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The *Sw-5b* NLR Immune Receptor Induces Early Transcriptional Changes in Response to Thrips and Mechanical Modes of Inoculation of *Tomato spotted wilt orthotospovirus*

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The NLR (nucleotide-binding leucine-rich repeat) class immune receptor *Sw-5b* confers resistance to *Tomato spotted wilt orthotospovirus* (TSWV). Although *Sw-5b* is known to activate immunity upon recognition of the TSWV movement protein NSm, we know very little about the downstream events that lead to resistance. Here, we investigated the *Sw-5b*–mediated early transcriptomic changes that occur in response to mechanical and thrips-mediated inoculation of TSWV, using near-isogenic tomato lines CNPH-LAM 147 (*Sw5b*$^{+/+}$) and Santa Clara (*Sw-5b*$^{-/-}$). We observed earlier *Sw-5b*–mediated transcriptional changes in response to thrips-mediated inoculation compared with that in response to mechanical inoculation of TSWV. With thrips-mediated inoculation, differentially expressed genes (DEGs) were observed at 12, 24, and 72 h postinoculation (hpi). Whereas with mechanical inoculation, DEGs were observed only at 72 hpi. Although some DEGs were shared between the two methods of inoculation, many DEGs were specific to either thrips-mediated or mechanical inoculation of TSWV. In response to thrips-mediated inoculation, an NLR immune receptor, cysteine-rich receptor-like kinase, G-type lectin S-receptor-like kinases, the ethylene response factor 1, and the calmodulin-binding protein 60 were induced. Fatty acid desaturase 2-9, cell death genes, DCL2b, RIPK/PBL14-like, ERF017, and WRKY75 were differentially expressed in response to mechanical inoculation. Our findings reveal *Sw-5b* responses specific to the method of TSWV inoculation. Although TSWV is transmitted in nature primarily by the thrips, *Sw-5b* responses to thrips inoculation have not been previously studied. Therefore, the DEGs we have identified in response to thrips-mediated inoculation provide a new foundation for understanding the mechanistic roles of these genes in the *Sw-5b*–mediated resistance.

Keywords: differentially expressed genes, NLR immune receptor, RNA-seq, *Sw-5b*, tomato, *Tomato spotted wilt orthotospovirus* (TSWV)

Tomato spotted wilt orthotospovirus (TSWV) is one of the most economically important plant viruses affecting tomato production worldwide (Oliver and Whitfield 2016; Zhu et al. 2019) and is the type member of the genus *Orthotospovirus*, family *Tospoviridae*, order *Bunyavirales*, 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 (Zhu et al. 2019). TSWV can be transmitted mechanically with infected sap (Rotenberg et al. 2015); however, in nature, the western flower thrips *Frankliniella occidentalis* (Pergande) is the primary thrips species transmitting orthotospoviruses. Transmission by the insect is in a circulative-propagative manner, and the orthotospoviruses 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 (Montero-Astua et al. 2016; Ullman et al. 1993a and b, 1995; Whitfield et al. 2005).

Several TSWV resistance genes (*Sw-1a*, *Sw-1b*, *Sw-2*, *Sw-3*, *Sw-4*, *Sw-5*, *Sw-6*, and *Sw-7*) have been described in tomatoes (Brommonschenkel et al. 2000; Dianese et al. 2011). The most effective among them, with regards to orthotospovirus resistance, is *Sw-5*, a single dominant resistance gene locus that was introgressed from *Solanum peruvianum* to a commercial tomato line, Stevens (Stevens 1964). The gene was mapped to the end of chromosome nine (Brommonschenkel et al. 2000; de Oliveira et al. 2018). Within the *Sw-5* gene cluster, *Sw-5a* has been shown

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to be nonfunctional against TSWV (De Oliveira et al. 2016; Hallwass et al. 2014; Spassova et al. 2001) 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 tomato-infecting orthotospoviruses (Boiteux and Giordano 1993; De Oliveira et al. 2016, 2018; Spassova et al. 2001). The *Sw-5b* gene encodes a coiled-coil nucleotide-binding leucine-rich repeat (CC-NLR) class of immune receptors (Brommonschenkel et al. 2000; Zhu et al. 2019). 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 cell-to-cell 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 (Hallwass et al. 2014; Peiro et al. 2014). Within NSm, *Sw-5b* specifically recognizes a conserved 21–amino acid motif (NSm^{21}) and confers resistance against most of the American-type orthotospoviruses (Zhu et al. 2017). *Sw-5b* adopts a unique two-step recognition mechanism to induce a robust immune response against TSWV (Li et al. 2019). In addition to the leucine-rich repeat (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 NLR 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 NLR region, and induction of HR cell death. The SD also facilitates recognition of low levels of NSm by the NLR domain of *Sw-5b* 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).

Although we have some insights on how the *Sw-5b* NLR recognizes the viral effector NSm, we know very little about the mechanisms involved immediately downstream of effector recognition, especially the early (less than 72 h) transcriptional changes that activate immune responses leading to cell death and containment of virus to the infection site. Gene expression changes that occur in *Sw-5b* plants in response to TSWV infection with thrips have not been studied, even though this is the primary manner of spread in the field. Therefore, we investigated the early transcriptional changes that occur during *Sw-5b* NLR recognition of TSWV in tomato when infected with TSWV via thrips and through mechanical inoculation. We found that the *Sw-5b* NLR induces earlier transcriptomic changes in response to thrips-mediated inoculation of TSWV compared with the response to mechanical inoculation, and that many differentially expressed genes (DEGs) were specific to either thrips-mediated or mechanical inoculation of TSWV. Our findings provide new insight into genes that could play a role in *Sw-5b*–mediated resistance to TSWV and highlight the importance of understanding the *Sw-5b*–regulated genes in response to thrips-mediated inoculation in mediating TSWV resistance.

Results and Discussion

Overview of transcriptomic changes observed in *Sw-5b*–resistant plants in response to TSWV infection

To determine the best developmental stage (DS) of plants to use for TSWV inoculation and to collect tissue for transcriptome analysis, we tested plants at six different DSs (Fig. 1A). Nearly 100% of the mechanically inoculated susceptible Celebrity tomato plants at stages DS2 to DS4 were systemically infected, as measured by enzyme-linked immunosorbent assay (ELISA) and developed symptoms by 12 days postinoculation (dpi) (Fig. 1B). The percentage of plants showing symptoms decreased when inoculation was done at later DSs (Fig. 1B). Only about 50 and 10% of the plants at DS6 and DS7, respectively, were symptomatic (Fig. 1B). Regression analysis showed a positive and statistically significant ($P = 0.0004$) correlation between DS and symptom expression (Fig. 1C). Based on these results, we selected DS2 plants for our studies.

