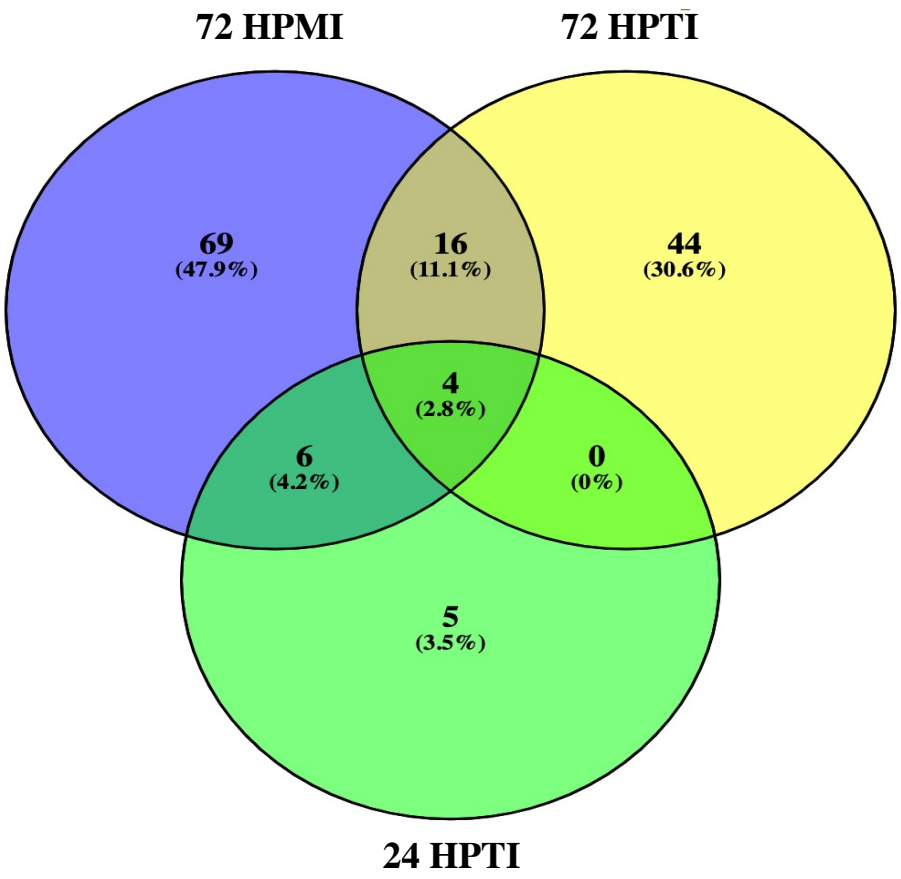


Figure 2.4. Comparative analyses of RNA-seq data sets from inoculation of *Solanum lycopersicum* 'CNPH-LAM 147', +*Sw5-b* (resistant isoline) with *Tomato spotted wilt orthotospovirus* (TSWV) using sap prepared from TSWV^{MR-01} infected tissue (mechanical) or TSWV infected western flower thrips (WFT), *Frankliniella occidentalis,* at 24 and 72 hours post inoculation revealed that 16 differentially expressed genes (DEGs) were detected at 72 HPMI and 72 HPTI, 6 DEGS were detected at 24 HPTI and 72 HPMI, and 4 were detected at 24 HPTI, 72 HPTI and 72 HPMI. HPTI = hours post thrips inoculation, HPMI = hours post mechanical inoculation.

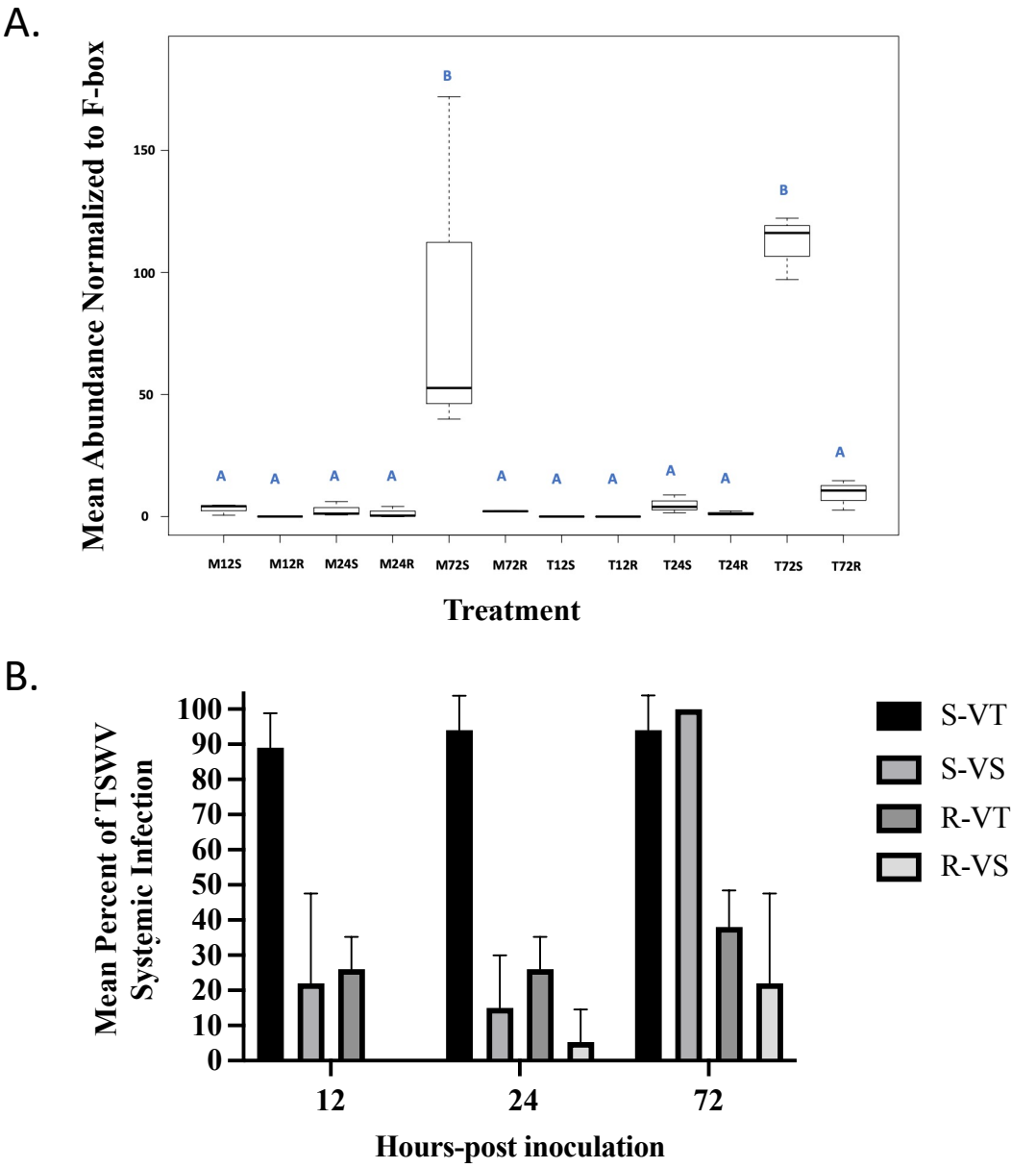


Figure 2.5. Characteristics of *Tomato spotted wilt orthotospovirus* (TSWV) infection of the near isogenic lines, *Solanum lycopersicum* 'Santa Clara' (*Sw-5b*$^{-}$, the TSWV-susceptible line) and *S. lycopersicum* 'CNP-LAM 147' (*Sw-5b*$^{+}$, the TSWV- resistant line), following inoculation with sap prepared with TSWV infected tissue (mechanical) or infected western flower thrips (WFT). (A) Quantification by RT-qPCR of mean TSWV titers in inoculated leaves from all treatments

used for preparation of RNA-seq libraries. Treatments: M= mechanical inoculation with sap prepared from infected TSWV tissue; T= thrips inoculation with TSWV infected thrips; 12, 24 and 72 hours post inoculation (HPI). Virus titer estimated by normalized abundance (ratio) of TSWV-N RNA to tomato F-box transcripts calculated by the Pfaffl (30) equation: normalized abundance = ECt(fbox)/ECt(N), where E = efficiency of PCR for each primer pair. (B) Assessment of systemic TSWV infection 12 days post inoculation on newly emerged leaves from treatments following 12, 24 and 72 HPI. Columns indicate mean percent of TSWV systemic infection with standard error bars. R = resistant, and S = susceptible isoline as in (A), VT= viruliferous thrips and VS= virus infected plants.

Table 2.1. Functional categories for differentially expressed genes DEGs) of interest detected with RNA-seq exclusively shared at12, 24 and 72 hours post-thrips inoculation in the resistant *Solanum lycopersicum* 'CNPH-LAM 147', +*Sw5-b* line with *Tomato spotted wilt orthotospovirus.* DEGs with their corresponding logFc and annotation.

Gene ID	Hours post thrips inoculation			Annotation
	12 LogFC	24 LogFC	72 LogFC	
NLR and receptor-like kinases				
Solyc04g007490			1.44	CC-NLR disease resistance protein
Solyc12g005720			4.80	Cysteine-rich receptor-like protein kinase
Solyc04g077340			7.08	G-type lectin S-receptor-like kinase
Solyc05g008310			2.20	G-type lectin S-receptor lke protein kinase
Solyc07g063770			1.83	G-type lectin S-receptor-like kinase
Transporters				
Solyc09g091670		3.95		Pleiotropic drug resistance protein
Solyc01g105450			4.45	ABC transporter G family
Solyc05g053600			4.15	Pleiotropic drug resistance protein
Transcription factors				
Solyc04g016000			5.56	Heat shock transcription factor protein 8
Solyc07g053140			4.84	Zinc finger protein/CONSTANS-like protein
Solyc06g069760			3.48	3.48 Dof zinc finger protein
Solyc05g051200			2.91	Ethylene-responsive factor 1

Table 2.1. (continued).

