

ABSTRACT

HOULE, JESSICA LEE. Consequences of Interactions between Thrips-Vectored *Tomato Spotted Wilt Virus* and Plants Carrying Resistance Genes. (Under the direction of Dr. George G. Kennedy).

Tomato spotted wilt virus (TSWV), a thrips-vectored agent of the disease tomato spotted wilt (TSW), causes significant damage to crops worldwide including tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*). Development of the resistance genes *Sw-5* in tomato and *Tsw* in pepper has helped to reduce epidemics of TSW. Unfortunately, *Sw-5* and *Tsw* have both failed to provide full protection from TSWV. In tomatoes carrying the *Sw-5* gene, late-season infections producing symptoms limited mostly to the fruit have led to major losses in marketable fruit. We hypothesized that viruliferous western flower thrips, *Frankliniella occidentalis*, feeding on blossoms or young, developing fruit could cause fruit-limited infections in tomatoes with *Sw-5* and that some infections could become systemic. In the greenhouse, 21% of blossom or fruit inoculated resistant plants developed infections compared to 68% of susceptible plants, with 71.4% of infections of resistant plants traveling systemically out of the inoculated tissue into foliage and/or other fruit. Field studies showed 38.9% of fruit were infected on resistant tomato plants when *F. occidentalis* was abundant in blossoms with occasional cases of systemic infection detected in foliage adjacent to infected fruit. Managing *F. occidentalis* populations during the bloom and fruiting stages of tomatoes is an important step in preventing major losses of marketable fruit.

A second major contributor to resistance gene failure is the emergence of resistance-breaking (RB) variants of TSWV. An experimental evolution approach was used to study the changes

in infectivity and virulence of a field-collected *Tsw* resistance-breaking (RB) isolate when it underwent selection by serial passages in resistant and susceptible peppers. It was predicted that selection in a host would lead to increased infectivity and virulence in the selecting host and decreased infectivity and virulence in the alternate host. Selection of a field collected TSWV isolate by serial passages in resistant hosts led to higher infectivity in resistant peppers while maintaining high infectivity in susceptible pepper. When the same TSWV isolate was selected by serial passages in susceptible pepper plants, infectivity increased in susceptible peppers but decreased in resistant peppers in 3 out of 4 replicates. Serial passages in a selecting host of either resistant or susceptible genotype led to higher virulence, manifested as an increase in chlorosis and mottling, leaf deformity, small necrotic lesions, and stunting. To examine the stability of RB following selection by resistant hosts, highly RB isolates were selected by serial passages in susceptible hosts. These isolates did not revert back to their original infectivity and virulence in resistant hosts. Repeated selection by plants expressing the *Tsw* gene could lead to the establishment of RB isolates in the landscape. Managing RB isolates by limiting continuous selection by resistant plants could potentially help improve the durability of this control strategy.

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Consequences of Interactions between Thrips-Vectored *Tomato spotted wilt virus* and Plants
Carrying Resistance Genes

by
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DEDICATION

To my friends who supported me, made me laugh, and put up with all the critters I brought around with me for show and tell.

To my mother, who always was there to support me and remind me that I could achieve anything I wanted.

BIOGRAPHY

Jessica Lee Houle was born in Attleboro, MA and raised near Ithaca, NY. Jessica liked to explore everything. She was an avid fort builder and nature explorer as a child and grew a love for insects early on. A small deviation occurred when she attended college at Cornell University, where she obtained her B.S. degree in Design and Environmental Analysis. While in college, she took entomology courses and remembered her love of insects. After college, she lived in a van, traveling the country rock climbing and found herself a few part-time jobs in the Department of Entomology at the University of Kentucky. The following year, she began her graduate studies at NC State and found her passion for science communication when she became active in outreach and teaching. She is still an explorer at heart, and can be found doing adventure races or out rock climbing, while also showing off the insects she finds to all those around her (whether they want to see them or not).

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INTRODUCTION

Tomato spotted wilt virus (family *Bunyaviridae*, genus *Tospovirus*, TSWV) is a major threat to crops worldwide and ranks among the top ten most damaging plant viruses (Goldbach and Peters 1994, Pappu et al. 2009). In the southeast US alone, it causes an estimated \$100 million/year in damages (Pappu 1997). Originally discovered early in the 20th century, TSWV has seen a major resurgence in the past three decades following the global spread of one of its vectors, *Frankliniella occidentalis* (Brittlebank 1919, Samuel et al. 1930, Greenough et al. 1985, Allen and Broadbent 1986, Kirk and Terry 2003, Dietzgen et al. 2005). TSWV and its relative tospoviruses are transmitted solely by thrips (Thysanoptera: Thripidae). There are 9 reported vector species of TSWV, with three present in North Carolina. *Frankliniella fusca* and *Frankliniella occidentalis* are considered the most serious vectors (Riley et al. 2011b), while *Thrips tabaci* is usually less of a threat because its ability to transmit different virus isolates is highly variable across populations (Jacobson and Kennedy 2013).

The extent of TSWV's impact can be largely attributed to its relationship with thrips. Thrips can only transmit TSWV if they acquire it as 1st instars when feeding on an infected plant (Ullman et al. 1992, van de Wetering et al. 1996). After reaching the midgut epithelium and replicating, TSWV migrates to the muscle cells and eventually reaches the salivary glands where it can be transmitted by the adult thrips during feeding for the remainder of its life (Wijkamp et al. 1993, Nagata et al. 2002). Thrips are highly polyphagous, which has enabled TSWV to infect over 1000 species of plants, including many important crops as well as common weeds (Parrella et al. 2003). The disease causes mottling, leaf and fruit deformity,

and when a plant is severely infected, death (Cho et al. 1996). Thrips overwinter in annual weeds, which may also be infected with TSWV, before dispersing in the spring to susceptible crops (Groves et al. 2001b, 2003, Morsello and Kennedy 2009). *F. fusca* is a significant early season vector (Groves et al. 2002, 2003), while *F. occidentalis* populations often build later in the season, contributing to secondary spread (Beaudoin 2011).

Managing TSWV is very challenging and often requires multiple tactics. Abundant weed hosts make it impossible to eliminate all reservoirs for virus and vectors. Managing vector populations is also difficult because they prefer tight, protected spaces like blossoms or new growth, which make pesticide applications less effective at reaching them (Hansen et al. 2003). Even if thrips are exposed to insecticides while feeding, they still have an opportunity to transmit the virus before they are affected. The use of antifeedant insecticides such as imidacloprid is effective at reducing *F. fusca* feeding and TSWV transmission (Groves et al. 2001a, Coutts and Jones 2005), but is not effective against *F. occidentalis* (Joost and Riley 2005, Riley 2006). Another important control strategy involves the use of reflective mulches (Greenough et al. 1990, Reitz et al. 2003, Momol et al. 2004), but these are expensive and may slow early season crop development by lowering soil temperature (Riley and Pappu 2004). One of the most effective single control methods is disease resistant cultivars (Riley and Pappu 2004, Gordillo et al. 2008, Riley et al. 2011a, UGA CAES 2012). In tomato and pepper, there are single dominant resistance (R) genes that provide protection against most TSWV isolates (Stevens et al. 1992, Boiteux 1995, Black et al. 1996, Moury et al. 1997, Jahn et al. 2000). The R gene proteins recognize an avirulence factor in the virus and induce a

hypersensitive response (HR) that leads to cell death surrounding the inoculation site (Brommonschenkel et al. 2000, Nimchuk et al. 2003). However, the emergence of resistance-breaking (RB) isolates has been reported and is a major problem threatening the long-term durability of their use (Boiteux and Giordano 1993, Hobbs et al. 1994, Cho et al. 1996, Latham and Jones 1998, Roggero et al. 2002, Aramburu and Martí 2003, Margaria et al. 2004, Ciuffo et al. 2005, Sharman and Persley 2006).