To investigate the transcriptome changes that occur early (12, 24 and 72 h postinoculation [hpi]) during *Sw-5b*–mediated resistance to TSWV infection, we used the near-isogenic susceptible Santa Clara ($Sw\text{-}5b^{-/-}$) and resistant CNPH-LAM 147 (CN147) ($Sw\text{-}5b^{+/+}$) tomato lines (Dianese et al. 2010; Hallwass et al. 2014). In addition, we followed two modes of TSWV infection, mechanical- and thrips-mediated inoculation (Fig. 1D). From a total of 92 RNA-seq libraries, 1.1 billion 50-bp reads were generated using Illumina sequencing. The number of reads per sample ranged from 5 to 30 million (Supplementary Table S1). Four libraries had a low number of reads and were excluded from the downstream analysis. After quality control and filtering of low-quality reads, 84% of the reads were mapped to the reference tomato complementary DNA (cDNA) sequences (version ITAG4.1 from the Solanaceae Genomics Network https://solgenomics.net). The mapped reads ranged from 4.5 to 25 million reads per sample (Supplementary Table S1).

The generalized linear model of the edgeR package in R was used to identify the DEGs in the resistant near-isogenic line CN147 compared with the susceptible Santa Clara for each inoculation method and timepoint (described below). Inoculations with sap prepared from noninfected plants (mock mechanical inoculation), noninfected thrips (nonviruliferous thrips, mock) and no thrips were included as controls (Supplementary Table S1).

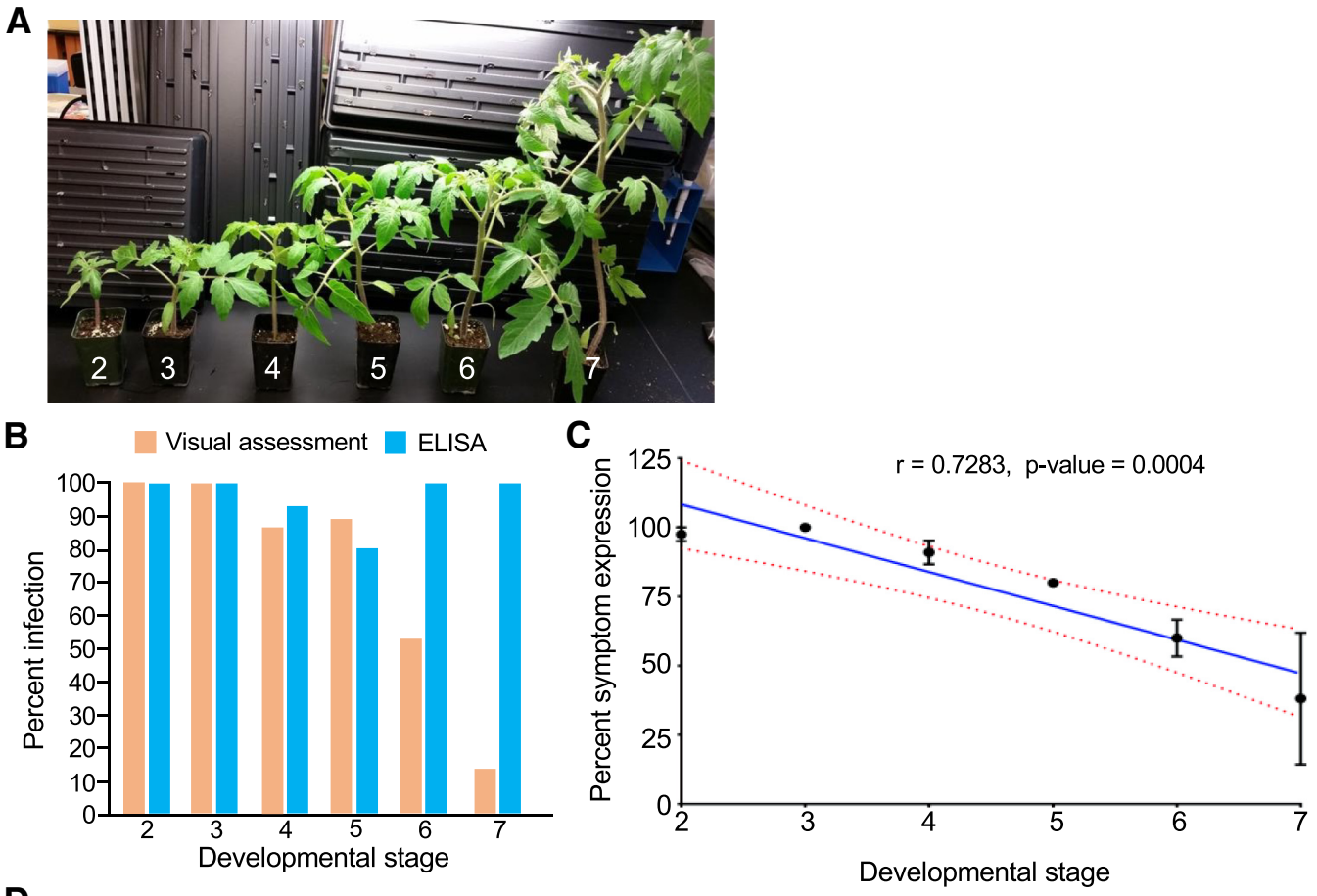
The following comparisons in gene expression were performed for each inoculation method separately. For mechanical inoculation control: TP0_R_M vs. TP0_S_M, TP12_R_M vs. TP12_S_M, TP24_R_M vs. TP24_S_M, and TP72_R_M vs. TP72_S_M; and for thrips inoculation control: TP0_R_no thrips vs. TP0_S_no thrips, TP12_R_Mock vs. TP12_S_Mock, TP24_R_Mock vs. TP24_S_Mock, TP72_R_Mock vs. TP72_S_Mock, and TP72_R_no thrips vs. TP72_S_no thrips. For mechanical inoculation with TSWV (treatment): TP0_R_T vs. TP0_S_T, TP12_R_T vs. TP12_S_T, TP24_R_T vs. TP24_S_T, and TP72_R_T vs. TP72_S_T; and for thrips inoculation with TSWV (treatment): TP12_R_TSWV vs. TP12_S_TSWV, TP24_R_TSWV vs. TP24_S_TSWV, and TP72_R_TSWV vs. TP72_S_TSWV (Supplementary Table S1).