Pathogenesis-related proteins				
Solyc10g055820			5.99	Chitinase
Solyc04g079230			2.29	Patatin
Cytochrome P450s				
Solyc10g083690			4.18	Cytochrome P450
Solyc09g092640			3.30	Cytochrome P450
Solyc02g084930			3.22	Cytochrome P450
Calmodulin-binding proteins				
Solyc03g113960			3.26	Calmodulin binding protein-like
Solyc12g008960			2.38	Calmodulin-binding protein
Solyc03g112700			1.80	Calmodulin-binding protein
Other genes				
Solyc09g082340	-8.19			Vicilin
Solyc05g051350		8.59		Rhamnogalacturonate lyase
Solyc08g079900			7.11	Subtilisin-like protease
Solyc01g079980			-1.48	Aspartyl protease family protein
Solyc11g022590			1.91	Trypsin inhibitor-like protein precursor
Solyc07g054600			1.20	F-box protein
Solyc01g099010			3.88	GDSL esterase/lipase
Solyc08g078870			3.51	Lipid-transfer protein
Solyc05g008220			2.62	PADRE gene family
Solyc02g081980			2.27	Apyrase

Table 2.2. Functional categories for differentially expressed genes DEGs) of interest detected with RNA-seq only at 72 hours post-mechanical inoculation (HPI) in the resistant *Solanum lycopersicum CNPH*-LAM 147, +*Sw-5b* line with *Tomato spotted wilt orthotospovirus.* DEGs with their corresponding logFc and annotation

Gene ID	Log FC	Annotation
Pathogenesis-related proteins		
Solyc08g080670	6.56	Pathogenesis-related 5-like protein
Solyc01g097280	2.39	Pathogenesis-related protein 4b
Solyc08g080640	4.63	NP24 protein precursor
Solyc04g016470	6.34	Beta(1,3) glucanase
Solyc08g080660\	3.76	Pathogenesis-related thaumatin family protein
Solyc02g090490	2.48	Patatin
Solyc10g055800	3.23	Chitinase
Transcription regulators		
Solyc12g009240	5.11	Ethylene-responsive transcription factor
Solyc12g010410	4.59	Homeobox protein knotted-1-like 3
Solyc05g015850	3.91	SlWRKY75
Solyc12g088400	3.25	Zinc-finger protein
Solyc02g091250	-3.41	Mediator of RNA polymerase II transcription subunit
Solyc04g064770	-5.65	Zinc finger transcription factor 34
Protein degradation		
Solyc04g008100	3.79	RING-type E3 ubiquitin ligase
Solyc05g055870	-2.61	F-box domain, Phloem protein 2-like protein
Solyc11g006740	-2.65	F-box domain, Phloem protein 2-like protein"
Cytochrome P450s		
Solyc04g083140	8.92	Cytochrome P450
Solyc12g045020	4.84	Cytochrome P450

Table 2.2. (continued).

Solyc12g042480	2.35	Cytochrome P450
Solyc07g006140	-1.66	Cytochrome P450
Transporters		
Solyc05g053610	6.66	Pleiotropic drug resistance protein
Solyc03g096540	6.64	PLAT/LH2 domain-containing protein
Solyc03g025250	-2.01	Multidrug resistance protein mdtK
Other genes		
Solyc12g100250	10.51	Fatty acid desaturase
Solyc09g008760	6.62	VQ motif-containing protein 29
Solyc01g101190	6.61	Terpene synthase
Solyc08g074682	6.22	Polyphenol oxidase
Solyc09g097810	5.26	SAR8.2 protein
Solyc02g077290	4.86	Glutamate receptor-like 1.2
Solyc01g105070	3.15	Peroxidase
Solyc04g071890	2.91	Peroxidase
Solyc05g005460	-1.87	Nucleoredoxin 2
Solyc03g098740	4.45	Kunitz trypsin inhibitor
Solyc04g079470	-3.04	Serpin
Solyc06g053710	1.92	Ethylene receptor homolog (ETR4)
Solyc11g008540	-1.34	Dicer-like 2b
Solyc05g055010	-1.39	RNA-binding protein 42
Solyc09g097860	-1.66	Kinesin-like protein KIN12B
Solyc05g025820	-4.23	Protein kinase

Table 2.3. Differentially expressed genes (DEGs) shared in the resistant *Solanum lycopersicum Sw-5b* isogenic line 'CNPH-LAM 147' (+*Sw-5b*) following inoculation with sap prepared from infected tissue (mechanical) or infected western flower thrips (Thrips) detected with RNA-seq. Six DEGs were shared between 24 HPTI and 72HPMI. Four DEGS were shared between 24- and 72-HPTI, and 72 HPMI. 16 DEGS shared between 72 HPTI and 72 HPMI. HPMI= hours post mechanical inoculation, HPTI = hours post thrips inoculation

Gene ID	Thrips inoculation		Mechanical inoculation	Annotation
	24 LogFC	72 LogFc	72 LogFc	
Solyc01g101180	6.33		5	Terpene synthase
Solyc02g070110	4.12		3.88	FAD-binding Berberine family protein
Solyc10g011925	2.63		3.14	Phenylalanine ammonia-lyase
Solyc03g115060	2.48		2.54	Suppressor SRP40-like protein
Solyc10g076510	2.25		2.22	Pyruvate decarboxylase
Solyc03g123540	-3.01		-3.09	Class III heat shock protein - HSP17.4
Solyc03g025670	4.55	3.7	5.19	Pathogenesis-related protein-1c
Solyc01g097240	4.13	4.48	6.4	Pathogenesis-related protein 4
Solyc09g006005	3.58	3.48	5.74	Pathogenesis-related protein 1
Solyc10g079860	2.81	2.23	4.68	Beta(1,3) glucanase
Solyc09g007010		8.26	6.48	Pathogenesis-related protein 1
Solyc04g064880		7.46	6.88	Pathogen-related protein
Solyc01g106620		6.02	6.26	Pathogenesis-related protein 1-like
Solyc08g080650		4.78	5.06	Pathogenesis related protein P23
Solyc02g082920		3.86	4.6	Class II chitinase
Solyc10g055810		3.16	4.92	Chitinase
Solyc05g054380		9.12	7.35	Major allergen Pru ar 1-like
Solyc02g078650		8.69	6.66	Polyphenol oxidase, chloroplastic
Solyc01g008620		8.46	6.86	Glucan endo-1,3-beta-glucosidase
Solyc01g095080		6.74	5.67	1-aminocyclopropane-1-carboxylic acid synthase-2
Solyc01g080570		5.78	5.32	Nucleoside hydrolase family protein
Solyc08g029000		5.77	7.24	Lipoxygenase
Solyc09g011870		5.33	4.49	Arogenate dehydrogenase 2
Solyc01g059965		5.32	5.76	Glucan endo-1,3-beta-glucosidase B
Solyc04g072280		5.01	5.88	Laccase
Solyc07g049530		4.56	3.61	1-aminocyclopropane-1-carboxylate oxidase 1

Chapter 3

Preliminary functional analysis of differentially expressed genes during *Sw-5b* NLR-mediated resistance to *Tomato spotted wilt orthotospovirus*

Abstract

The *Sw-5b* gene encodes a coiled-coil nucleotide-binding leucine-rich repeat receptor (CC-NLR) class of immune receptor that contains an extended N-terminal Solanaceae domain (SD) that is present in some CC-NLRs from solanaceous species. The *Sw-5b* NLR recognizes the movement protein, NSm, of *Tomato spotted wilt orthotospovirus* (TSWV), and induces a localized cell death response, the hypersensitive response (HR), at the site of infection that limits virus spread. Recent studies have revealed a unique two-step recognition process in which the *Sw-5b* NLR specifically recognizes a conserved 21 amino acid motif of NSm (NSm^{21}), and the N-terminal SD also directly interacts with NSm before resistance can be induced. These studies provided important insights on how *Sw-5b* recognizes the viral effector (NSm), but little information exists about players involved immediately downstream of effector recognition. Chapter 2 of this dissertation describes biological experiments and transcriptomic analyses aimed at elucidating the transcriptional changes that activate *Sw-5b*-mediated immune responses leading to HR, and virus containment at the site of infection. From this transcriptome analysis, my collaborators and I selected 15 differentially expressed genes (DEGs) of interest. In this chapter, I describe use of a *Tobacco rattle virus* (TRV)-based virus-induced gene silencing system (VIGS) and transgenic *Sw-5b Nicotiana benthamiana* plants to investigate the potential role of DEGs of interest in the downstream defense pathway following *Sw-5b* activation. In addition, I used this VIGS system to investigate the roles of the SGT1 and EDS1 genes in the *Sw-5*-mediated signaling pathway. I found that considerable variation occurred in the responses I obtained using the TRV-based VIGS system; therefore, I explored various experimental conditions that are described herein. While the outcomes of these experiments were not fully conclusive, TRV-based VIGS revealed two DEGs of interest, ERF1 and a receptor-like kinase

family protein, that are potentially required for *Sw-5b*-mediated resistance against TSWV and are worthy of further investigation. In addition, I confirmed that the chaperone SGT1 is required for *Sw-5b* function, while the *Sw-5b*-mediated signaling pathway was independent of EDS1. Based on the difficulty in replicating results from the TRV-based VIGS system, ERF1, the receptor-like kinase family protein, and other candidate DEGs should be further evaluated by using VIGS, RNAi and CRISPR/Cas9 approaches to definitively establish their role in *Sw-5b*-mediated resistance.

Introduction

Tomato spotted wilt orthotospovirus (TSWV) is the type member of the genus *Orthotospovirus* in the family *Tospoviridae*, order *Bunyavirales* (Nilon et al. 2021). TSWV is one of the most economically important plant viruses in global agriculture due to its broad host range of over 1500 plant species (Parrella et al. 2003; Scholthof et al. 2011; Gilbertson et al. 2015). One of the most important vectors of TSWV is the insect, *Frankliniella occidentalis* (Pergande), the western flower thrips (Wijkamp et al. 1996). A very important mode of crop protection against TSWV infection is through use of resistant varieties that carry *Sw-5b*. The *Sw-5b* gene encodes a coiled-coil nucleotide-binding leucine-rich repeat (CC-NLR) class of immune receptor that contains an extended N-terminal Solanaceae domain (SD) that is present in some CC-NLRs from solanaceous species. (De Oliveira et al. 2016; Zhu et al. 2017; Spassova et al. 2001; Chen et al. 2016).