Attention has been drawn to the factors leading to infections in tomatoes and peppers carrying the R genes *Sw-5* and *Tsw*, respectively. Researchers have sought to find the genetic underpinnings that enable TSWV to break resistance (Jahn et al. 2000, Hoffman et al. 2001, Margaria et al. 2007, Lovato et al. 2008, Lopez et al. 2011). Other studies have looked at the fitness costs to having these mutations to better understand the durability of these genes (Latham and Jones 1998, Thomas-Carroll and Jones 2003).

R genes may also fail due to reasons other than virus mutations. Expression of R genes may vary across plant tissue, developmental stages, and environmental conditions (White et al. 1995, Wang et al. 2009, Kathiria et al. 2013). Epidemics of apparently fruit-limited infections in *Sw-5* tomatoes is one common problem seen that leads to major losses in marketable fruit (Moriones et al. 1998, Culbreath 2003). Aramburu et al. (2000) showed that viruliferous thrips feeding on harvested green tomato fruit leads to the development of ringspots as the fruit ripens.

The objective of this study was to understand how interactions between TSWV and plants with R genes lead to the disease epidemics we see in the field and the potential consequences of these infections over time. For the first objective, we asked if *F. occidentalis* feeding on blossoms or newly developing fruit of *Sw-5* tomatoes could result in not only fruit infections, but also systemic infections in the foliage and other fruit. While late season infections of resistant fruit are common, the severity of the problem is not well explored. Through laboratory and field experiments, we looked at how frequently fruit infections appeared when *F. occidentalis* were present at the blossom and fruiting stage of development. We also observed the occurrence of non-resistance-breaking isolates systemically infecting resistant hosts through blossom or fruit tissue to determine if this is a potential mechanism for TSWV to evolve RB to the *Sw-5* gene. Our second objective was to study the response of a naturally occurring RB TSWV isolate to selection by serial passages through peppers with and without the *Tsw* R gene. We looked at the impact of host selection on infectivity and virulence of the virus to make predictions about the fitness of RB variants and the long-term durability of the *Tsw* R gene. These studies enhance understanding of the mechanisms for how TSWV infects resistant hosts and the consequences of interactions between TSWV and hosts with R genes. By uncovering the weakness of R genes, we can develop more targeted strategies to protect resistant cultivars when they are most vulnerable.

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CHAPTER 1

***TOMATO SPOTTED WILT VIRUS* INFECTS RESISTANT TOMATO WHEN
THRIPS FEED ON BLOSSOM OR DEVELOPING FRUIT**

ABSTRACT

Tomato spotted wilt is a major viral disease of crops. The most effective control method in tomato (*Solanum lycopersicum*) is the use of resistant cultivars carrying the *Sw-5* resistance gene. However, fruit-limited infections of *Sw-5* plants suggest that the resistance gene may not work effectively in fruit-associated tissue. The objective of this study was to determine if the insect vector, *Frankliniella occidentalis*, can transmit the virus when confined to blossoms or newly set fruit in tomatoes with and without *Sw-5*. The second objective was to test if the virus can move systemically out of these tissues. We observed fruit infections in 21% of *Sw-5* plants and 68% of susceptible plants when inoculated at the blossom or newly set fruit stage. Systemic infections also occurred in both host genotypes, causing foliage and fruit on other branches to develop symptoms in 71.4 and 76.5% of the infections, respectively. These results were further supported by field experiments that showed high proportions of infected fruit in *Sw-5* plants when *F. occidentalis* were abundant in blossoms as well as limited foliar infections surrounding infected fruit. These findings help to explain observations of abundant late season infections of *Sw-5* cultivars in commercial plantings.

INTRODUCTION

Tomato spotted wilt virus (family *Bunyaviridae*, genus *Tospovirus*, TSWV) continues to threaten the production of many crops worldwide despite ongoing control efforts (Goldbach and Peters 1994, Pappu et al. 2009). At least nine species of thrips (family Thripidae) are known to transmit TSWV to over 1000 plant species, including important crops such as tomatoes, peppers, tobacco, and peanuts (Mound 1996, Parrella et al. 2003, Riley et al. 2011). Controlling the spread of TSWV is challenging because immature thrips can acquire the virus from many common weed hosts and disperse into crops as adults, transmitting the virus for the duration of their lives (Ullman et al. 1997; Groves et al. 2001, 2002). In many production areas, *Frankliniella occidentalis* is considered the most significant vector in tomato (Cho et al. 1989, German et al. 1992, Wijkamp et al. 1995, Eckel et al. 1996, Latham and Jones 1997, Persley et al. 2006, Pappu et al. 2009). Its high competence as a vector and capacity for rapid population growth makes it a threat throughout the growing season (Lublinkhof and Foster 1977, Katayama 1997). Techniques such as reflective mulch (Greenough et al. 1990, Brown and Brown, 1992, Riley et al. 2012), applications of plant defense activators (Pappu et al. 2000, Riley et al. 2012) and use of pesticides (Brown and Brown 1992, Riley and Pappu 2000, 2004) have all shown limited success at controlling the disease when used singly (Cho et al. 1989, Reitz et al. 2003, Momol et al. 2004). The development of cultivars carrying a resistance (R) gene is currently considered one of the most effective methods for controlling TSWV in tomatoes, peppers, and peanuts (Riley and Pappu 2004, Gordillo et al. 2008, Riley et al. 2011, UGA CAES 2012). *Sw-5* is a single, dominant gene in tomato that belongs to a common family of disease R genes that encode

nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins. It is hypothesized that the *Sw-5* proteins act as inhibitors that recognize the virus and launch a signal transduction pathway causing a local lesion (called a hypersensitive response) to form at the site of the inoculation, which prevents further spread of the virus to other cells (Brommonschenkel et al. 2000, Nimchuk et al. 2003). The *Sw-5* gene has been very effective both in the greenhouse and field against most virus isolates (Stevens et al. 1992, 1994, Boiteux and de Giordano 1993, Gordillo et al. 2008, Riley et al. 2011).