Four libraries had a low number of reads that were excluded from the downstream analysis. These included samples from mock, no thrips, and time zero of virus infection by mechanical inoculation (TP12_R_M replicate 1, TP24_R_M replicate 1, TP0_S_T replicate 3, and TP72_R_nothrips replicate 3) (Supplementary Table S1). Since we did not perform the comparisons between mock and virus-infected plants, low reads of these samples in one replication will not impact identification of the DEGs identified in virus-infected samples. DEGs were not identified between any resistant and susceptible genotypes with mock mechanical inoculation at all timepoints. Comparison between resistant and susceptible genotypes treated with nonviruliferous thrips (mock) revealed some DEGs; however, none of these overlapped with DEGs identified in response to viruliferous thrips (TSWV), with the exception of one DEG. Genes with more than a twofold expression difference (false discovery rate [FDR] < 0.05) between resistant and susceptible genotypes after mechanical- or thrips-mediated inoculation of TSWV were considered significantly different. We identified a total of 80 and 95 DEGs in the resistant line across three different time-

points post-thrips and post-mechanical inoculation, respectively (Fig. 2A; Supplementary Table S2). Of the 145 nonredundant DEGs identified, 111 were up-regulated and 34 were down-regulated across different timepoints and inoculation methods (Fig. 2A; Supplementary Table S2).

The *Sw-5b* NLR induces early transcriptomic changes to thrips-mediated inoculation of TSWV

We observed DEGs as early as 12 and 24 h post–thrips inoculation (HPTI) (Fig. 2A; Supplementary Table S2). In contrast, DEGs were not detected until 72 h post–mechanical inoculation (HPMI). At 12 HPTI, we observed a single downregulated DEG (Solyc09g082340; −8.18 $\log_2$ fold) (Supplementary Table S2) that is predicted to encode a vicilin-like protein. The vicilin-like gene was not differentially expressed at later timepoints in thrips-inoculated treatments nor in mechanically inoculated treatments, suggesting the importance of this gene in early *Sw-5b*-mediated resistance to thrips inoculation of TSWV. Vicilins are plant-specific proteins and structurally belong to the cupin superfamily of proteins (Chen et al. 2013; Dunwell et al.



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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)	

Fig. 1. Virus inoculation and tissue collection scheme used for RNA-seq analysis. **A,** Plants at six developmental stages (DSs) used for mechanical inoculation of *Tomato spotted wilt orthotospovirus* (TSWV) to determine the optimal DS for tissue collection for RNA-seq experiments. **B,** Comparison of mean percent infection based on visual assessment and enzyme-linked immunosorbent assay of susceptible Celebrity tomato 12 days post–mechanical inoculation (dpi) of TSWV at six DSs. **C,** Correlation between plant DS and symptom expression at 12 dpi (regression analysis, mean percentage of plants with symptoms). Data in B and C are from two experimental replicates (rep 1, $n = 20$ to 21 plants per DS and rep 2, $n = 5$ to 15 plants per DS). **D,** Schematics of treatments used for RNA-seq experiments. Isogenic susceptible tomato line Santa Clara and resistant tomato line CNPH-LAM 147 were inoculated with TSWV by mechanical inoculation or through thrips, and tissue samples were collected at 0, 12, 24, and 72 h postinoculation.

2001). The cupin domain represents a conserved β barrel fold that was originally identified in plant germin and germin-like proteins (Dunwell et al. 2004). Structure-guided biochemical analysis of vicilin from pepper and tomato indicate that vicilin may function as superoxide dismutase (SOD) to regulate oxidative stress (Shikhi et al. 2018, 2020). Furthermore, the C-terminal cupin 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. 2018, 2020). Given that reactive oxygen species (ROS) play an important early defense role (Castro et al. 2021), *Sw-5b*-mediated recognition of TSWV could downregulate vicilin to suppress SOD activity. In *Arabidopsis*, *PAP85* that encodes a vicilin-like protein is upregulated early during tobacco mosaic virus (TMV) infection, and virus accumulation was reduced in *AtPAP85* knockdown plants (Chen et al. 2013). Since TMV replication was reduced in protoplasts prepared from RNA interference plants, it was proposed that *AtPAP85* might be involved in virus replication by facilitating endoplasmic reticulum (ER) membrane transition that is important for TMV replication. Notably, the NSm movement protein of TSWV that is recognized by *Sw-5b* to activate defense has been shown to physically interact with the ER membrane, and disruption of this interaction inhibits the cell-to-cell movement 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* as a defense strategy could downregulate Solyc09g082340 that encodes vicilin-like protein early during the defense response to limit TSWV to the infection site.

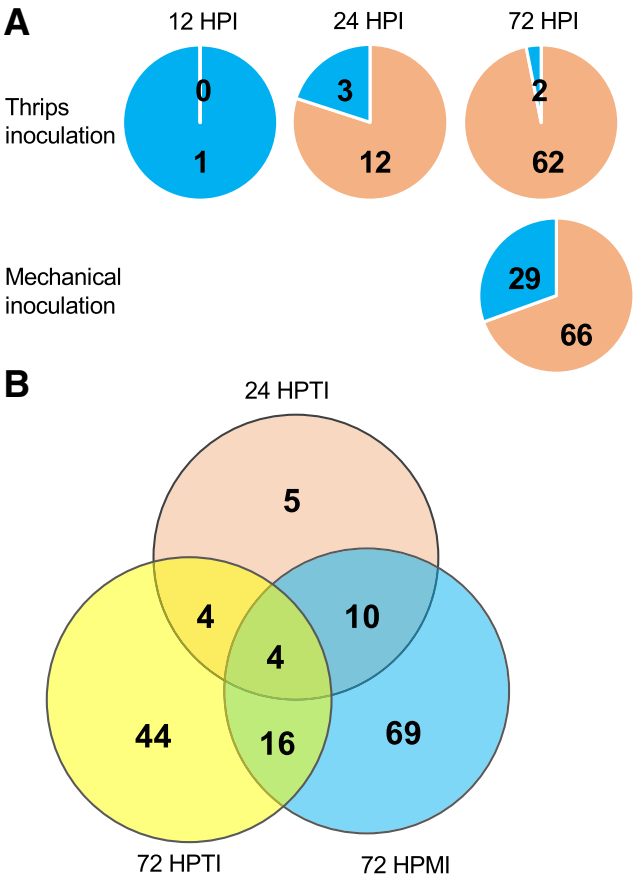

Fig. 2. Venn diagrams showing the number of differentially expressed genes (DEGs). **A,** Number of DEGs detected at 12, 24 and 72 h post–thrips inoculation (HPI) (top panels) and mechanical inoculation (bottom panel) of *Tomato spotted wilt orthotospovirus* (TSWV) in the *Sw-5b* resistant tomato line compared with the susceptible line. Orange, upregulated DEGs; blue, downregulated DEGs. **B,** Number of unique and common DEGs detected among different timepoints and different inoculation methods. HPTI = hours post–thrips-mediated inoculation of TSWV; HPMI = hours post–mechanical inoculation of TSWV.