Although some recent studies have provided insights on molecular mechanisms by which *Sw-5b* recognizes the TSWV movement protein (NSm), and activates immune responses, there is a paucity in understanding the processes that occur post-NSm recognition (Zhu et al. 2017; De Oliveira et al. 2016; de Oliveira et al. 2018). The SGT1 (Suppressor of the G2 allele of SKP 1)

chaperone required for the function of all studied plant NLRs to-date, is also required for the function of *Sw-5b* (Chen et al., 2021). It is widely accepted that the NLRs that contain the Toll-interleukin 1 receptor (TIR) homology domain at the N-terminus (TIR-NLRs) signal through EDS1 (Enhanced disease susceptibility 1), but not CC-NLRs (Aarts et al. 1998; Wiermer et al. 2005; Dongus and Parker 2021). Consistent with this, EDS1 is not required for the function of *Sw-5b* (Chen et al., 2021). Interestingly, NDR1 (Non-race-specific disease resistance 1) that is required for CC-NLR function does not play a role in *Sw-5b*-mediated immune signaling (Chen et al., 2021).

To uncover transcriptional changes that occur in *Sw-5b* plants in response to TSWV infection, I performed a time course RNA-seq analyses of mechanically inoculated and thrips-mediated inoculated TSWV samples on near isogenic lines of tomato (see Chapter 2 for details). The analysis identified a number of interesting genes that are differentially expresed in the resistant *Sw-5b* isoline in response to TSWV infection. In order to start evaluating the possible role of these differentially expressed genes (DEGs) in *Sw-5b*-mediated resistance, my collaborators and I selected 15 interesting candidates, and used a virus-induced gene silencing approach (VIGS) in transgenic *N. benthamiana* plants expressing *Sw-5b* to begin a functional analysis.

The VIGS approach has been widely used in plants for gene function studies (Kant and Dasgupta 2019; Dommes et al. 2019; Senthil-Kumar and Mysore 2011; Burch-Smith et al. 2004; Lacomme 2014; Baulcombe 1999). VIGS takes advantage of the natural defense mechanism plants have against viral dsRNA, which triggers post-transcriptional gene silencing (PTGS) (Robertson 2004; Becker and Lange 2010; Baulcombe 1999). In the VIGS approach, a short fragment of a gene that is targeted for silencing is cloned into a recombinant viral vector. When

the viral vector carrying the host gene fragment infects plants, it knocks down the expression of that gene by the RNAi. Several viruses have been utilized to develop VIGS vectors. The *Tobacco rattle virus* (TRV)-based VIGS vector is the most widely used system for gene function studies in dicotyledonous plants (Shi et al. 2021; Burch-Smith et al. 2004).

In this Chapter, I describe my attempts to optimize a TRV-based VIGS system to explore the function of DEGs of interest during the *Sw-5b*-mediated response to TSWV. Using VIGS, I confirmed that *Sw-5b*-mediated resistance to TSWV is dependent on SGT1 and independent of EDS1 as recently reported by Chen et al., (2021). Among the 15 candidate DEGs tested using TRV-based VIGS, the ethylene response factor 1 (ERF1) and a receptor-like kinase family protein are potentially required for *Sw-5b*-mediated resistance against TSWV. These two candidates and several other candidate DEGs that resulted in potentially interesting plant phenotypes should be further evaluated by using VIGS, RNAi and CRISPR/Cas9 approaches to firmly establish their role in *Sw-5b*-mediated resistance.

Materials and Methods:

Plasmid Construction

The tomato sequence of each DEG was used to search the *N.benthamiana* database at SOL Genomics Site (https://solgenomics.net/) to identify homologous genes in *N. benthamiana*. The target region for each of the genes was selected by using the VIGS tool at the SOL Genomics site (https://vigs.solgenomics.net/) (Supplementary Table 3.1). The synthesized VIGS target fragments (Genewiz Inc) were cloned into the TRV2 vector pYL156 as described in (Supplementary Table 3.2) (Liu et al. 2002). All clones were sequenced to confirm the insert identity.

Host plant and virus

The transgenic *Sw-5b N. benthamiana* line (*Nb::Sw-5b*) was provided by Dr. Xiaorang Tao, Nanjing Agricultural University, and was described in (Chen et al. 2021; Zhu et al. 2017). The *Nb::Sw-5b* plants were grown from seed in a plant growth cart at a temperature of 23°C and used for VIGS four weeks post-germination. The $TSWV^{MR-01}$ strain was used for all experiments (Adegbola et al. unpublished data) (see further description in Chapter 2), and the virus was maintained on *Datura stramonium.*

VIGS assay

VIGS using pTRV1 and pTRV2 was performed as described in (Liu et al. 2002). pTRV1, pTRV2 and its derivatives were introduced into *Agrobacterium* strain GV3101 by a heat shock method. A 4-milliliter (ml) culture with appropriate antibiotics was grown for 16 hours at 28°C and processed as described in (Liu et al. 2002). Agrobacterium cultures at $O.D._{600}$ =1.0 containing TRV1 and TRV2 or TRV2 derivatives were mixed (1:1 ratio), and infiltrated onto the two lower leaves of 4-week-old *Nb::Sw-5b* plants using a 1 ml-needle-less syringe. Three controls were included in all silencing experiments: TRV2-*Phytoene Desaturase* (*PDS*) gene fragment, TRV2 with the *Sw-5b* gene fragment, and a TRV2 empty vector. The photobleaching phenotype due to silencing of PDS was used as a visual sign of silencing being successful. The successful silencing of *Sw-5b* was revealed by the loss of resistance against TSWV resulting in a loss of HR and systemic infection. The TRV2 empty vector control was used to confirm that TRV alone has no effect on TSWV resistance. The infiltration of *Nb::Sw-5b* with TRV2 produced a range of phenotypes ranging from yellowing to leaf distortion and in other cases there were no TRV symptoms observed. At ten days post-VIGS, the plants were rub inoculated with sap prepared from $TSWV^{MR-01}$ infected plants. For experiments 1 and 2, virus inoculum was

prepared by grinding one gram of infected TSWV^{MR-01} *D. stramonium* leaf tissue in 15 ml of inoculation buffer (0.1M potassium phosphate, 1 mM sulphite, 1% celite, pH 7) with a mortar and pestle on ice. For experiments 3 and 4, the quantity of infected TSWV^{MR-01} *D. stramonium* leaf tissue was doubled. Inoculated plants were maintained in a controlled environment at 30°C under 16 light/8 dark photoperiod and 50% relative humidity, the phenotypes were recorded every day up to 12 days post-TSWV infection. TSWV symptoms ranged from necrosis with yellowing, stunting and ringspots. A striking difference from the yellowing sometimes observed with TRV2 alone.

ELISA assay

At 12 days post mechanical inoculation with TSWV^{MR-01}, newly emerged leaves of *Nb::Sw-5b* plants were collected and tested for TSWV infection with enzyme linked-immunosorbent assay (ELISA) using the reagent set from Agdia (Agdia, Elkhart, IN). ELISA was used to verify TSWV infection on TSWV symptomatic plants. Extracts of leaf samples were processed using a general extraction buffer. Optical density was read using a microplate reader (Bio-Rad, model 550) at 405n (A_{405}). TSWV infection was indicated when the A_{405} was ≥2-fold than the average of ELISA-controls (non-infected leaf sample).

Results

Silencing of *Sw-5b* leads to loss of resistance to TSWV

Since VIGS is relatively easy to perform in *N. benthamiana* and the transgenic *Sw-5b N. benthamiana* line (*Nb::Sw-5b*) has been shown to confer resistance to TSWV (Zhu et al. 2017; Chen et al. 2021), I used this plant line for the VIGS analysis. To determine whether *Nb*::*Sw-5b* could be used to examine the function of the selected DEGs that were differentially regulated during *Sw-5b*-mediated resistance, I first targeted *Sw-5b* with VIGS. Silencing of *Sw-5b* should

lead to a loss of resistance to TSWV (no HR and systemic TSWV infection, denoted as +TSWV).

Four-week-old *Nb::Sw-5b* plants were infiltrated with TRV1 and TRV2-*Sw-5b*, TRV2-*PDS* or TRV2 alone (empty vector control). The photobleaching *PDS* phenotype was visible by 10 days (Supplementary Figure 3.1) and was used as a visual indicator that the silencing procedure was successful. When photobleaching was observed in the PDS control, which consistently occurred at 10 days post-infiltration, plants from all treatments were then rub inoculated with $TSWV^{MR-01}$ onto upper systemic leaves (5-6 true leaves). In plants infiltrated with TRV2 alone (shown prior to challenge with TSWV in Supplementary Figure 3.2A), we observed necrotic cell death classically referred to as HR by 72 hours post TSWV inoculation (Figure 3.1, Supplementary Figure 3.2B). The symptoms we observed in *Nb::Sw-5b* challenged with TSWV (Supplementary Figure 3.3A) differ from previous studies by other researchers (Zhu et al., 2017), in that we observed classic HR lesions, while Zhu et al. (2017), who infiltrated with NSm rather than TSWV, observed cell death without the necrosis of HR. The phenotype of TRV2-*Sw-5b* plants prior to challenge with TSWV is shown in Supplementary Figure 3.3B. By seven days post TSWV inoculation, the TRV2-*Sw-5b* plants either had a complete loss of HR or had significantly reduced numbers of HR lesions compared to TRV2 alone controls (Supplementary Figure 3.3C). TSWV symptoms ranging from necrosis, stunting, and ringspots were observed on the TRV2-*Sw-5b* plants. Decreased HR, rather than a complete loss of HR when *Sw-5b* was silenced in the *Nb::Sw-5b* plants, could be attributed to variation in silencing efficiencies, which is one of the pitfalls of using VIGS (Burch-Smith et al. 2004). ELISA testing of the plants 12 days post TSWV inoculation (DPI) showed that all plants infiltrated with TRV2-*Sw-5b* and challenged with TSWV were systemically infected with TSWV, including those with

reduced HR. In contrast, the control plants infiltrated with the TRV2 alone and challenged with TSWV did not develop systemic TSWV infections nor symptoms (Supplementary Figure 3.4). These results showed that silencing of *Sw-5b* in *Nb::Sw-5b* plants via VIGS leads to loss of TSWV resistance. Therefore, I proceeded with use of TRV-based VIGS as an approach for functional characterization of selected DEGs identified in Chapter 2.