TSWV can cause substantial losses to growers by reducing the quantity and quality of fruit produced. Control efforts are focused on protecting plants at the early stages of development when they are most susceptible to TSWV infections and suffer the largest effects on yield (Chaisuekul et al. 2003, Beaudoin et al. 2009). Symptoms in susceptible cultivars include discoloration and deformity of the leaves and fruit, stunting, and even death of the plant (Cho et al. 1996). Despite carrying the *Sw-5* gene, resistant tomato cultivars can also develop symptoms, which are usually fruit-limited and appear later than the majority of infections seen in susceptible cultivars (Fig. 2). Even though infected resistant cultivars continue to produce fruit, the appearance of ringspots and deformities on the tomatoes make them unmarketable, resulting in major losses to growers (Moriones et al. 1998, Culbreath 2003). Infected fruit has reached as high as 90% of total fruit produced on resistant cultivars in growers' fields (Kennedy, unpublished) and occasionally, foliar symptoms surrounding infected fruit test positive for TSWV (Houle, personal observation).

Frequent observations of *Sw-5* plants expressing only symptomatic fruit late in the season led to the hypothesis that direct feeding on the fruit by viruliferous thrips can cause localized fruit infections (de Haan et al. 1996, Aramburu and Rodriguez 1999). Aramburu et al. (2000) tested this hypothesis by placing viruliferous thrips on harvested, green fruit. The appearance of ringspots occurred on several of the inoculated fruit, confirming this phenomenon. In the field, they observed *Sw-5* plants with systemic infections at low titers, but attributed them to resistance-breaking (RB) isolates or incomplete penetration of *Sw-5*. This study left many questions unanswered about the full extent of this problem. Since only harvested fruit were used, the possible consequences of disease progression within the plant, especially if initiated earlier in the fruiting stage, are unknown. Furthermore, their study did not evaluate whether the *Sw-5* resistance gene increases protection against TSWV when fruit are fed upon by viruliferous thrips as compared to susceptible genotypes. Since resistance-breaking (RB) isolates of TSWV have emerged around the world (Cho et al. 1996, Latham and Jones 1998, Aramburu and Martí 2003, Ciuffo et al. 2005, Gordillo et al. 2008) it is also important to confirm that fruit-associated tissue is providing a gateway for non-resistance breaking (NRB) isolates to infect *Sw-5* plants, both in the fruit and systemically.

Our study focuses on the potential consequences of feeding by viruliferous *F. occidentalis* on fruit-associated tissue at the blossom and newly set fruit stages as it relates to the occurrence of late-season TSWV infections commonly seen in the fruit of tomato cultivars carrying the *Sw-5* gene. Our primary objective was to determine if NRB isolates of TSWV could be transmitted by *F. occidentalis* into blossoms or newly set fruit in resistant tomato cultivars.

Our second objective was to see if systemic infections could develop following inoculation of these tissues by *F. occidentalis*. Finally, we wanted to compare the infection rates between resistant and susceptible cultivars to determine if the *Sw-5* gene provides any protection against TSWV relative to susceptible cultivars when *F. occidentalis* feed on blossom or newly set fruit tissue. We explored the occurrence of late season infections in *Sw-5* tomatoes to understand how resistance is circumvented and emphasize the importance of control strategies that reduce exposure of resistant plants to *F. occidentalis* throughout the blossom and fruiting stages.

MATERIALS AND METHODS

Plants. Greenhouse and field experiments used *Solanum lycopersicum* cultivars ‘Mountain Glory’ and ‘Mountain Spring’ (Clifton Seeds, Faison, NC). ‘Mountain Glory’ (R) contains the *Sw-5* gene and ‘Mountain Spring’ (NR) has no resistance against TSWV.

Verification that TSWV Isolates Were NRB. Three TSWV isolates (designated John10a, Cart10, and John11) were collected from TSWV-susceptible cultivars in growers’ fields in Moore and Montgomery Counties in North Carolina in 2010 and 2011. These isolates were mechanically inoculated into *Emilia sonchifolia* and maintained in a greenhouse. The isolates were tested for the ability to break *Sw-5* resistance by mechanically inoculating into the foliage of tomato seedlings of R and NR cultivars. Inoculum was prepared by grinding infected leaf tissue from *E. sonchifolia* in a buffer containing 10 mM Tris·HCl (pH 7.8), 10mM Na₂SO₃, and 0.1% L-cysteine. All fully expanded leaves were coated with 600-mesh

Carborundum and gently rubbed using a cotton swab saturated with inoculum. To further confirm that isolates were NRB, thrips inoculations were conducted on R and NR seedlings using the same viruliferous thrips as were used in the experimental trials (described below). A subsample of 30 thrips were released onto 2 to 3 week-old R and NR tomato seedlings growing in thrips-proof cages within a greenhouse in each of the 4 trials. Thrips were allowed to feed for 1 week on seedlings before plants were sprayed with spinosad insecticide to kill the thrips. Leaf tissue samples were collected 3 weeks after thrips were released and tested for TSWV using double sandwich enzyme linked immunosorbent assay (DAS-ELISA) for the nucleocapsid protein following the manufacturer's protocol (AGDIA, Elkhart, IN, USA). Samples were classified as TSWV positive when the optical density exceeded the mean plus four standard deviations of the negative controls (3 non-infected samples from same tissue type). Measurements were taken at 405 nm using a THERMOmax microtiter plate reader (Molecular Devices Corp., Menlo Park, CA).

Greenhouse Transmission Assays. A virus-free lab colony of *F. occidentalis* was maintained on *Phaseolus vulgaris* bean pods at ca. 26°C, 55% RH, and continuous light. Neonate *F. occidentalis* were placed on excised *E. sonchifolia* leaves that were infected with 1 of the 3 field-collected NRB TSWV isolates. The leaves and thrips were maintained inside a 50 mm diameter x 9mm Parafilm[®] sealed petri dish (Fisher Scientific, Pittsburg, PA) containing moist filter paper and placed in an incubator for a 48-72 hour acquisition period at 30°C. The thrips were transferred onto *P. vulgaris* bean pods in plastic containers with thrips-proof mesh tops and bottoms and reared to adult.

Adult *F. occidentalis* were confined on individual blossom clusters or clusters of newly set fruit enclosed in thrips-proof cages constructed from 100-micron screen (Midwest Filter Corp, Lake Forest, IL). Each cage was sealed around the peduncle of individual blossom clusters prior to blooming using putty (Oatey, Cleveland, Ohio) or poster putty (Duck, Avon, Ohio). All uncaged blossom clusters were removed regularly prior to blossoms opening. Putatively viruliferous adults were aspirated in groups of 5 into 1.5-ml microcentrifuge tubes 2–4 d after emergence and then released into 1 inflorescence cage per plant containing either freshly opened blossoms or newly set fruit (approx. diameter: 2-5mm). The experiment was run across 4 trials with 3 isolates (Table 1). In trials 1-3, five thrips per individual blossom or fruit were released into an inflorescence cage containing 2-4 blossoms or fruit. In trial 4, 5 to 10 thrips were released into a cage containing 2-4 blossoms. A systemic insecticide (aldicarb) was added to the soil 5-7 days after the thrips release to kill all thrips for the remainder of the experiment.