At 24 HPTI, 12 genes were up-regulated and three genes were down-regulated. Among these 15 DEGs, five were uniquely detected in response to thrips inoculation at this timepoint and were not detected at 72 HPTI nor at any mechanical inoculation timepoints (Fig. 2B; Table 1; Supplementary Table S2). These five unique DEGs included rhamnogalacturonate lyase (Solyc05g051350), pleiotropic drug resistance protein (Solyc09g091670), WEB family protein (Solyc11g010210), NOD26-like intrinsic protein 2.1 (Solyc03g013340), and a class II heat-shock protein HSP17.6 (Solyc03g007890) (Supplementary Table S2). Pleiotropic drug resistance protein and the NOD26-like intrinsic protein are known to function as transporters facilitating the movement of diverse molecules across membranes in response to stimuli and stresses (Nuruzzaman et al. 2014; Wallace et al. 2006; Xie et al. 2021). In tobacco, pleiotropic drug resistance proteins have been shown to be regulated by defense hormones jasmonic acid (JA) and SA, suggesting a regulatory role for these proteins in defense (Xie et al. 2021). The above-discussed five DEGs are of interest for further characterization because they were specifically observed only in the thrips-inoculated treatments.

Among the 64 DEGs identified at 72 HPTI, 62 were up-regulated and two were down-regulated. In 72-HPMI plants, 66 genes were up-regulated and 29 genes were down-regulated. Only four DEGs were shared between two inoculation methods and different timepoints (Fig. 2B; Table 1). Ten genes that were differentially expressed at 24 HPTI were only observed at the later timepoint in mechanical inoculation (72 HPMI) (Fig. 2B; Table 1). A significant number of DEGs at 72 hpi were uniquely expressed either in the thrips-inoculated or mechanically inoculated plants (Fig. 2B; Supplementary Table S2). Together, these results indicated that thrips inoculation induced earlier *Sw-5b* NLR-mediated transcriptome changes and that many of the DEGs induced were unique to inoculation method.

Specific transcriptomic responses are shared among thrips and mechanical inoculations

Some of the DEGs detected at 24 HPTI were also detected in the 72-HPTI and 72-HPMI DEGs (Fig. 2B, Table 1; Supplementary Table S2). Among the 12 upregulated DEGs at 24 HPTI, four were also upregulated in the 72-HPTI and 72-HPMI treatments (Table 1). These DEGs are predicted to encode pathogenesis-related (PR) proteins (Table 1), suggesting an important role for these well-known defense response genes in *Sw-5b*-mediated resistance. The upregulation of these DEGs indicates that, as early as 24 HPTI, the *Sw-5b* NLR had initiated the defense response. These same genes are also induced at 72 HPMI (Table 1), supporting their importance in the *Sw-5b*-mediated resistance response independent of which inoculation method was used.

Of the 15 DEGs detected at 24 HPTI, 11 were not detected at 72 HPTI (Table 1). Among these 11 DEGs, six were detected at 72 HPMI, indicating that these DEGs are responding to TSWV either delivered through thrips or through mechanical inoculation (Table 1). The four upregulated DEGs encode enzymes that are known to play a role in defense, and these include terpene synthase, pyruvate decarboxylase, phenylalanine ammonia lyase (PAL), and fatty acid desaturase (FAD)-binding berberine family protein (Table 1). Overexpression of terpene synthase in maize has been shown to enhance resistance to a fungal pathogen

(Chen et al. 2018). The upregulation of pyruvate decarboxylase is known to induce PR proteins (Rojas et al. 2014; Tadege et al. 1998). In lettuce and sunflower, FAD-binding berberine family proteins and berberine bridge–like enzymes are upregulated after infection (Benedetti et al. 2018; Daniel et al. 2017). Although isochorismate synthase 1 (ICS1) is a major player in pathogen-induced SA biosynthesis, PAL has also been implicated in SA biosynthesis (Ding and Ding 2020). Knockout of all PAL genes in *Arabidopsis* resulted in a 50% decrease in pathogen-induced SA accumulation and increased susceptibility to *Pseudomonas syringae* infection (Huang et al. 2010). In addition, both ICS and PAL pathways contribute to SA biosynthesis in soybean (Shine et al. 2016). PAL is also required for the biosynthesis of many other secondary metabolites, including lignin (Pascual et al. 2016).

Sixteen DEGs were shared between thrips and mechanical inoculation methods at 72 hpi (Table 2; Supplementary Table S2). Many of these DEGs are indicators of activation of plant defense and eight encode PR proteins (Table 2). We observed upregulation of arogenate dehydrogenase (Solyc09g011870) and polyphenol oxidase (Solyc02g078650), which are known to play a role in coordinating ROS levels, leading to a hypersensitive cell-death response (Huang et al. 2019; Kaur et al. 2022). Two of the upregulated DEGs encode 1-aminocyclopropane-1-carboxylic acid (ACC) synthase 2 (Solyc01g095080) and ACC oxidase 1 (Solyc07g049530) enzymes that participate in ethylene production (Table 2) (Houben and Van de Poel 2019). We also observed upregulation of a gene that encodes lipoxygenase (LOX) (Solyc08g029000). LOX is generally associated with responses to wounding from mechanical damage or thrips feeding damage resulting in the synthesis of JA (Yan et al. 2013). Given our experimental setup included controls such as mock inoculations with sap prepared from noninfected plants and inoculation with noninfected thrips, the observed upregulation of the LOX is specific to the *Sw-5b*-mediated resistance to TSWV infection and not due to wounding response. It is possible that induced LOX during *Sw-5b*-mediated defense could increase JA synthesis, which is known to impart resistance against thrips. In pepper, LOX2 is involved in JA biosynthesis and induces defense against western flower thrips (Sarde et al. 2019). We did not measure resistance to thrips in our experiments; however, this finding suggests that *Sw-5b*-mediated resistance results in outcomes that could negatively impact thrips biology as well as TSWV infection.

Genes differentially expressed uniquely in *Sw-5b* in response to thrips-mediated inoculation of TSWV

Of the 64 DEGs, 44 were detected specifically in response to thrips inoculation at 72 hpi (Fig. 2A; Supplementary Table S2). Some of the DEGs that are specifically regulated at 72 HPTI are shown in Table 3. We detected upregulation of a CC-NLR, a cysteine-rich receptor-like kinase (CRK), and three G-type

Table 1. Differentially expressed genes in *Sw-5b* resistant plants[a]

	Thrip inoculation		Mechanical inoculation	
Gene ID	**24 hpi**	**72 hpi**	**72 hpi**	**Annotation**
Solyc03g025670	4.55	3.70	5.19	Pathogenesis-related (PR) protein 1c
Solyc01g097240	4.13	4.48	6.40	PR protein 4
Solyc09g006005	3.58	3.48	5.74	PR protein 1
Solyc10g079860	2.81	2.23	4.68	β-(1,3) Glucanase
Solyc01g101180	6.33		5.00	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	SRP40-like protein
Solyc10g076510	2.25		2.22	Pyruvate decarboxylase
Solyc03g123540	−3.01		−3.09	Class III heat-shock protein HSP17.4
Solyc05g051350	8.59			Rhamnogalacturonate lyase
Solyc09g091670	3.95			Pleiotropic drug resistance protein
Solyc11g010210	2.52			WEB family protein
Solyc03g013340	−1.23			NOD26-like intrinsic protein 2.1
Solyc03g007890	−1.50			Class II heat-shock protein HSP17.6

[a] Log_2 fold change after thrip-inoculation of *Tomato spotted wilt orthotospovirus* (24 and 72 h postinoculation [hpi]) and after mechanical inoculation (72 hpi).