***Sw-5b*-mediated resistance to TSWV requires SGT1, but not EDS1**

I next used TRV-based VIGS to determine the function of two known immune regulators, SGT1 and EDS1. For this experiment, four-week-old transgenic *Nb::Sw-5b* plants were infiltrated with TRV1 and TRV2-*NbEDS1*, TRV2-*NbSGT1,* TRV2-*Sw-5b,* TRV2-*PDS* or TRV2 alone. At 12 days post-infiltration of TRV, newly emerged leaves of TRV2-*PDS* plants developed the strong silencing photobleaching indicating that silencing was functional. At that time all the treatment were rub inoculated with $TSWV^{MR-01}$. Figure 3.2 shows the TRV2 infiltrated plants developed HR by 72 HPI. Equivalent TRV2-*Sw-5b* plants inoculated with $TSWV^{MR-01}$ failed to develop HR (Figure 3.2) and systemic infection was confirmed from the newly emerged symptomatic leaves (stunting, yellowing, and ringspots) using ELISA (Supplementary Figure 3.5). By 72 HPI, TRV2-*NbEDS1* plants developed HR (Figure 3.2), and at 12 days post challenge, no systemic TSWV infection was observed nor detected with ELISA (Supplementary Figure 3.5). In contrast, HR was not observed for the TRV2-*NbSGT* plants inoculated with $TSWV^{MR-01}$ (Figure 3.2) and newly emerged symptomatic leaves confirmed systemic TSWV infection with ELISA (Supplementary Figure 3.5). These results indicate that functionality of *Sw-5b*-mediated resistance to TSWV requires SGT1, but not EDS1. These findings are consistent with a recently published report (Chen et al. 2021).

Silencing of selected DEGs reveals the potential importance of ERF1 and a receptor-like kinase, and several other DEGs for functionality of *Sw-5b*-mediated resistance to TSWV.

The silencing experiments described above for TRV2-*Sw-5b*, TRV2-*NbEDS1*, and TRV2-*NbSGT1* experiments established that VIGS should be useful in determining the role of DEGs identified from RNA-seq (Chapter 2) in *Sw-5b*-mediated resistance. The fifteen DEGs selected for screening with VIGS are shown in Table 3.1. Specific information about the selected DEGs can be found in Chapter 2 and this is summarized in the following paragraphs. Among the 15 selected DEGs, vicilin, ERF1, and the receptor-like kinase were only detected in response to thrips inoculation of TSWV. Five DEGs (alpha-dox, lipoxygenase, ALD-1, viroid-inducible protease II, and subtilisin-like protease) were detected in mechanical and thrips TSWV inoculations. The remaining seven DEGs (kinase family protein, mediator of RNA polymerase II transcription subunit, F-box protein PP2, F-box protein family, fatty acid desaturase, ethylene response factor A1, an R2R3 MYB transcription factor) were only detected in plants that were mechanically inoculated with TSWV. Of the 15, five DEGs were identified to be downregulated (F-box PP2, vicilin, F-box protein family, kinase family protein, and mediator of RNA polymerase II transcription subunit), whereas 10 DEGs were upregulated.

Experiment 1, VIGs of 15 selected DEGs

TRV1 and TRV2 with each DEG were infiltrated onto transgenic *Nb::Sw-5b* plants. The TRV2-*PDS* photobleaching phenotype typically occurred 10 days post silencing and served as a visual indicator of silencing (Supplemental Figure 3.1). Hence, 10-day post infiltration, when photobleaching occurred in TRV2-*PDS*, all treatments (2 plants/treatment), were challenged by mechanical inoculation of TSWV. Assessment of HR over time was recorded and testing for

systemic TSWV infection on upper leaves with ELISA was done at 12 DPI. The HR phenotypes are summarized in Table 3.1, and ELISA results are summarized in Supplemental Figure 3.6A.

The TRV2 infiltrated plants, with which an HR response would be expected, were used to verify that the inoculum method used for the mechanical inoculation was successful. When TRV2 infiltrated plants were challenged, they developed HR and newly emerged leaves did not develop symptoms, nor was TSWV infection detected with ELISA. In contrast, as expected, the phenotype of TRV2-*Sw-5b* plants was a loss of HR when challenged with TSWV, yellowing and ringspots in the newly emerging leaves, and systemic TSWV infections that were confirmed with ELISA.

Silencing of two of the DEGs, ERF 1 and the receptor like kinase, led to apparent loss of full *Sw-5b* functionality as indicated by the newly emerged leaves developing infection symptoms (stunting, yellowing and ringspots) and infection was confirmed with ELISA (Table 3.1, Supplementary Figure 3.6A). The TRV2- receptor-like kinase led to no HR on the inoculated leaves and systemic TSWV infection, suggesting silencing of this gene results in loss of *Sw-5b* functionality. HR was visible among TRV2-*ERF1* plants; however, a systemic infection also occurred, indicating that *Sw-5b*-mediated resistance was not fully functional, even though some HR was observed. It is possible that HR occurred on TRV-*ERF1* plants due lack of complete silencing efficiency, which is common with VIGS. These results suggested that ERF1 and the receptor like kinase may play a role in the signal transduction pathway of *Sw-5b*.

The six additional DEGs that were silenced (kinase family protein, mediator of RNA polymerase II transcription subunit, f-box PP2, fatty acid desaturase, R2R3MYB transcription factor, and lipoxygenase) resulted in loss of HR and systemic TSWV infection in one of the two plants used for silencing. Although loss of *Sw-5b* function was observed in only one of the two

plants silenced, these results suggest that these DEGs could play a role in *Sw-5b*-mediated resistance to TSWV. Insufficient silencing efficiency is one explanation for the difference we observed in phenotypes of these plants. When silencing occurred, we observed partial loss of *Sw-5b* functionality (HR, systemic TSWV infection), and where it did not occur, *Sw-5b* remained functional (HR, no systemic TSWV infection). In the future, measuring silencing efficiencies would be advisable, and it is possible that improvements could be made to the silencing constructs to obtain less variation in silencing efficiency.

Silencing of seven DEGs (vicilin, f-box protein family, ethylene response factor A1, ALD-1, subtilisin-like protease, alpha-DOX1 and viroid inducible protease inhibitor) did not alter the *Sw-5b* phenotype. These plants developed HR in the inoculated leaves by 72 HPI and no signs of systemic infection were observed, nor were they detected with ELISA by 12 DPI. It is possible that silencing of these genes may have minor effects that did not result in loss of resistance, or they may only be functional when TSWV is inoculated by thrips (e.g. vicilin), or their function may be better studied in tomato rather than *N. benthamiana*.

Experiment 2: Selection of the eight most interesting DEGs from Experiment 1, and increased numbers of silenced plants

Based on the results of Experiment 1, a second VIGS experiment was designed and repeated in two independent experiments with the eight most promising DEGs (ERF1, F-box protein PP2, R2R3MYB transcription factor, fatty acid desaturase, kinase family protein, lipoxygenase, mediator of RNA polymerase II transcription subunit, and receptor-like kinase protein) (Table 3.2). In this experiment, I inoculated 4 plants per silencing construct in each experiment. All the controls worked as expected in these experiments: TRV2-*PDS* resulted in the photobleaching phenotype, and TRV2-*Sw-5b* plants lacked production of HR in the TSWV

inoculated leaves (Table 3.2) and developed systemic TSWV infections as detected with ELISA (Supplementary Figure 3.6B). However, when the DEG silenced plants were challenged with $TSWV^{MR-01}$, all plants produced HR by 72 HPI (Table 3.2) and the newly emerged leaves did not develop infection symptoms nor was TSWV detected by ELISA (Supplementary Figure 3.6B). Thus, the findings regarding the DEGs of interest from Experiment 1 were not observed in Experiment 2, although the controls performed as expected, suggesting the silencing conditions were adequate and transmission of TSWV was successful.

Experiments 3 and 4: Increased TSWV inoculum and increased plant number/construct did not lead to more conclusive results

I hypothesized, with advice from my collaborators, that issues around silencing efficiencies, and inoculum concentration could explain the results from Experiment 2. Therefore, I performed two additional experiments (Experiments 3 and 4), in which the number of plants was increased to 12 plants/construct and the virus inoculum was increased from one gram ($TSWV^{MR-01}$ infected leaf tissue/15ml (buffer) to two grams /15ml. Experiment 3 included the mediator of RNA polymerase II transcription subunit, lipoxygenase, alpha-DOX1, and R2R3MYB transcription factor, and Experiment 4 included the two DEGs of greatest interest from Experiment 1 (receptor-like kinase family protein, ERF1), and the fatty acid desaturase,

The increase in plant number and virus inoculum led to unexpected phenotypes, even among the controls, which makes it difficult to interpret Experiments 3 and 4 (Table 3.3, 3.4). In Experiment 3 (Table 3.3, Supplementary Figure 3.6C), none of the treatments resulted in a complete loss of *Sw-5b* function (i.e., no HR and systemic infection), including the TRV-*Sw-5b* control that had caused the expected phenotype for loss of *Sw-5b* infection in previous experiments. In contrast, all of the treatments and the controls caused multiple phenotypes,

including a phenotype in which HR and systemic TSWV infection was observed. Whereas this result could suggest that these genes play a role in *Sw-5b*-mediated resistance, the observation of this phenotype in the TRV2 alone was unexpected and suggests that the virus pressure resulting from the increase in TSWV inoculum may have instead simply overwhelmed the *Sw-5b* immune response. Notably, the expected phenotype for the TRV2 alone was observed in one plant (HR, no systemic TSWV infection) and one plant failed to produce HR and was not systemically infected with TSWV. Further concerns about Experiment 3 results were raised by observations that were different from results of previous experiments. For example, in previous experiments TRV2-*Sw-5b* plants lost *Sw-5b* functionality as indicated by no development of HR and systemic TSWV infection. In contrast, in Experiment 3, the majority of TRV-*Sw-5b* control plants developed HR and systemic TSWV infection (4 plants) or developed no HR or systemic TSWV infection (2 plants). These findings are equally supportive of the possibility that poor silencing efficiency occurred, or that the increase in TSWV inoculum overwhelmed the *Sw-5b* immune response. Among the DEGs of interest screened in this experiment, *Sw-5b* function remained apparently fully intact for the lipoxygenase (1 plant), alpha-DOX1 (4 plants), and R2R3MYB transcription factor 62 (1 plant) (HR, -TSWV), but not for the mediator of RNA polymerase II transcription subunit. The unexpected results in the experimental controls make conclusive interpretation of this experiment impossible. However, the experiment does support further study of the DEGs of interest assayed.