To minimize the risk of thrips escaping from the blossom / fruit cages and moving to other plants, each experimental plant was placed inside large thrips-proof enclosures (2 ½' x 2 ½' x 4') along with a yellow sticky trap to detect the presence of thrips outside of the blossom / fruit cages. To detect unintended virus spread, an indicator plant (*E. sonchifolia*) was also placed inside each enclosure and replaced every 2 weeks. In addition, 5 TSWV- and thrips-free tomato plants of each cultivar, were placed randomly throughout the greenhouse as sentinels to detect virus spread within the greenhouse. No *F. occidentalis* were caught on the

yellow sticky traps or observed outside of the blossom/fruit cages, nor did any of the *E. sonchifolia* or sentinel tomato plants become infected by TSWV.

To determine if TSWV infections spread systemically from inoculated blossoms or fruit to non-inoculated fruit, additional blossom clusters were enclosed in thrips-proof cages prior to the blossoms opening to exclude thrips. At least one blossom cluster was caged to exclude thrips prior to the thrips release on the same plant and additional blossom clusters that were initiated by the plant during weeks 3 and 6 following the thrips release were caged to increase the chances of picking up systemic movement of the virus into newly developing fruit. All other blossoms were removed prior to opening.

To determine TSWV infection, tissue samples were collected from ripened fruit inside the blossom / fruit cages into which the putatively viruliferous thrips were released as well as from cages that excluded thrips. In addition, systemic spread of the infection into foliage was tested 3 and 6 weeks after thrips were released using samples of leaf tissue growing on the same branch one node above and outside the cage and of leaf tissue growing on other randomly selected branches. These samples were tested for TSWV infection using DAS-ELISA as described previously.

Analysis. Data were analyzed with a generalized linear mixed model using the GLIMMIX procedure with Logit link function and a normal response distribution (SAS Institute 2005) to test for significant effects of inoculation site (blossom or fruit), genotype (R or NR), and their

interaction on the frequency of fruit-limited and systemic infections. In this analysis, trial was treated as a random effect and host genotype (R or NR) and inoculation site (blossom or fruit) were treated as fixed effects. Because the number of thrips released per blossom / fruit cage differed across trial runs, a preliminary analysis was conducted to examine the effect of number of thrips released on infection rates using the GLIMMIX procedure of SAS as described above. This analysis did not reveal a significant effect of the number of thrips released on incidence of fruit-limited or systemic infections ($F_{1,1}=2.29$, $p=0.37$); hence, this variable was removed from the analysis.

Field Experiments. Small plot, field experiments were conducted in 2011 and 2012 to monitor the prevalence of fruit-limited and systemic infections of TSWV in R and NR cultivars. In both years the experimental plantings were located in Candor, NC along one edge of a commercial planting of the TSWV-resistant tomato cultivar ‘Redline’, which expresses the *Sw-5* gene. The R and NR treatments were included along with other tomato cultivars in a larger randomized complete block experiment to evaluate performance. Detailed data on TSWV incidence were not collected from the other cultivars and hence they were not included in the analysis of results reported here. The 2011 experiments consisted of 4 replicates, each consisting of 3 plants of R or NR cultivars, transplanted on 2 May. The 2012 experiment consisted of 5 replicates, each consisting of 5 plants of R or NR cultivars, transplanted on 16 May.

During both years, all plots were inspected at ca. 2-week intervals to identify TSWV-infected plants. To determine the presence of systemic infections, foliage samples were taken from each plant and subjected to DAS-ELISA. In 2011, samples were taken on 5 July and 25 August. In 2012, foliage samples were collected from plants with suspicious foliar or fruit symptoms every 2 weeks from 30 May through 31 July, at which time foliage from all plants not previously classified as infected were tested. In addition, during both years, symptomatic fruit were collected at the time that they were first observed and the plants from which they were collected were classified as either expressing or not expressing foliar symptoms. On 25 August, 2011, all remaining fruit present on the surviving 10 R plants were removed and tested for infection using DAS-ELISA. In 2012, all fruit present on 30 July were collected from each plot and classified as TSWV-infected or non-infected based on presence or absence of symptoms and then subjected to DAS-ELISA to verify infection status and detect asymptomatic infections.

Natural thrips population. Thrips populations present in tomato blossoms during 2011 were assessed to determine their general abundance and species composition. Samples of blossoms were collected in groups of 10 by random selection across all cultivars and placed in 70% ethanol on 14, 22, and 30 June, and 22 July, 2011. The blossoms were taken to the laboratory where they were dissected and all thrips counted. Adult thrips were then slide-mounted and identified to species. In samples containing more than 25 thrips, a subsample of 25 were identified to species. On 28 June and 17 and 30 July, 2012, each sample of 10

blossoms contained 2 randomly selected blossoms from each of the 5 plots for R and NR cultivars. Samples were processed as in 2011. Results are presented with standard errors.

RESULTS

Verification that TSWV Isolates Were NRB. In addition to the mechanical inoculation tests for RB, inoculation tests using a subset of thrips during each trial were done to test if each isolate could infect 2-3 week old seedlings from each cultivar. No R seedlings became infected but each isolate was able to infect NR seedlings (Table 2). Trial 4 used the same isolate as Trial 3 for thrips inoculations, but had low transmission efficiency relative to the other trials.

Greenhouse Transmission Assays. Transmission of TSWV to both R and NR genotypes occurred when viruliferous thrips were caged on blossoms and on newly set fruit in greenhouse trials (Fig. 1, Table 3). Symptomatic fruit infections developed from the inoculated blossom or fruit cluster as well as systemic infections in the foliage and other fruit of the same plant. With the exception of 2 R and 2 NR plants, systemic infections were only seen on plants exhibiting infections at the site of inoculation. In one of these cases, the inoculated tissue never developed into fruit. In the other three cases, they were blossom inoculated and not all of the blossoms developed into fruit. Tomato plants commonly abort flowers, both in healthy conditions and in response to stress, including extensive thrips feeding (Childers 1997). It is possible that infections spread into the plant from infected tissue that did not develop into mature fruit. Infections, defined as a positive DAS-ELISA

test from any part of the plant, differed significantly between R and NR hosts ($F_{1,54}=4.49$, $p=0.04$), with 21% of R plants and 68% of NR plants becoming infected over the 4 trial runs (Table 3). The incidence of infected fruit did not differ significantly between treatments in which thrips were released onto blossoms or newly set fruit ($F_{1,3,2}=1.02$, $p=0.38$) nor was there a significant interaction between host genotype and inoculated tissue ($F_{1,54}=2.87$, $p=0.10$).