Table 2. Differentially expressed genes in *Sw-5b* resistant plants[a]

Gene ID	Thrips inoculation	Mechanical inoculation	Annotation
Solyc05g054380	9.12	7.35	Pathogenesis related (PR) protein STH-2
Solyc02g078650	8.69	6.66	Polyphenol oxidase
Solyc01g008620	8.46	6.86	Glucan endo-1,3-β glucosidase A precursor
Solyc09g007010	8.26	6.48	PR protein PR1a
Solyc04g064880	7.46	6.88	PR protein-like
Solyc01g095080	6.74	5.67	1-Aminocyclopropane-1-carboxylic acid synthase 2
Solyc01g106620	6.02	6.26	PR1
Solyc01g080570	5.78	5.32	Inosine-uridine preferring nucleoside hydrolase family
Solyc08g029000	5.77	7.24	Lipoxygenase
Solyc09g011870	5.33	4.49	Arogenate dehydrogenase 2
Solyc01g059965	5.32	5.76	Glucan endo-1,3-β-glucosidase B
Solyc04g072280	5.01	5.88	Laccase
Solyc08g080650	4.78	5.06	PR protein P23
Solyc07g049530	4.56	3.61	1-Aminocyclopropane-1-carboxylate oxidase 1
Solyc02g082920	3.86	4.60	Endochitinase (PR3)
Solyc10g055810	3.16	4.92	Chitinase (PR3)

[a] Log_2 fold change in genes that are shared between thrips and mechanical inoculation at 72 h after *Tomato spotted wilt orthotospovirus* infection.

lectin S-receptor-like kinases (G-LecRLK) (Table 3). Given the emerging evidence that the helper or other NLRs function downstream of sensor NLRs to activate immunity (van Wersch et al. 2020), it will be interesting to test, in the future, the precise role of the upregulated CC-NLR (Solyc04g007490) in *Sw-5b*-mediated defense. CRKs have been shown to play an important role in cell death and disease resistance (Bourdais et al. 2015; Saintenac et al. 2021; Yadeta et al. 2017). LecRLKs are important regulators of defense responses against various pathogens and pests (Sun et al. 2020). Overexpression of G-LecRLK has been shown to increase plant defense to biotrophic pathogens (Zhao et al. 2018). G-LecRLKs in *Nicotiana attenuata* have been demonstrated to be involved in recognition of insect feeding and required to induce a full defense response against *Manduca sexta* (Gilardoni et al. 2011). Therefore, it is possible that the upregulated G-LecRLKs in *Sw-5b* plants could play a role in defense against thrips.

We detected upregulation of five transcription factors at 72 HPTI in *Sw-5b* plants (Table 3). Among these ethylene response factors (ERFs) and members of calmodulin-binding protein 60 (CBP60) are important players in defense (Huang et al. 2016; Zheng et al. 2022). ERFs are known to bind a *cis* element in the promoter region known as the GCC box (AGCCGCC) and promote expression of some PR genes (Ohme-Takagi and Shinshi 1990). The ERF1 that we detected in this study, when overexpressed in tobacco, has been shown to induce PR genes and ethylene responses (Huang et al. 2004). In *Arabidopsis*, CBP60a and CBP60b function as positive regulators of immunity and overexpression of CBP60b induces constitutive defense responses (Huang et al. 2021).

Other classes of genes that were differentially regulated during thrips-mediated inoculation belong to the proteases and protease inhibitor family (Table 3). We detected significant upregulation of a gene encoding subtilisin-like protease (Solyc08g079900) (Table 3). Subtilisin proteases have an established role in plant immunity against different pathogens (Figueiredo et al. 2018). The gene Solyc11g022590 that is upregulated is predicted to encode trypsin inhibitor–like protein and shares significant homology with the *Arabidopsis* gene At1g73260 that regulates cell death triggered in response to infection by *Pseudomonas syringae* expressing AvrB effector (Li et al. 2008). In contrast, Solyc01g079980 that encodes aspartyl protease was downregulated in response to thrips-mediated inoculation, indicating that it could play a negative regulatory role during *Sw-5b*-mediated resistance to TSWV.

We also observed upregulation of other interesting genes at 72 HPTI (Table 3). The *Arabidopsis* homologs of Solyc01g105450, which encodes ABC transporter family protein, and Solyc05g053600, which encodes pleiotropic drug resistance protein, have been shown to transport cuticular lipids (McFarlane et al. 2010) and abscisic acid (Kang et al. 2010), respectively. The lipid transfer protein (Solyc08g078870) homolog DRN1 (DISEASE RELATED NONSPECIFIC LIPID TRANSFER PROTEIN 1) in *Arabidopsis* is required for resistance against various phytopathogens (Dhar et al. 2020). The calmodulin binding protein (Solyc12g008960) homolog in *Arabidopsis* is known to bind catalase 2 and regulates JA biosynthesis (Lv et al. 2019).

Genes differentially expressed uniquely in *Sw-5b* in response to mechanical inoculation of TSWV

Of the 95 DEGs detected, 69 were unique to 72 HPMI (Fig. 2; Supplementary Table S2). The number of genes that were down-regulated (28 genes) was greater in 72 HPMI treatment than in any other treatment (Supplementary Table S2). Among five DEGs belonging to the transcription factor family, three were up-regulated and two were down-regulated (Table 4). Compared with ERF1, which is upregulated in thrips-inoculated treatment at 72 h (Table 3), we detected upregulation of a different ERF family member, ERF017, in response to mechanical inoculation of TSWV in *Sw-5b* plants. The WRKY75 that we detected during *Sw-5b* mediated resistance is regulated at the epigenetic level in response to biotic and abiotic stresses in *Solanaceae* plants (Lopez-Galiano et al. 2018).