In Experiment 4 (Table 3.4, Supplementary 3.6D), the expected phenotypes for the controls, TRV2 alone and TRV2-*Sw-5b* were not observed. There were four TRV2 alone infiltrated plants with no HR and no systemic infection. The other two TRV2 alone infiltrated plants developed in HR and were systemically infected with TSWV, a result certainly not

expected for the empty vector control. All six TRV2-*Sw-5b* controls exhibited an unexpected phenotype of no HR or systemic TSWV infection. Initially, I interpreted the results from the TRV2-*Sw-5b* controls as a problem with infectivity of the TSWV inoculum; however, systemic infection of some TRV2 infiltrated plants (2 plants) and multiple plants in each of the experimental treatments show that the inoculum was infectious. There is no simple explanation for the lack of both HR and systemic infection among the controls. As in Experiment 3, plants in each of the experimental treatments had HR and systemic TSWV infection, a phenotype that could suggest either that these genes play a role in *Sw-5b*-mediated resistance or could support a conclusion that the increase in virus inoculum overwhelmed *Sw-5b*. At the same time, a relatively large number of plants in each experimental treatment also failed to develop HR and were not systemically infected with TSWV, a counter intuitive finding when increased inoculum was used.

Among the TRV2-*receptor-like kinase family protein* plants; six produced HR followed by detection of systemic infection, whereas the other six plants lacked HR and no systemic infection was observed. Five of the TRV2-*ERF1* plants were observed to produce HR and lacked systemic infection of TSWV, whereas an additional five TRV2-*ERF1* plants lacked both HR systemic TSWV infection. The remaining two TRV2-*ERF1* plants had HR and were systemically infected with TSWV. Among the 12 TRV2-*fatty acid desaturase* plants, five produced HR and were systemically infected with TSWV. A lack of both HR and systemic infection was observed in four of the TRV2-*fatty acid desaturase* plants, and HR and systemic TSWV infection were observed in the other four plants. As with other treatments in this experiment, these results may have occurred due to poor silencing efficiencies, or because the high inoculum amount overwhelmed *Sw-5b* functionality.

Overall, the results from experiments 3 and 4 were inconclusive because of the diverse and largely inexplicable results. Doubling of the inoculum concentration relative to Experiments 1 and 2 may have overwhelmed *Sw-5b* resistance. It could also have resulted in concentration of plant compounds that could inhibit consistent mechanical inoculation of the virus in some cases. The increased number of plants being silenced in Experiment 3 and 4 raises the possibility that technical issues occurred in implementation of the VIGS system, resulting in poor silencing efficiencies. In future experiments, it will be important to estimate silencing efficiencies experimentally to control for these potential problems and to better optimize the VIGS system. It is also possible that inconsistencies arose from trying to examine genes of interest using *N. benthamiana* rather than tomato. Although the experiments described in this chapter were not fully repeatable, they do suggest that exploration of the potential role of the tested DEGs in *Sw-5b*-mediated resistance are warranted. It was my intention to pursue studies on silencing efficiencies with a goal of repeating these experiments; however, the campus-wide lockdown due to COVID prevented my access to the laboratory, and upon my return, there was limited access to the plates required for RT-qPCR. Thanks to the laboratory of Joanna Chui, I acquired plates for validation of my RNA-seq results and TSWV quantification reported in Chapter 2; however, as replacement supplies failed to be delivered from multiple sources, I did not have plates for assessment of silencing efficiencies. VIGS in tomato can be difficult to optimize, but future investigations might consider using the isogenic lines described in Chapter 2 or tomato transgenic for *Sw-5b*, to explore the potential role of the DEGs I have described. Although the experiments described in this Chapter were not fully conclusive, they provide an important foundation for continuing work aimed at exploring the role of these DEGs in *Sw-5b*-mediated resistance.

Discussion

TRV-based VIGS has been used in various pathosystems as a molecular screening tool to perform functional analysis of genes of interest (Robertson 2004; Ratcliff et al. 2001; Lacomme and Chapman 2008). The results of my experiments show that TRV-based VIGS using *Nb::Sw-5b* plants can be used to study the roles of genes in functionality of *Sw-5b* with some caveats. This conclusion is supported by two lines of evidence. First, silencing of *Sw-5b* resulted in a complete loss of resistance that included loss of HR and development of typical spotted wilt symptoms (stunting, yellowing and ringspots) followed by systemic TSWV infection (Figure 3.1). Secondly, silencing of SGT1 resulted in complete loss of *Sw-5b* immune signaling, whereas silencing of EDS1 did not affect *Sw-5b* function (Figure 3.2). These latter findings also confirm the work published from another laboratory that shows that SGT1 silencing compromises *Sw-5b*-mediated resistance to TSWV, while EDS1 is not required for *Sw-5b* function (Chen et al., 2021). Although most TIR-NLRs require EDS1, only few CC-NLRs, such as HRT and RPW8 have been shown to require EDS1 (Wiermer et al. 2005; Xiao et al. 2005; Chandra-Shekara et al. 2004). Silencing of EDS1 had no effect on HR production or resistance to TSWV infection indicating that like many CC-NLRs, *Sw-5b* also does not require EDS1 for immune signaling.

Silencing Experiment 1 showed that eight of the 15 DEGs chosen for testing showed phenotypes suggesting a potential functional role in *Sw-5b*-mediated resistance (loss of HR followed by systemic TSWV infection or HR followed by systemic TSWV infection). Most interesting were the ethylene responsive factor 1 (ERF1) and the receptor-like kinase, because silenced plants were 100% systemically infected with TSWV (Supplementary Figure 3.6A). Although silencing of ERF1 did not hinder production of HR, plants developed typical spotted

wilt symptoms, and systemic infection of TSWV, making this a gene of interest for further studies. Silencing of the receptor-like kinase resulted in a complete loss of HR and a systemic TSWV infection in both silenced plants. These results indicate that silencing of the receptor-like kinase family protein and ERF1 can drastically alter the functionality of *Sw-5b*. ERF1 is a transcription factor known to play a role in jasmonic acid and ethylene-dependent defense signaling pathway (Huang et al. 2016; Kim et al. 2014). Receptor-like kinase family proteins have been found to play a role in directly interacting or for pathogen detection (Zeng et al. 2012; Kim et al. 2009). A study found Bti9, a receptor-like kinase protein in tomato, directly interacted with AvrPtoB, and when silenced, immunity response was compromised, resulting in enhanced disease symptoms (Zeng et al. 2012). I hypothesize that the receptor-like kinase family protein might be required for S*w-5b* resistance, resulting in a complete loss of function when silenced. Whereas these were exciting results, my inability to replicate these findings in Experiments 2 and 3 do not allow for strong conclusions about the role of these genes in *Sw-5b*-mediated resistance. They do however, suggest that further experimentation is warranted. Although many TRV-VIGS systems have been optimized for host and vector systems, my data indicates that careful attention to silencing efficiencies with different constructs may be important for consistent replication of results. Before going forward with additional experiments, it will be important to measure silencing efficiency of each construct.

Experiments 3 and 4 also produced interesting findings where systemic TSWV infection and symptoms were observed in more plants than in all other experiments. However, systemic infection phenotypes could also be explained by the increase in the inoculum concentration. Since the virus inoculum was doubled, the systemic infection observations could have occurred because the high inoculum overcame the functionality of *Sw-5b*, and may not have occurred

specifically due to silencing of particular genes. This is observed in the field with *Sw-5b* tomatoes under high thrips and virus pressure. Taken together, the observations from all the silencing experiments do suggest that the 15 selected DEGs, (vicilin, ERF1, receptor-like kinase, Alpha-dox, lipoxygenase, ALD-1, viroid-inducible protease II, subtilisin-like protease, kinase family protein, mediator of RNA polymerase II transcription subunit, F-box protein PP2, F-box protein family, fatty acid desaturase, ethylene response factor A1, an R2R3 MYB transcription factor) could have an impact on the functionality of *Sw-5b*, and should continue to be investigated. Further investigations may profit from use of other molecular tools, i.e. CRISPR/Cas9 approaches, to firmly establish the role of these DEGs in *Sw-5b*-mediated resistance.

Since thrips do not feed on *N. benthamiana,* VIGS in this plant species limited our assessment of gene functionality to mechanically inoculation. Some of the DEGs of interest may only be important when thrips inoculate TSWV (see Chapter 2, e.g. vicilin); hence, evaluation of the DEGs in tomato will be important so thrips inoculations can be used in following up on the function of specific DEGs. Although the silencing constructs used with *Nb*::*Sw-5b* were created by identifying the homologous genes from tomato, there is also a possibility that the genes in *Nb*::*Sw-5b* might not play the same roles during *Sw-5b*-mediated resistance against TSWV as they do in tomato.

In summary, I successfully used VIGS to silence *Sw-5b* and established that VIGS could be used as a preliminary genomic tool to perform functional assessment of genes that may play an integral role in *Sw-5b*-mediated resistance against TSWV. I was able to explore the role of SGT1 and EDS1 in the *Sw-5b* immune response and found that silencing SGT1 compromised *Sw-5b*-mediated resistance. These data indicated that SGT1 is required for *Sw-5b* to mediate

resistance against TSWV, while EDS1 is not. Preliminary functional assessment using VIGS showed silencing of 8 out of 15 DEGs of interest compromised *Sw-5b*-mediated resistance against TSWV. ERF1 and a receptor-like kinase family protein are of particular interest because the silencing of these genes resulted in a compromised HR and systemic TSWV infection, similar to when *Sw-5b* was silenced. However, other silencing experiments were highly variable, perhaps due to silencing efficiency, a common pitfall of VIGS. Based on the difficulty in replicating results I observed with the TRV-based VIGS system, ERF1, the receptor-like kinase family protein, and other candidate DEGs should be further evaluated by using VIGS, RNAi and CRISPR/Cas9 approaches to firmly establish their role in *Sw-5b*-mediated resistance.