Systemic infections, defined as a positive DAS-ELISA test from either leaf tissue samples or from other non-inoculated fruit that was caged on the same plant to exclude thrips were seen in trials 1-3 for R hosts. The frequency of infections that went systemic did not differ between the R and NR genotypes ($F_{1,54}=1.50$, $p=0.23$) or between blossom or fruit releases of thrips ($F_{1,3,7}=0.13$, $p=0.74$), nor was the genotype by inoculated tissue interaction effect significant ($F_{1,54}=2.96$, $p=0.09$). Out of the total infections, 71.4% spread systemically in R hosts and 76.5% spread systemically in NR hosts (Table 3). Of the infections resulting from thrips released on blossoms, 88.2% were accompanied by systemic infections compared to 42.9% when thrips were released on small fruit. Of the systemic infections, 3 R and 6 NR plants had infections that traveled to other fruit that were caged to avoid any possible feeding by escaped thrips. All of the 9 infections in other caged fruit originated from blossom inoculations.

Typical symptoms of ringspots, necrosis and abnormal growth were observed on the fruit (Fig 2). Symptom severity was often extreme, in some cases causing fruit to wither and die.

Other fruit that became infected systemically also suffered severe necrosis and deformity. The most severe symptoms were seen only on blossom-inoculated fruit, suggesting that earlier infections in reproductive tissue may lead to more severe symptoms (Fig. 2 A & B). Foliage infections in R hosts showed small necrotic lesions in both greenhouse transmission assays and field cultivars, suggesting possible hypersensitive response due to detection by the plant (Fig. 3 A & C).

Field Experiments. In both years, fruit-limited infections in R plants were common. In 2011, all R plants had at least some fruit that were infected as compared to 28% in 2012 (Table 4). The percentage of infected fruit per R plant averaged $38.9 \pm 4.41\%$ (n=10) in 2011 and $12.1 \pm 4.05\%$ (n=18) in 2012. Because most of the NR plants developed severe systemic infections early in the season, they rarely produced fruit. In 2012, 3 NR plants that had infected fruit did not express systemic infections in the foliage as evidenced by negative DAS-ELISA tests, suggesting infections may have originated in fruit tissue. The percentage of infected fruit per NR plant averaged $31.9 \pm 13.6\%$ (n=8).

Systemic infections were common in the NR cultivar in both years, but infrequent in the R cultivar. All NR plants in 2011 and 64% in 2012 were systemically infected. None of the R plants were systemically infected in 2011, but 12% had systemic infections in 2012 (Table 4). One of the R plants became infected early in the season and developed symptoms typical of those observed in NR plants. The other two infections were detected late in the season and were limited to the fruit and foliage located directly above the developing fruit on the same

branch. Sampling in the grower's planting of a TSWV-resistant cultivar in 2011 showed a similar phenomenon. There were 2 cases of infections found in the foliage directly above symptomatic fruit. These systemic infections in the foliage were located on the same branch as symptomatic fruit, suggesting systemic movement may have occurred from the blossom or fruit tissue into surrounding foliar tissue.

Natural Thrips Population. *F. occidentalis* was the predominant vector present, comprising $89 \pm 6.5\%$ of the thrips population within the blossoms during 2011 and $49 \pm 12.2\%$ in 2012. No other TSWV vectors were present in the blossoms, and the only other species identified was *Frankliniella tritici*. In 2011, *F. occidentalis* numbers remained high throughout the season, averaging 4.25 ± 0.25 adults per blossom while in 2012, *F. occidentalis* were less abundant, averaging 1.2 ± 0.33 adults per blossom (Fig. 4). Overall thrips populations were extremely low in 2012 relative to 2011, likely due to a rainy, cool spring (Chappell et al. 2013).

DISCUSSION

We demonstrated that NRB isolates of TSWV are transmittable through blossom and young, developing fruit tissue into tomatoes with the *Sw-5* gene. This study extends previous research on TSWV transmission to nearly mature fruit (Aramburu et al. 2000) by demonstrating that these infections also result from feeding by viruliferous *F. occidentalis* on young tissue at the blossom and newly set fruit stage and can even spread into foliage and other fruit tissue. The *Sw-5* gene was effective at preventing infections resulting from

inoculation of foliar tissue directly and demonstrated some effectiveness at reducing the percentage of infections resulting from inoculation of blossom and fruit relative to susceptible hosts in our experiments. However, in conditions where virus and vector are present during the blossom and fruiting stages, the *Sw-5* gene may not be adequate in preventing major losses from TSWV. For this reason, management of *F. occidentalis* populations in late-season tomato plantings is important for reducing losses of marketable fruit in both resistant and susceptible tomato.

Systemic infections caused by NRB isolates were frequently seen in our experiment. Previous studies have attributed systemic infections either to RB isolates or incomplete penetration of *Sw-5*, which is estimated to result in 2% of plants failing to fully express *Sw-5* (Stevens et al. 1992, Aramburu et al. 2000). Our experiment saw a much higher incidence of infection. To ensure that this was not unique to a specific TSWV isolate, we successfully used 3 different isolates that failed to infect *Sw-5* plants following mechanical or thrips inoculation of foliar tissue. In the field, we also found infections of *Sw-5* plants that were limited to foliage directly above symptomatic fruit where virus is likely to move in a systemic infection. Furthermore, several isolates were collected from a grower's resistant tomato planting in Candor, NC to be tested for RB. While most inoculations out of field-collected resistant plant tissue were unsuccessful, mechanical inoculations using isolates collected in 2 tomato fruit and 1 leaf from different *Sw-5* plants were able to infect susceptible tomato seedlings, but failed to infect resistant tomato seedlings (Houle,

unreported data). This suggests that the isolates infecting the resistant plants are not likely to be RB and that most NRB are capable of causing fruit-initiated infections.

In our field experiments, *F. occidentalis* were abundant in the blossoms throughout the entire reproductive stage of the crop during 2011 and proved to be the predominant vector species in the tomato field during both years. A large percentage of the fruit on all *Sw-5* plants in the field had symptomatic infections in 2011, while 2012 saw a much lower percentage of fruit infections. This could be explained by a decline of the thrips population in 2012 just prior to the main bloom stage and low numbers of *F. occidentalis* that were present in the blossoms. Our results suggest that controlling *F. occidentalis* populations throughout the season to prevent their feeding on blossoms and newly set fruit may prove to be important in reducing late season spread and the incidence of infected fruit. Although tomato plants that become infected later in the season produce significantly more fruit than plants infected early, the fruits often express symptoms and are unmarketable (Moriones et al. 1998). A further concern with thrips populations is the potential for secondary spread. Since immature *F. occidentalis* can be consistently found in tomato blossoms throughout the season (Beaudoin 2011), it may be possible for them to acquire TSWV and spread it among fruit clusters within a field.

The failure of *Sw-5* to prevent late season fruit infections is not understood, but we hypothesize that *Sw-5* expression in blossoms and developing fruit may differ from foliar tissue. While currently no work has been done to look at *Sw-5* gene expression in different

plant tissues, recent work in tobacco has shown variation in expression of reporter genes controlled by the *N* gene promoter, an R gene against *Tobacco mosaic virus* (Kathiria et al. 2013). Reporter gene expression was variable in both the organs of the plant and during different developmental stages. It is not surprising that plant tissues express R genes differently and emphasizes the importance of understanding when and where R genes are active in a plant if we want to protect it during its most vulnerable stages.