Some of the other potentially significant genes regulated at 72 HPMI are shown in Table 4. FAD2-9 was significantly induced (10.51 $\log_2$ fold). FADs are known to regulate ROS signaling

Table 3. Selected interesting differentially expressed genes in *Sw-5b* resistant plants specifically in response to 72 h post–thrips inoculation of *Tomato spotted wilt orthotospovirus*

Gene ID	$\log_2$ fold change	Annotation
Coiled-coil nucleotide-binding leucine-rich repeat (CC-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 like protein kinase
Solyc07g063770	1.83	G-type lectin S-receptor-like kinase
Transcription factors		
Solyc04g016000	5.56	Heat-shock transcription factor protein 8
Solyc07g053140	4.84	Zinc finger protein/CONSTANS-like protein
Solyc06g069760	3.48	Dof zinc finger protein
Solyc05g051200	2.91	Ethylene-responsive factor 1
Solyc03g113960	3.26	CBP60-like
Proteases and protease inhibitor		
Solyc11g022590	7.11	Subtilisin-like protease
Solyc08g079900	1.91	Trypsin inhibitor-like protein precursor
Solyc01g079980	−1.48	Aspartyl protease family protein
Other interesting genes		
Solyc01g105450	4.45	ABC transporter G family
Solyc05g053600	4.15	Pleiotropic drug resistance protein
Solyc01g099010	3.88	GDSL esterase/lipase
Solyc08g078870	3.51	Lipid-transfer protein
Solyc05g008220	2.62	PADRE gene family
Solyc12g008960	2.38	IQM1-like protein
Solyc02g081980	2.27	Apyrase
Solyc03g112700	1.80	Herbivore elicitor-regulated
Solyc07g065660	1.69	Cellulose synthase-like protein E1
Solyc07g054600	1.20	F-box protein

and fluidity of cell membranes during defense (Xiao et al. 2022). Some genes involved in cell death, such as the gene encoding biotic cell death associated protein (Solyc03g098740), were upregulated and a gene that encodes serpin (Solyc04g079470) was downregulated. The *Arabidopsis* homolog of serpin we detected is known to inhibit proteases involved in cell-death induction (Lampl et al. 2013; Lema Asqui et al. 2018). Hence, it is possible that, during the *Sw-5b*-mediated response, tomato serpin is downregulated, allowing the pro–cell death proteases to induce a hypersensitive cell-death response to TSWV infection.

Protein degradation processes play important roles during defense (Linden and Callis 2020). At 72 HPMI of TSWV, a gene encoding the U-box protein (Solyc04g008100) was upregulated and two genes that encode F-box proteins (Solyc05g055870 and Solyc11g006740) were down-regulated (Table 4). The Solyc05g025820 gene that encodes protein kinase was significantly down-regulated (Table 4). In contrast to our study, a homolog of this kinase, *Arabidopsis* RIPK/PBL14 is known to phosphorylate RIN4 and function as a positive regulatory role in RPM1 NLR-mediated immunity against *P. syringae* expressing avrB and avrRpm1 effectors (Liu et al. 2011). Another gene that is down-regulated encodes a dicer-like (DCL) protein, DCL2b (Solyc11g008540). In tomato, DCL2b is required for defense against tomato mosaic virus, TMV, and potato virus X (Wang et al. 2018a and b). Since DCL2 proteins generally function in antiviral defense, it will be interesting to study why DCL2b is down-regulated during *Sw-5b*-mediated resistance to TSWV.

Table 4. Selected interesting differentially expressed genes in *Sw-5b* resistant plants specifically in response to 72 h post–mechanical inoculation of *Tomato spotted wilt orthotospovirus*

Gene ID	Log_2 fold change	Annotation
Transcription factors		
Solyc12g009240	5.11	Ethylene-responsive transcription factor (ERF017)
Solyc12g010410	4.59	Homeobox protein knotted-1-like 3
Solyc05g015850	3.91	WRKY DNA binding protein
Solyc02g091250	−3.41	Mediator of RNA polymerase II transcription subunit
Solyc04g064770	−5.65	Zinc finger transcription factor 34
Other interesting genes		
Solyc12g100250	10.51	Fatty acid desaturase 2-9 (FAD2-9)
Solyc05g053610	6.66	Pleiotropic drug resistance protein (ABCG40)
Solyc03g096540	6.64	Lipase/lipooxygenase
Solyc09g097810	5.26	SAR8.2
Solyc02g077290	4.86	Glutamate receptor-like 1.2
Solyc01g107390	4.74	Auxin-responsive GH3 product
Solyc03g098740	4.45	Biotic cell death-associated protein
Solyc04g008100	3.79	U box protein I(PUB21/CMPG5-like)
Solyc01g105070	3.15	Peroxidase precursor
Solyc04g071890	2.91	Peroxidase
Solyc06g053710	1.92	Ethylene receptor homolog
Solyc11g008540	−1.34	DCL2b
Solyc01g008220	−1.51	Stress-induced protein
Solyc09g097860	−1.66	Kinesin-like protein KIN12B
Solyc06g008820	−1.70	Na^+/H^+ antiporter 1
Solyc05g005460	−1.87	Nucleoredoxin
Solyc03g025250	−2.01	Protein detoxification
Solyc05g055870	−2.61	F-box domain, Phloem protein 2-like protein
Solyc11g006740	−2.65	F-box domain, Phloem protein 2-like protein
Solyc04g079470	−3.04	Serpin (AtSerpin1-like)
Solyc05g025820	−4.23	Protein kinase superfamily protein (PBL14/RIPK-like)

Validation of RNA-seq results of some selected genes

To validate the RNA-seq data, we performed reverse transcription-quantitative PCR (RT-qPCR) analysis of selected DEGs. We observed a similar trend in expression of selected genes between RT-qPCR and RNA-seq, with some difference in magnitude (Fig. 3). Furthermore, the fold changes in gene expression observed between RNA-seq and RT-qPCR were strongly correlated ($r = 0.74$; $P = 0.0004$).

Differences in gene expression due to *Sw-5b* responses to thrips and mechanical inoculation of TSWV are not caused by virus titer

Because TSWV must replicate for production of the NSm protein that triggers *Sw-5b* resistance responses, we hypothesized that the earlier response that we observed in thrips-inoculated *Sw-5b* plants may be due to faster or more-efficient viral replication in thrips-inoculated leaves. qRT-PCR of the TSWV *N* gene in plant materials that were used for RNA-seq analysis detected TSWV in thrips- and mechanically inoculated leaves from susceptible and resistant lines as early as 12 hpi (Fig. 4). Both inoculation methods resulted in a significant increase in virus titer in the susceptible line at 72 hpi, but virus titers remained low at all timepoints in the *Sw-5b*-resistant line with both inoculation methods (Fig. 4). Furthermore, the abundance of TSWV *N* gene transcripts was not significantly different between thrips- and mechanically inoculated resistant plants. Based on these results, the differences we observed in gene expression profiles using the two inoculation methods are not due to differential rates of virus replication.