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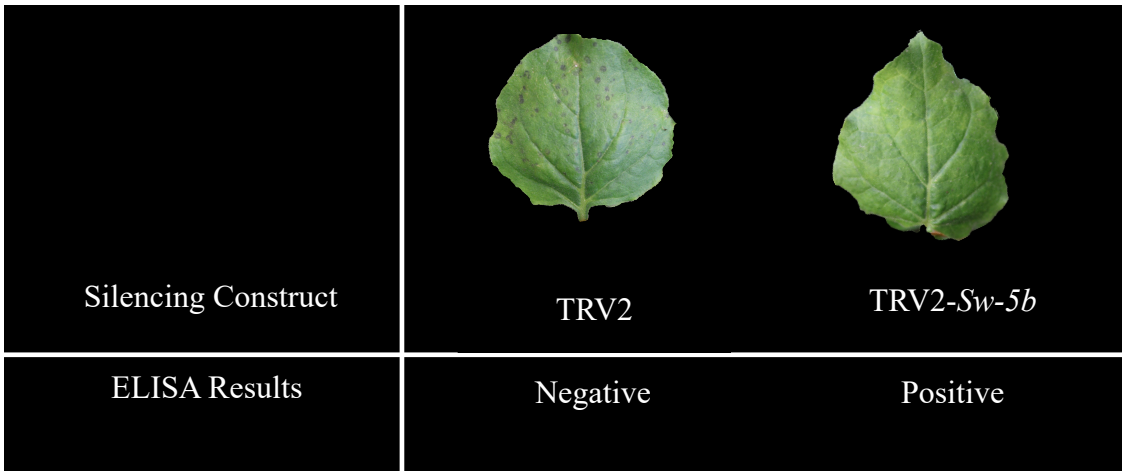


Figure 3.1. Phenotype of transgenic *Sw-5b Nicotiana benthamiana* used for virus-induced gene silencing of *Sw-5b*. At 12 days post *Tomato spotted wilt orthotospovirus* (TSWV) inoculation, new growth from the silenced plant was tested for systemic infection using enzyme-linked immunosorbent assay (ELISA). The TRV2 has an empty multiple cloning site and this allowed us to verify that TRV does not affect the *Sw-5b* immune response to TSWV. Consequently, a hypersensitive response (HR) occurred and there was no systemic TSWV infection detected by ELISA. Infiltration with TRV2-*Sw-5b* resulted in a complete loss of function resulting in no HR and systemic TSWV infection of TSWV detected by ELISA.

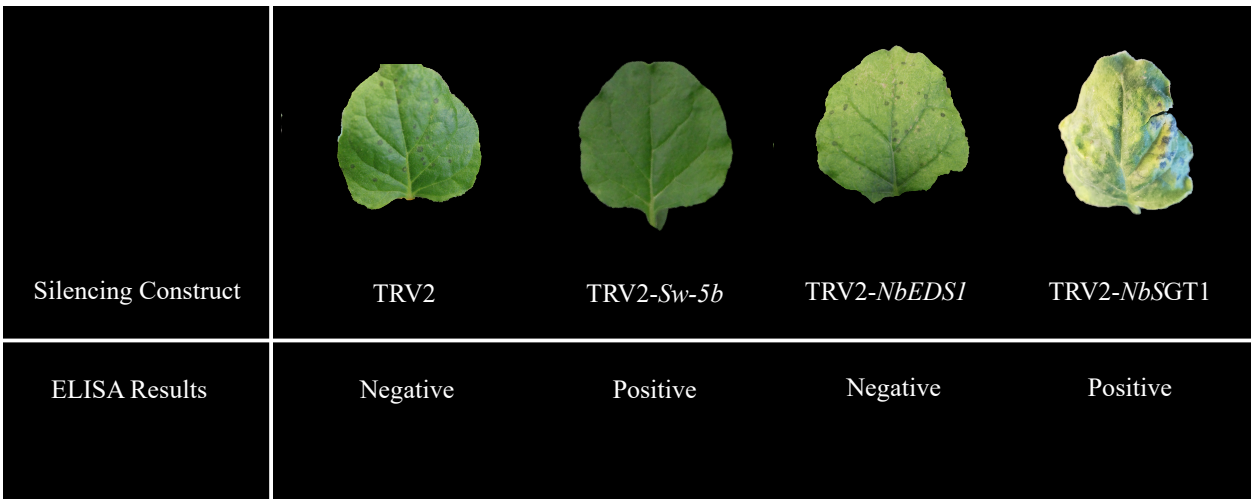
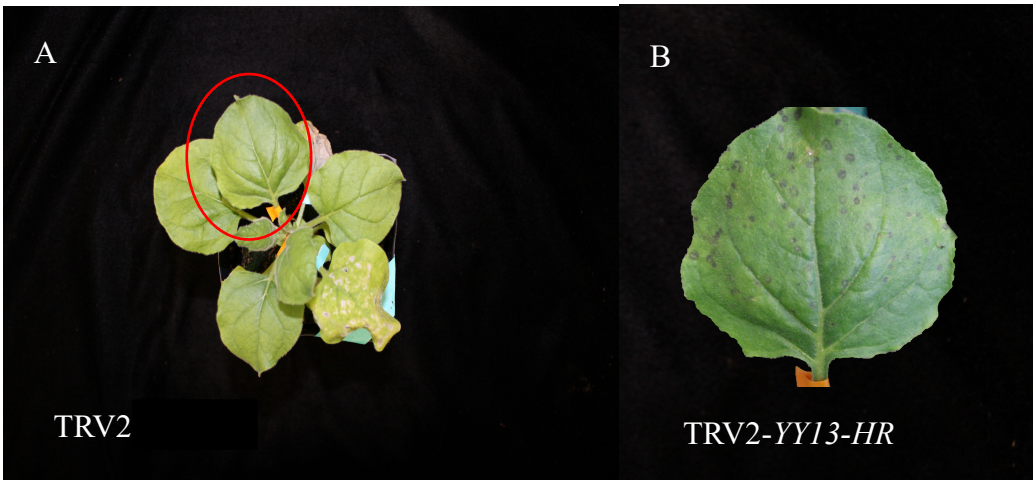
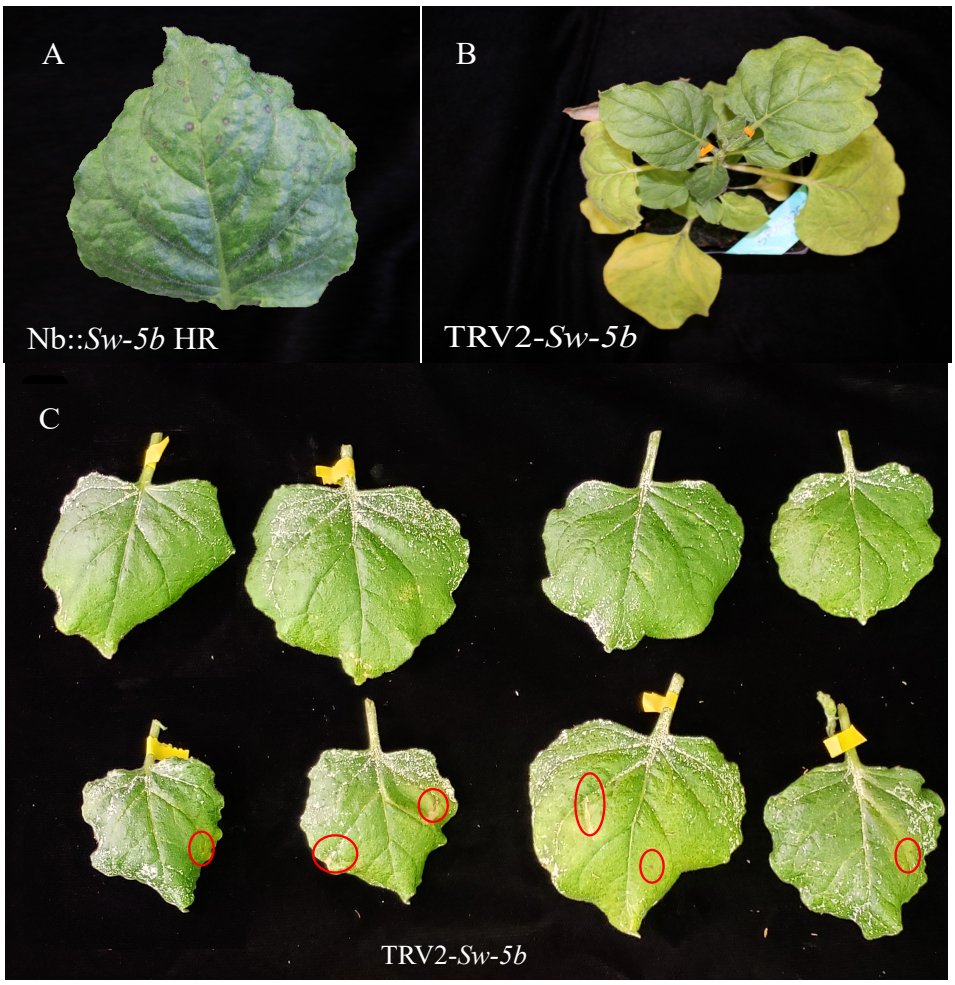


Figure 3.2. Phenotype of the transgenic *Sw-5b Nicotiana benthamiana* following silencing of EDS1 and SGT1. Silenced plants were mechanically inoculated with *Tomato spotted wilt orthotospovirus (*TSWV) and 12 days post inoculation assessed for systemic TSWV infection via enzyme-linked immunosorbent assay. Silencing controls included: TRV2 and TRV2-*Sw-5b.* When *TRV2-NbEDS1* plants were challenged with TSWV, a hypersensitive response (HR) based immunity response preventing systemic infection occurred. In comparison, TVR-*NbSGT1* failed to produce HR and resulted in a systemic TSWV infection.

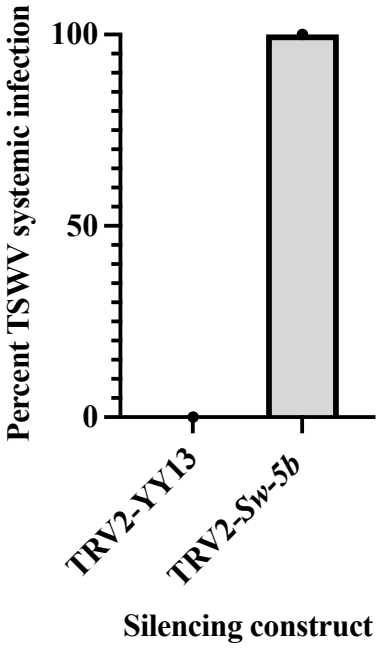
Supplementary Figure 3.1. Transgenic *Sw-5b Nicotiana benthamiana* infiltrated with TRV2-*Phytoene Desaturase* (*PDS*) exhibiting the photobleaching phenotype used as a visual sign of successful silencing (10 days post infiltration).