The *Sw-5* gene has provided significant protection against TSWV in tomato, but continued success depends on recognizing its weaknesses and protecting resistant plants when they are most vulnerable. To promote long-term durability of R genes, it is important to understand the consequences of different mechanisms that viruses use to circumvent plant defenses. The ability of NRB TSWV isolates to infect hosts carrying an R gene and travel systemically into foliar tissue has important implications for TSWV evolution. The appearance of necrotic lesions on the foliage of R plants suggests that the virus is being detected and the plant is initiating a hypersensitive response. This interaction may be applying selective pressure on variants with improved fitness in the R host, increasing the opportunities for RB to emerge. It is important to reduce the opportunities viruses have to interact with R genes to decrease the chances of new RB isolates from emerging. The *Sw-5* gene has been an effective tool in preventing TSW epidemics, but should be used with multiple control tactics that reduce risk of TSWV exposure during the vulnerable blossom and fruiting stages.

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Table 1. Trial descriptions showing TSWV isolate and number of R and NR plants used.

Trial	Isolate	# R plants	# NR plants
1	John10a	5	5
2	Cart10	6	5
3	John11	10	10
4	John11	12	5

Table 2. Inoculation tests to verify that TSWV isolates were non-resistance-breaking (# infected/# inoculated). Mechanical inoculations were done for each isolate and thrips inoculations were done at the time of each trial.

Isolate	Mechanical inoculation		Trial	Thrips inoculation	
	R	NR		R	NR
John10a	0/22	8/21	1	0/45	28/48
Cart10	0/18	12/20	2	0/48	23/46
John11	0/24	13/24	3	0/49	32/48
John11			4	0/48	6/49

Table 3. Greenhouse transmission assays showing number of plants that developed infections in each host (R and NR) when blossom or fruit were inoculated out of the total number of plants inoculated. Any infection includes fruit-limited and systemic infections.

Inoculated tissue	R			NR		
	Any infection	Systemic	Total Plants	Any infection	Systemic	Total Plants
Blossom	3	3	26	14	12	20
Fruit	4	2	7	3	1	5
Total	7	5	33	17	13	25

Table 4. Field data showing the number of plants for each host (R and NR) that had fruit-limited infections or systemic infections in 2011 and 2012.

Year	R			NR		
	Fruit-limited	Systemic	Total Plants	Fruit-limited	Systemic	Total Plants
2011	10	0	10	0	12	12
2012	6	3	25	3	16	25

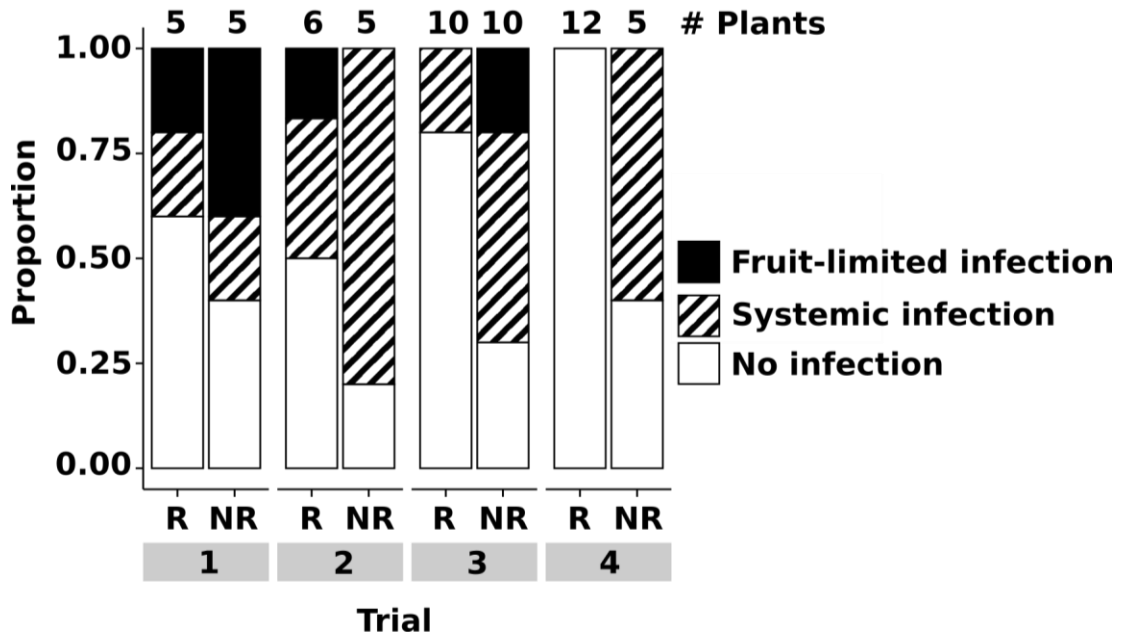


Figure 1. Proportion of resistant (R) and non-resistant (NR) tomato infections in greenhouse trials 1-4. Fruit-limited infection= DAS-ELISA positive infections that never traveled beyond the fruit that were inoculated either at the blossom or fruit stage. Systemic infection= DAS-ELISA positive infections that moved outside of inoculated tissue into foliage or other non-inoculated fruit. No infection = no positive DAS-ELISA infections in any part of the plant.

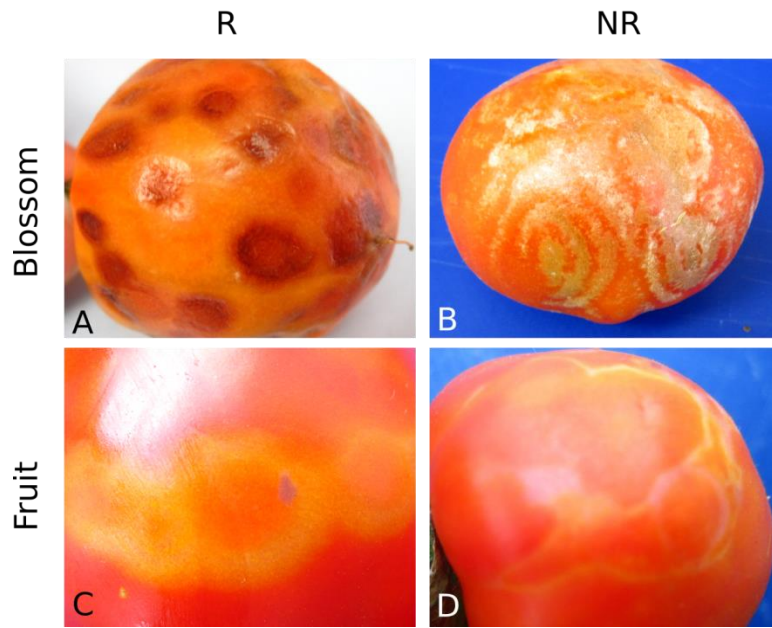


Figure 2. Photos of typical fruit symptoms in blossom (BI) and fruit (FI) inoculated infections in R and NR hosts: A) BI, R host B) BI, NR host C) FI, R host D) FI, NR host.