Conclusions

Our findings revealed that the *Sw-5b* NLR immune receptor induces transcriptome responses to thrips-mediated inoculation of TSWV at timepoints 12 and 24 hpi. In contrast, tran-

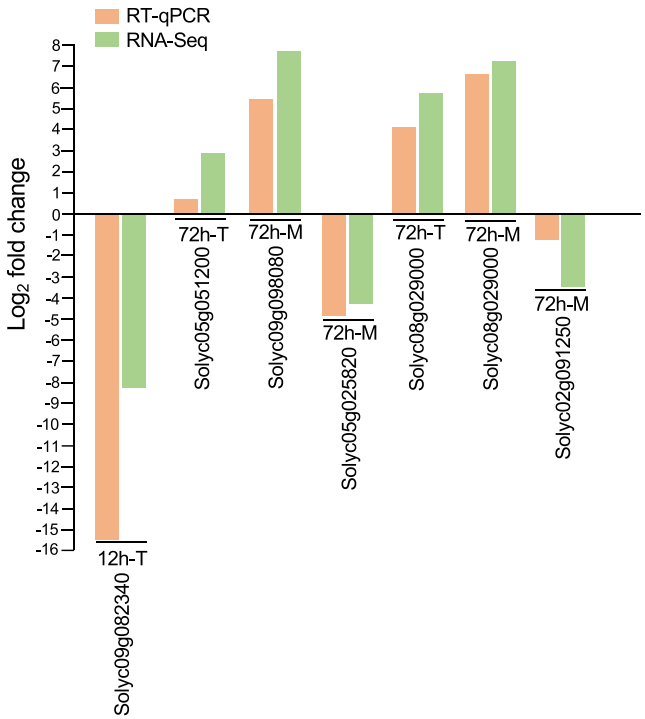

Fig. 3. Comparison of reverse transcription-quantitative PCR (RT-qPCR) with RNA-seq for selected differentially expressed genes. Expression (log_2 fold change) of selected genes quantified by RT-qPCR shows the same trend as RNA-seq. Supplementary Table S2 shows gene annotation. T = thrips inoculation, M = mechanical inoculation, h = hours postinoculation.

scriptome responses were not detected in mechanically inoculated treatments until 72 hpi. The manner of virion placement and synchronization of infection in plant cells likely differs between thrips and mechanical modes of inoculation. Thrips salivate inside the plant cell, depositing virions directly in the cytoplasm, presumably relatively undamaged. When plants are mechanically inoculated, virions enter via the wounds created by rub inoculation and may be in various states when they arrive in the cytoplasm. Since activation of the *Sw-5b*-mediated defense requires recognition of TSWV NSm (Chen et al. 2016; Zhu et al. 2017) and this nonstructural protein is produced following virus replication (Zhu et al. 2019), we hypothesized that the earlier response observed in the thrips-inoculated treatment could be because TSWV replicated more quickly and efficiently, making NSm available for interaction with *Sw-5b* earlier than in the mechanical inoculation. RT-qPCR analysis of the plant materials used for the RNA-seq library preparation did not support this hypothesis, instead indicating no difference in the virus titer between mechanical and thrips-inoculated samples. Thus, one or more yet-to-be-identified mechanisms associated with thrips inoculation of TSWV promotes a more rapid response with *Sw-5b*.

Traditionally, breeders searching for orthotospovirus resistance have done so using mechanical inoculation of plants, due to the ease of screening using this method (Qi et al. 2021). Potentially resistant plants are not screened by thrips inoculation except during field testing, largely because thrips inoculation is complex and difficult. Our findings revealing the differences in *Sw-5b* responses to thrips and mechanical inoculation, suggest that more effort may be warranted in examining potential virus resistance using thrips inoculation. Based on our findings, while designing screening assays, it is important to consider timing of sample collection depending on the inoculation method used.

In conclusion, our findings described here provide new insight into the genes that are differentially regulated in *Sw-5b*-mediated resistance, the importance of the inoculation method, and provide a foundation for future functional analyses to determine the roles of these genes in *Sw-5b*-mediated immunity.

Materials and Methods

Plant and virus material for RNA-seq experiments

To investigate the transcriptomic changes that occur during *Sw-5b*-mediated resistance to TSWV infection, we used near-isogenic TSWV-susceptible tomato Santa Clara and TSWV-resistant CN147, which have been described previously (Dianese et al. 2010; Hallwass et al. 2014). Seeds of these lines were planted in individual pots with SunGro professional mix. All treatments of each replicate were planted at the same time of day (7:00 AM) to fully synchronize growth and avoid differences between replicates or treatments. Plants of all treatments and replicates were grown in the same growth chamber set at 26°C with 16 h of light and 8 h of dark, 50% relative humidity, and 300 microEinsteins light intensity. TSWV isolate MR-01 (TSWV $^{MR-01}$) was collected originally from infected radicchio in Monterey County, California and was flash frozen and stored at −80°C. Complete sequences of this strain can be found in the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/nuccore), using accession numbers MG593199 (S RNA), MG593198 (M RNA), and MG593197 (L RNA) (R. O. Adegbola, S. T. Adkins, and R. A. Naidu, *unpublished data*).

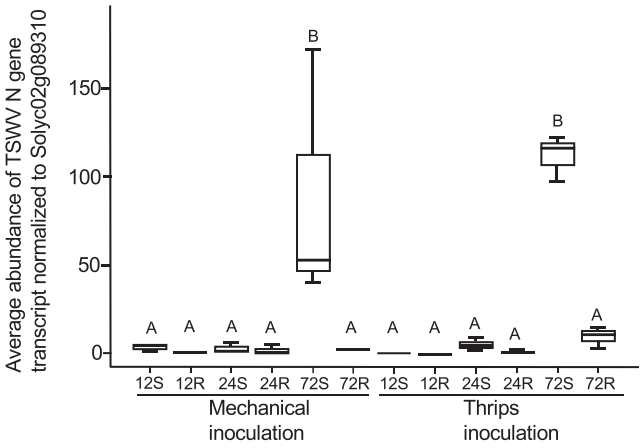

Fig. 4. There was no significant difference of *Tomato spotted wilt orthotospovirus* (TSWV) titer in mechanical- and thrips-inoculated resistant and susceptible tomato plants. Quantification of TSWV by reverse transcription-quantitative PCR of resistant (R) and susceptible (S) tomato lines that were infected mechanically or by thrips at 12, 24, and 72 h postinoculation.

Thrips colony and maintenance

Frankliniella occidentalis, from a lab colony originally collected from the Kamiloiki valley on the Hawaiian island of Oahu, was reared on green pods of *Phaseolus vulgaris* as previously described (Bautista 1993; Ullman et al. 1992).