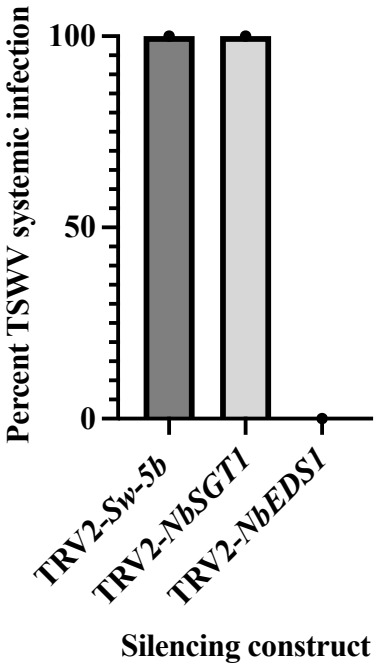
Supplementary Figure 3.2. Phenotype of the transgenic *Sw-5b Nicotiana benthamiana* (Nb::*Sw-5b*) infiltrated with TRV. (A) Phenotype of a Nb::*Sw-5b* plant at 10 days post infiltration with TRV2. The red circle denotes the silenced leaf to be challenged with *Tomato spotted wilt orthotospovirus* (TSWV) inoculation. (B) Hypersensitive response (HR) visible on the leaf within the red circle from (A) at 72 hours post TSWV inoculation.



Supplementary Figure 3.3. Phenotypes of the transgenic *Sw-5b Nicotiana benthamiana* (Nb::Sw-5b) following infiltration with TRV2-Sw-5b. (A) Inoculation of Nb::Sw-5b with *Tomato spotted wilt orthotospovirus* (TSWV) developed hypersensitive response (HR) by 72 hours post inoculation. (B) Nb::*Sw-5b* at 10 days post infiltration with TRV2-Sw-5b, but prior to TSWV inoculation. (C) Phenotypes observed with Nb::*Sw-5b* plants infiltrated with TRV2-*Sw-5b* and challenged with TSWV. Plants in the top row exhibited a complete loss of HR, while others, shown in the lower row significantly reduced HR (denoted by red circles).

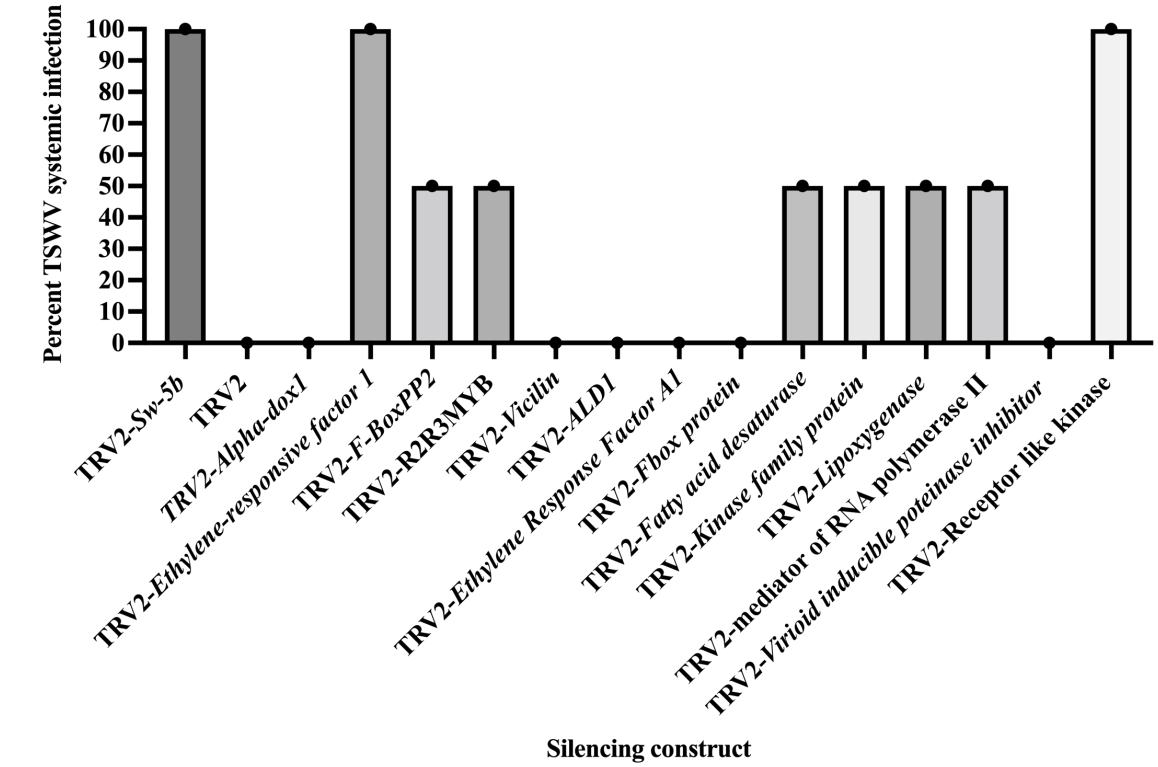


Supplementary Figure 3.4. Percent systemic infection with *Tomato spotted wilt orthotospovirus* (TSWV) in transgenic *Sw-5b Nicotiana benthamiana* infiltrated with TRV2 and TRV2-*Sw-5b* as indicated by testing of new growth with enzyme-linked immunosorbent assay 12 days post inoculation.

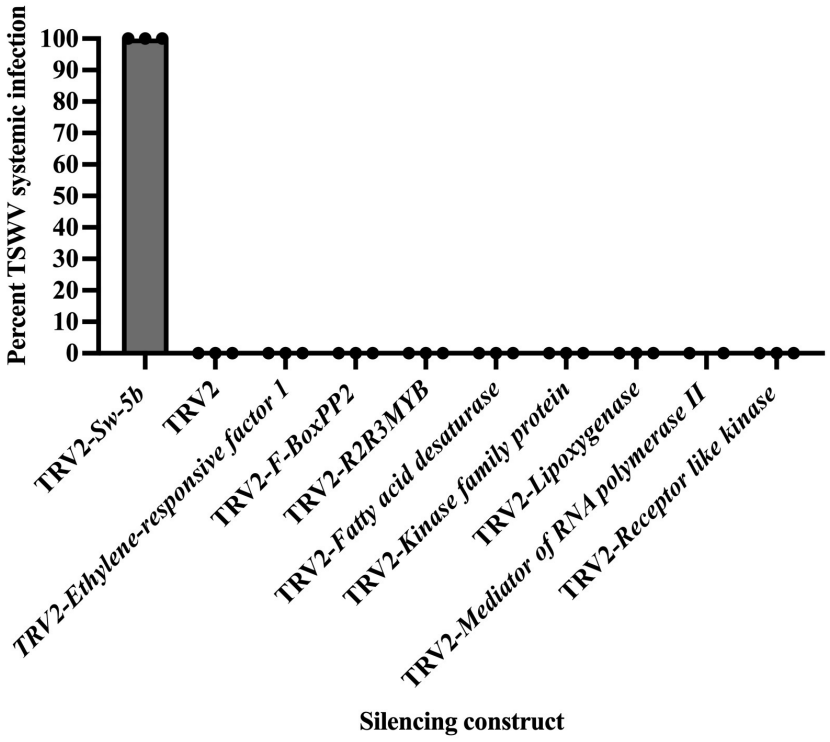


Supplementary Figure 3.5. Percent systemic infection of *Tomato spotted wilt orthotospovirus* (TSWV) on *Sw-5b Nicotiana benthamiana* plants infiltrated with TRV2-*Sw-5b*, TRV2-*NbSGT1*, and TRV2-*NbEDS1* at 12 days post inoculation assayed by enzyme-linked immunosorbent assay. n=10 plants /silencing construct.

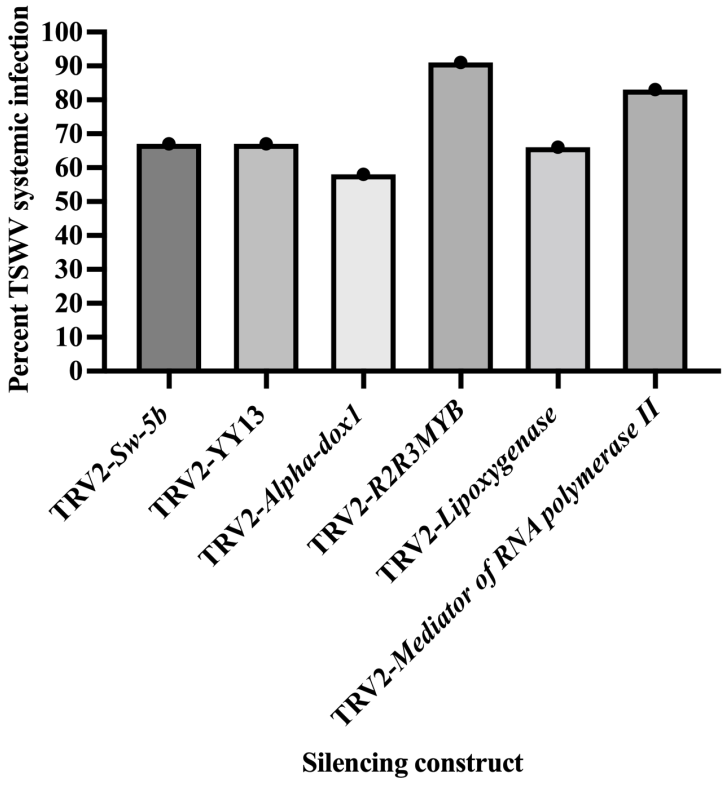
A


Percent TSWV systemic infection
100
90
80
70
60
50
40
30
20
10
0
TRV2-Sw-5b
TRV2
TRV2-Alpha-dox1
TRV2-Ethylene-responsive factor 1
TRV2-F-BoxPP2
TRV2-R2R3MYB
TRV2-Vicilin
TRV2-ALD1
TRV2-Ethylene Response Factor A1
TRV2-Fbox protein
TRV2-Fatty acid desaturase
TRV2-Kinase family protein
TRV2-Lipoxygenase
TRV2-mediator of RNA polymerase II
TRV2-Virioid inducible poteinase inhibitor
TRV2-Receptor like kinase
Silencing construct