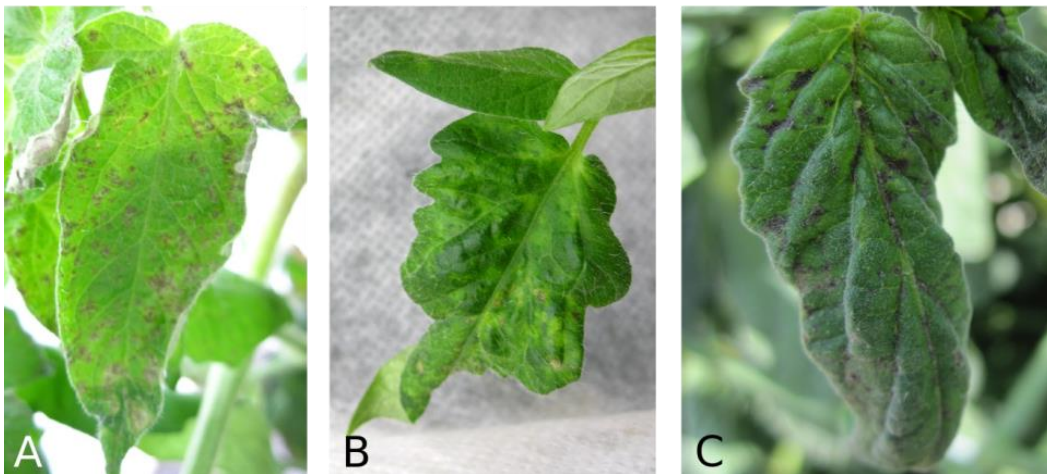


Figure 3. Photos of typical leaf symptoms in greenhouse trials for A) R and B) NR hosts. C) Infected leaf symptoms on field *Sw-5* cultivar

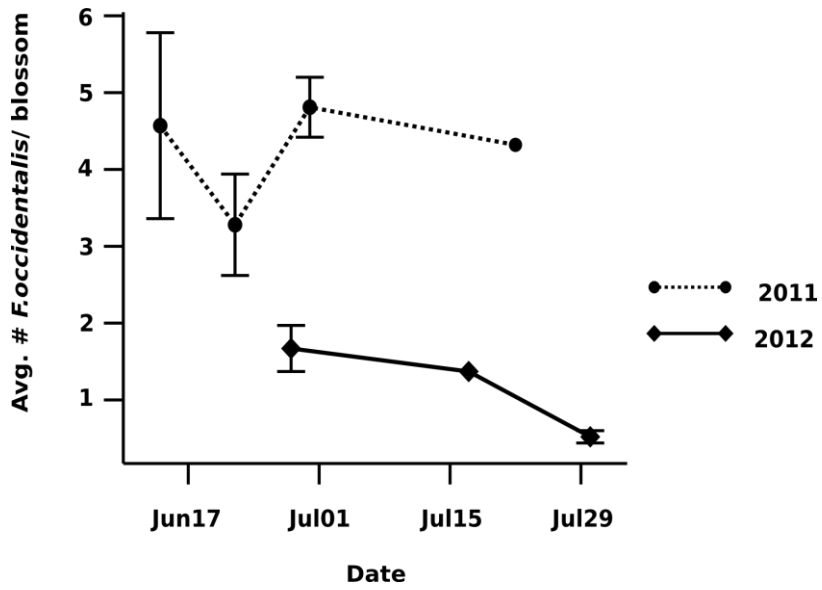


Figure 4. Average number of *F. occidentalis* thrips per blossom in 2011 and 2012. Error bars represent standard error. Where standard error bars are not shown, there is only one sample of ten blossoms represented.

CHAPTER 2

EVOLUTION OF *TOMATO SPOTTED WILT VIRUS* IN RESPONSE TO THE *TSW* RESISTANCE GENE

ABSTRACT

The use of resistance (R) genes in crops is an important control strategy for plant diseases. However, prolonged exposure to hosts with an R gene can lead to major genetic and phenotypic changes in the disease agent. Resistance-breaking (RB) variants of *Tomato spotted wilt virus* (TSWV) have emerged that can overcome pepper cultivars carrying the *Tsw* R gene. To study the effects of host genotype on RB evolution, an isolate with limited RB abilities was subjected to independent serial passages in resistant and susceptible pepper host lines to look for changes in infectivity and virulence. We hypothesized that infectivity and virulence should increase with increasing number of serial passages in the selecting host, but decrease in an alternate host. Selection of the parent virus population by serial passage through resistant hosts resulted in an increase in infectivity in resistant hosts, while also maintaining high infectivity in susceptible hosts. Selection of the same parent virus population through susceptible hosts resulted in an increase in infectivity in susceptible hosts, but infectivity remained low in resistant hosts. We found that virulence also increased with serial passages in the selecting host but did not significantly change in the alternate host. In a second experiment, we looked at the stability of RB after selection by 6 serial passages in resistant hosts. We found that after 5 serial passages through susceptible hosts, no reversion to lower infectivity or virulence was detected in R hosts. This study lays the foundation for understanding how RB isolates of TSWV respond to selection by the *Tsw* gene and supports management strategies that reduce extended exposure to the gene to increase R-gene durability.

INTRODUCTION

Tomato spotted wilt virus (TSWV) infects a wide range of host plants causing significant damage worldwide (Pappu et al. 2009). The virus is transmitted horizontally by thrips (Thysanoptera: Thripidae) that acquire the virus as 1st instars and transmit for the duration of their adult lives (Ullman et al. 1992, Wijkamp et al. 1993, van de Wetering et al. 1996, Nagata et al. 2002). The use of resistance (R) genes is one major strategy for preventing plant virus epidemics. The *Sw-5* and *Tsw* R genes have been employed in tomato and pepper, respectively, to prevent TSWV infections. While both have been important tools for preventing epidemics, the evolution of resistance-breaking (RB) has emerged repeatedly, threatening the durability of these R genes (Boiteux and Giordano 1993, Hobbs et al. 1994, Cho et al. 1996, Latham and Jones 1998, Roggero et al. 2002, Aramburu and Martí 2003, Margaria et al. 2004, Ciuffo et al. 2005, Sharman and Persley 2006).

TSWV is a single-stranded, tripartite RNA virus with ambisense coding. When TSWV infects a host with *Tsw*, the R gene products identify the virus' nucleocapsid protein as the avirulence factor (Lovato et al. 2008) and trigger a hypersensitive response (HR) to form a local lesion at the site of the inoculation, which prevents further spread of the virus to other cells (Brommonschenkel et al. 2000, Nimchuk et al. 2003). RB, allowing TSWV to overcome the *Tsw* R gene in pepper, has been mapped to the gene on the S RNA strand coding for the non-structural protein (NS_s) (Margaria et al. 2007). It was determined that the NS_s gene suppresses the plant's TSWV gene silencing response (Takeda et al. 2002, Bucher et al. 2003).