Mechanical inoculation of TSWV

For mechanical inoculation of tomato lines, TSWV^{MR-01} inoculum was prepared by grinding 1 g of TSWV-infected *Datura stramonium* leaf tissue in 15 ml of buffer (0.1 M potassium phosphate, 1 mM sulphite, 1% celite, pH 7) with a mortar and pestle on ice. The equal amount of resulting sap was rub-inoculated onto leaves using a pestle. Ten minutes after the inoculations, the inoculated leaves were rinsed with distilled water and were maintained in the growth chamber, with conditions as described above, until the samples were collected for RNA extraction. The presence of TSWV^{MR-01} was confirmed by ELISA, following manufacturer protocol (Agdia, Inc.).

TSWV inoculation through thrips

Thrips transmission involves three phases,i.e., acquisition, latency, and inoculation. Importantly, acquisition only occurs during the larval stages, with inoculation occurring primarily during the adult stage. Thrips inoculations were standardized and optimized by collecting thrips larvae less than 6 h post-eclosion, then placing them on TSWV^{MR-01}-infected *Datura stramonium* for a 24-h acquisition access period (AAP). After the AAP, thrips were reared on green bean pods and at 24-h post–adult eclosion were used for thrips inoculations. Noninfected control thrips were created using the same strategy, except they were given acquisition access to noninfected *Datura stramonium*. During experiments to prepare materials for RNA-seq, single tomato plants and thrips were placed in cages made of two 32-ounce plastic cups placed on the pot, with the bottom cut out and covered with no-thrips insect screens (BioQuip) (Supplementary Fig. S1). The pot was covered using parafilm to prevent thrips movement into the soil. Ten thrips (five females and five males) were placed on leaves 1 and 2 on each plant, and the cage was closed. Thrips remained on the plants until samples were collected for RNA extraction.

Optimization of plant stage to be used for RNA-seq experiments

Since robust infection with TSWV is important to induce *Sw-5b* responses, we first optimized the DS of plants that would best support virus infection, using susceptible cultivar Celebrity tomato plants and TSWV^{MR-01}. Celebrity tomato seeds were planted weekly in a greenhouse on the University of California Davis campus (26°C with 16-h of light and 8 h of dark) to produce a cohort of plants at six stages (DS2, DS3, DS4, DS5, DS6, and

DS7) (Fig. 1A). Mechanical inoculation with $TSWV^{MR-01}$ was done using the methods described above. Plants were observed daily for symptom development and infection was measured by ELISA 12 dpi.

Tissue collection for RNA sequencing

Based on results from our TSWV inoculation optimization study, we used DS2 stage Santa Clara and CN147plants for inoculation with $TSWV^{MR-01}$, using optimized conditions for thrips and mechanical inoculations. Samples were collected at 0, 12, 24, and 72 h HPTI and HPMI. At each collection time, the two inoculated leaves from each plant ($n = 10$) were collected from each treatment, i.e., viruliferous thrips, nonviruliferous thrips (mock), mechanical inoculation with sap from virus-infected plants, mechanical inoculation with sap from non–virus infected plants (Fig. 1D). TSWV infection of the positive controls that were inoculated with viruliferous thrips and sap from virus-infected plants were used to verify inoculation success, using ELISA. The no-thrips control was used to test for cross-contamination during the biological experiments. The collected tissue samples were flash-frozen in liquid nitrogen and were stored at −80°C until total RNA was extracted, and RNA-seq libraries were prepared and sequenced as described below. Additional confirmation of TSWV presence and abundance was assayed, using RT-qPCR with primers that anneal to part of the *N* gene of TSWV (Supplementary Table S3). RT-qPCR was performed for three biological replicates and six technical and biological replicates, using SYBR green mix on a Bio-Rad CFX96 machine, with the following conditions: 95°C for 30 s, followed by 39 cycles of 95°C for 10 s, 55°C for 10 s, and 60°C for 20 s.

RNA extraction and RNA-seq library preparation

Tissue samples collected as described above were used for the preparation of RNA-seq libraries, using a modified protocol described by Nagalakshmi et al. (2010). Messinger RNA (mRNA) was isolated from 200 mg of tissue, using a Dynabeads mRNA DIRECT kit (Invitrogen) and was treated with RNase-free DNase I (New England Biolabs). First-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and second-strand cDNA synthesis was performed using DNA Pol I and RNaseH (New England Biolabs). The Ampure XP beads (Beckman Coulter) purified cDNA was fragmented using Fragmentase (New England Biolabs). The fragmented cDNA was used to prepare Illumina sequencing libraries, using a KAPA Hyper prep kit (Kapa Biosystems) and Bioo barcode adapters (Bioo Scientific). A total of 96 barcoded libraries were prepared, pooled, and sequenced, using the Illumina HiSeq4000, 50SR.

Differential gene expression analysis

The sequencing adaptors and low-quality bases were trimmed from the raw reads using Trimmomatic version 0.36 (Bolger et al. 2014). Four libraries (TP0_S_T_3.fq, TP12_R_M_1.fq, and TP24_R_M_1.fq, TP72_R_nothrips_3.fq [Supplementary Table S1]) had a low number of reads and were excluded from the downstream analysis. The high-quality reads were mapped to the tomato cDNA sequences (version 4.1, Sol Genomics https://solgenomics.net) using Salmon version 0.8.1 (Patro et al. 2017). The quality control and the mapping runs were performed using the resources of CyVerse Discovery environment (Merchant et al. 2016). Differential gene expression analysis was performed using the generalized linear model functionality of the edgeR package (Robinson et al. 2010). The model counts data using an overdispersed Poisson model and uses an empirical Bayes procedure to moderate the degree of overdispersion across genes. The inputs for edgeR are i) a table of counts (based on the reads mapped to the tomato transcripts), ii) a vector showing the total number of reads for each sample, and iii) a factor specifying the experimental group or condition for each sample (Robinson et al. 2010). The samples were grouped according to their genotype (Santa Clara and CN147), time after inoculation (0, 12, 24, or 72 h), method of inculcation (thrips and mechanical inoculation), and treatment (control and inoculated). Tomato genes with at least a twofold expression difference between the susceptible and resistant genotypes and FDR < 0.05 were considered differentially expressed.

RT-qPCR

Gene-specific primers were designed using Primer3Plus for amplification of 93- to 180-bp fragments from each target gene (Supplementary Table S3). The F-box gene (Solyc02g089310) was used to normalize the data (Liu et al. 2012). Total RNA was extracted from frozen leaf material, using TRIzol (Invitrogen) according to the manufacturer instructions. RNA was treated with DNase I (Invitrogen), followed by cDNA synthesis using the Verso cDNA synthesis kit (Thermo Fisher Scientific). Three biological replicates were run on Bio-Rad CFX96 machine, using the following conditions: 95°C for 30 s, followed by 39 cycles of 95°C for 10 s, 55°C for 10 s, and 60°C for 20 s. The gene expression fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

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