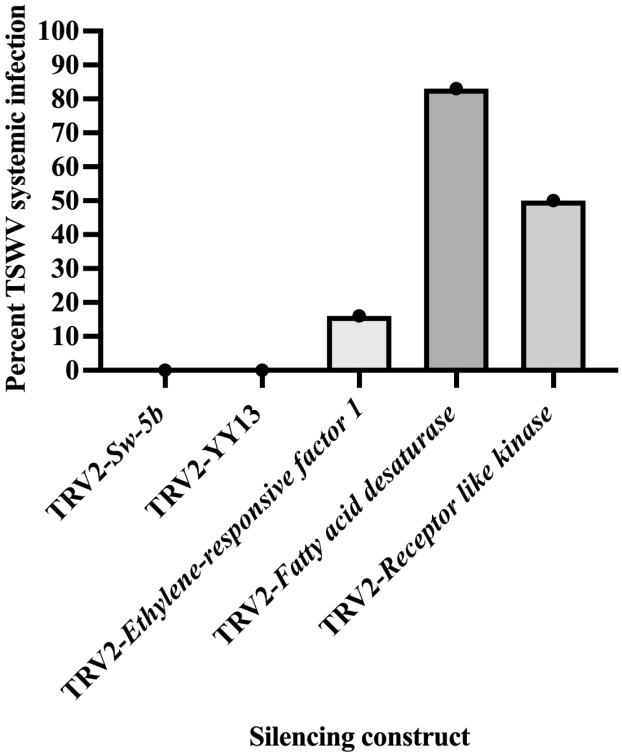
B


Percent TSWV systemic infection
100
90
80
70
60
50
40
30
20
10
0
TRV2-Sw-5b
TRV2
TRV2-Ethylene-responsive factor 1
TRV2-F-BoxPP2
TRV2-R2R3MYB
TRV2-Fatty acid desaturase
TRV2-Kinase family protein
TRV2-Lipoxygenase
TRV2-Mediator of RNA polymerase II
TRV2-Receptor like kinase
Silencing construct

C


Percent TSWV systemic infection
100
90
80
70
60
50
40
30
20
10
0
TRV2-Sw-5b
TRV2-YY13
TRV2-Alpha-dox1
TRV2-R2R3MYB
TRV2-Lipoxygenase
TRV2-Mediator of RNA polymerase II
Silencing construct

D


Percent TSWV systemic infection
100
90
80
70
60
50
40
30
20
10
0
TRV2-Sw-5b
TRV2-YY13
TRV2-Ethylene-responsive factor 1
TRV2-Fatty acid desaturase
TRV2-Receptor like kinase
Silencing construct

Supplementary Figure 3.6. Percent systemic infection with Tomato spotted wilt orthotospovirus (TSWV) of the transgenic *Sw-5b Nicotiana benthamiana* plants with genes of interest silenced. Infection status was assayed with enzyme linked-immunosorbent assay at 12 days post inoculation. All experiments included TRV2-*Sw-5b* and TRV2 as controls. (A). Systemic TSWV infection of the silenced plants from Experiment 1 (n=2/silencing construct). The eight DEGs showing partial or complete loss of *Sw-5b* function were selected for further testing as shown in (B). (B) Results of Experiment 2 (Replicate 1 and Replicate 2) examining systemic TSWV infection following infiltration with eight silencing constructs of interest from Experiment 1 (n=4 plants/silencing construct). (C) Systemic TSWV infection in Experiment 3, in which plants were infiltrated with silencing constructs and the inoculum used to challenge them, and number of plants was increased (n=6 plants/control, n=12 plants/silencing construct). (D) Systemic TSWV infection from Experiment 4, in which plants were infiltrated with different silencing constructs than Experiment 3, but the other treatment conditions were the same as Experiment 3 (n=6 plants/control, n=12 plants/silencing construct).

Table 3.1. Experiment 1: phenotypes observed with transgenic *Sw-5b Nicotiana benthamiana* using *Tobacco rattle virus* (TRV) based virus-induced gene silencing of 15 differentially expressed genes selected for functional assessment. Controls included: TRV2 and TRV2-*Sw-5b.* Silenced plants were mechanically inoculated with *Tomato spotted wilt orthotospovirus* (TSWV). Visual assessment of a hypersensitive response (HR) was recorded daily. Systemic TSWV infection was verified using enzyme-linked immunosorbent assay. Systemic infection= +TSWV; non-systemic/non-infected= -TSWV; n= 2 plants/construct.

Construct	Gene annotation	Phenotype
YY13	TRV2	2HR/2-TSWV
SPDK3255	TRV2-*Sw-5b*	2 no HR/2+TSWV
SPDK3921	Alpha-DOX1	2HR/2-TSWV
SPDK3922	Ethylene-responsive transcription factor 1	2HR/2 +TSWV
SPDK3923	F-box protein PP2	1HR/-TSWV; 1 no HR/+TSWV
SPDK3924	R2R3MYB transcription factor 62	1HR/-TSWV; 1 no HR/+TSWV
PDK3925	Vicilin	2HR/2-TSWV
SPDK3926	ALD1 - Aspartate aminotransferase, putative	2HR/2-TSWV
SPDK3927	Ethylene Response Factor A.1	2HR/2-TSWV
SPDK3928	F-box protein family	2HR/2-TSWV
SPDK3929	Fatty acid desaturase	1HR/-TSWV; 1 no HR/+TSWV
SPDK3930	Kinase family protein	1HR/-TSWV; 1 no HR/+TSWV
SPDK3931	Lipoxygenase	1HR/-TSWV; 1 no HR/+TSWV
SPDK3932	Mediator of RNA polymerase II transcription subunit	1HR/-TSWV; 1 no HR/+TSWV
SPDK3933	Viroid-inducible proteinase inhibitor II	2HR/2-TSWV
SPDK3934	Receptor-like kinase family protein	2 no HR/ +TSWV
SPDK3935	Subtilisin-like protease	2HR/2-TSWV

Table 3.2. The phenotypes from Experiment 2 (Rep 1 and Rep 2) of the transgenic *Sw-5b Nicotiana benthamiana* using *Tobacco rattle virus* (TRV) based virus-induced gene silencing of the eight differentially expressed genes showing promise in Experiment 1. The silencing controls included TRV2, TRV2-*Sw-5b.* Silenced plants were mechanically inoculated with *Tomato spotted wilt orthotospovirus (TSWV)* (n= 4 plants/construct). Visual assessment of a hypersensitive response (HR) was recorded daily. Systemic infection of TSWV was assayed using enzyme-linked immunosorbent assay. Systemic infection= +TSWV, non-systemic/non-infected= -TSWV.

Construct	**Gene annotation**	**Phenotype**	
		Rep 1	**Rep 2**
YY13	TRV2	4HR/-TSWV	4HR/-TSWV
SPDK3255	TRV-*Sw-5b*	4NO HR/ +TSWV	4NO HR/ +TSWV
SPDK3922	Ethylene-responsive transcription factor 1	4HR/-TSWV	4HR/-TSWV
SPDK3923	F-box protein PP2	4HR/-TSWV	4HR/-TSWV
SPDK3924	R2R3MYB transcription factor 62	4HR/-TSWV	4HR/-TSWV
SPDK3929	Fatty acid desaturase	4HR/-TSWV	4HR/-TSWV
SPDK3930	Kinase family protein	4HR/-TSWV	4HR/-TSWV
SPDK3931	Lipoxygenase	4HR/-TSWV	4HR/-TSWV
SPDK3932	Mediator of RNA polymerase II transcription subunit	4HR/-TSWV	4HR/-TSWV
SPDK3934	Receptor-like kinase family protein	4HR/-TSWV	4HR/-TSWV

Table 3.3. Phenotypes of Experiment 3 on the transgenic *Sw-5b Nicotiana benthamiana* using virus-induced silencing of the mediator of RNA polymerase II transcription subunit, lipoxygenase, alpha-DOX1 and R2R3MYB transcription factor. The silencing controls included TRV2 and TRV2-*Sw-5b.* Silenced plants were mechanically inoculated with *Tomato spotted wilt orthotospovirus (TSWV)* at 2g of infected material/15 ml of buffer. Visual assessment of hypersensitive response (HR) was recorded daily. Systemic infection of TSWV was assayed using enzyme-linked immunosorbent assay. Systemic infection= +TSWV; non-systemic/non-infected= -TSWV; n=12/construct and n=6/controls

Construct	Gene annotation	HR/ -TSWV	No HR/ -TSWV	HR/ +TSWV	No HR/ +TSWV
YY13	TRV2	1	1	4	0
SPDK3255	TRV2-*Sw-5b*	0	2	4	0
SPDK3932	Mediator of RNA polymerase II transcription subunit	0	2	10	0
SPDK3931	Lipoxygenase	1	3	8	0
SPDK3921	Alpha-DOX1	4	1	7	0
SPDK3924	R2R3MYB transcription factor	1	0	11	0

Table 3.4. Phenotypes of Experiment 4 on the transgenic *Sw-5b Nicotiana benthamiana* using virus-induced silencing of the receptor like-kinase family protein, ethylene-responsive transcription factor 1and fatty acid desaturase. The VIGS controls included TRV2, *Phytoene Desaturase* (PDS) and the *Sw-5b.* Silenced plants were mechanically inoculated with sap prepared from *Tomato spotted wilt orthotospovirus* (TSWV)infected leaves. Two grams of infected material/15 ml of buffer. Visual assessment of a hypersensitive response (HR) was recorded daily. TSWV symptoms were observed and confirmed using enzyme-linked immunosorbent assay. Systemic infection= +TSWV; non-systemic/non-infected= -TSWV; n=12/construct and n=6/control.

Construct	Gene annotation	HR/ -TSWV	No HR/ -TSWV	HR/ +TSWV	No HR/ +TSWV
YY13	TRV2	0	4	2	0
SPDK3255	TRV2-*Sw-5b*	0	6	0	0
SPDK3934	Receptor-like kinase family protein	0	6	6	0
SPDK3922	Ethylene-responsive transcription factor 1	5	5	2	0
SPDK3929	Fatty acid desaturase	3	4	5	0