The durability of R genes depends on the complexity of the resistance gene(s) and consequently, the number and type of mutations in the virus needed to overcome them (Harrison 2002). *Tsw* is a single dominant allele, which puts it at high risk for TSWV to overcome it. As an RNA virus with a high mutation rate, short replication time, and high yield, TSWV is capable of adapting quickly (Domingo and Holland, 1997). Natural populations of viruses often lack the genetic diversity one would expect from a high mutation rate, implying that purifying selection may be playing a significant role, weeding out less fit variants (French and Stenger, 2003). For a particular variant to become pathogenic in a new host and remain viable in the population, it must remain competitive within the virus population, especially if the novel host is rare. Specialist viruses are expected to be more successful than generalist viruses because they are predicted to evolve faster within a niche (Woolhouse et al. 2001). However, adaptation to one host is expected to result in a fitness cost in an alternate host (Levins 1968, Vanderplank 1984, Kawecki 1994, Jenner et al. 2002). Fitness costs associated with RB in TSWV have not been consistently observed. Using serial subculture, it was shown that TSWV RB isolates able to infect pepper expressing the *Tsw* gene maintained the ability to break resistance after four passages in susceptible peppers and remained competitive with a non-resistance-breaking (NRB) isolate in a mixed infection. However, after a fifth passage, some reversion to NRB was seen, suggesting the possibility of a fitness cost associated with the RB mutation (Thomas-Carroll and Jones 2003). In tomato with the *Sw-5* gene for resistance, RB isolates have shown greater stability. No reversion to NRB was seen after as many as 10 serial passages in susceptible tomato plants (Aramburu et al. 2010, Houle, unpublished data). Furthermore, RB isolates were thrips

transmitted equally as efficiently as NRB isolates (Aramburu et al. 2010) and shown to be competitive enough with NRB isolates to be present in mixed infections after co-inoculation (Latham and Jones 1998).

As viruses increase infectivity on a host containing an R gene, their effects on the new host and the ancestral host may change. The evolution of virulence, defined as the degree of damage caused to the host, is a highly disputed topic (Alizon et al. 2009). In systems where viruses are transmitted horizontally by vectors, virulence is predicted to increase, even at the cost of plant mortality because higher virus titer increases the chance of vectors acquiring the virus during feeding (Lipsitch et al. 1996, Doumayrou et al. 2012). This assumes that virulence increases with virus titer due to the additional nutrient burden on the plant and increased damage due to pathogenicity and host responses (Ewald 1983, Brown et al. 2006). However, in TSWV, viral concentrations have been correlated with symptom expression in susceptible but not resistant plants, demonstrating that high virus concentration can be present in plants that express mild symptoms (Resende et al. 2000). It is also argued that evolution should favor decreased virulence (Alexander 1981). If virulence is very high, it causes host mortality, shortening the time period in which a vector can acquire the virus. In some cases, more virulent infections may also make hosts less desirable to vectors, decreasing the opportunities for vector transmission (Escriu et al. 2003). The ‘transmission-virulence trade-off hypothesis’ argues that virulence is an unavoidable consequence of increasing transmission success (Anderson & May 1982). Due to the trade-offs between transmission and virulence, it has been argued that virulence should evolve to an intermediate

level in order to maximize the infectious period (May and Anderson 1983). The evolution of virulence is an important aspect of viral fitness due to the consequences it can have on vector transmission, infectious period, competition within the virus population, and replication (Frank 1996).

Our main objective was to determine how selection in resistant and susceptible peppers affects the infectivity and virulence of an isolate with limited ability to infect pepper expressing the *Tsw* R gene. We hypothesize that selection through serial passages in either resistant or susceptible pepper will result in an increase in infectivity and virulence in the selecting host and decrease in the alternate host. We also predict that selection in resistant hosts will lead to a more stable RB isolate. This experiment provides the groundwork for understanding the phenotypic consequences of exposing TSWV to repeated selection by hosts with the *Tsw* R gene and informs management strategies that reduce extended exposure to the gene to increase R gene durability. Furthermore, samples collected throughout this experiment can provide information on specific mutations in the virus that enable it to break resistance.

MATERIALS AND METHODS

Isolate. In 2010, an isolate was collected from tomato ('Celebrity') in a grower's field in Apex, NC. This isolate was discovered to have limited infectivity in R peppers during a mechanical inoculation from the original tomato tissue into R pepper seedlings. Infectivity averaged 46% in resistant peppers and 98% in susceptible peppers at the beginning of the

experiment. Peppers containing the *Tsw* gene had never been planted on this farm. Isolates were maintained in *Emilia sonchifolia* and preserved in -80°C freezer until their time of use.

Plants. Experiments used *Capsicum annuum* cultivars ‘Aristotle’ and ‘PS 099422815’ (Seminis Vegetable Seed). ‘PS 099422815’ contains the *Tsw* allele for resistance and ‘Aristotle’ has no resistance against TSWV.

Environment. Experiments were conducted in either a climate-controlled greenhouse (Raleigh, NC) or climate-controlled chambers within the North Carolina State University Phytotron. Greenhouse conditions were maintained at a temperature range of 18 to 24°C. In the greenhouse, plants were grown in pairs in 0.664 gallon pots (‘Classic 300 S’, Nursery Supplies Inc., Fairless Hills, PA) under a shade cloth to reduce natural lighting by 50%. There were a total of 12 plants in each treatment group and 8 control plants for each cultivar grown in the same conditions as treatment plants. Plants in the Phytotron were grown under conditions of 21/24°C, 12L/12D. A combination of fluorescent and incandescent lights provided an illuminance of 32.6 and 1.8 klx, respectively. Each plant was grown individually in a 114 mm (660 ml) pot with a 50/50 mixture of river bottom sand and peat-lite (Redi Earth, W.R. Grace Co.). A total of 12 plants were used for each treatment group and 8 control plants for each cultivar were grown in the same conditions as treatment plants. Plants were fertilized twice daily using the NCSU Phytotron nutrient solution (Saravitz et al. 2009).

Experimental design. To test our hypotheses, a two-part experiment was run to determine how the presence or absence of the *T_{sw}* gene affects virus infectivity and virulence across serial passages in each host background (Fig. 1). In Part A, the original isolate was inoculated into a group of resistant (R) and susceptible (S) pepper plants. Source inoculum was taken from the infected R plants and inoculated into a new set of R and S peppers as indicated by the direction of the arrows. Using inoculum from the second R passage, an R host line was created through serial passages from R hosts to R hosts (R_A-Line). Using inoculum from the S group inoculated in the second passage, an S host line was created through serial passages from S hosts to S hosts (S_A-Line). In Part B, isolates collected following the 6th serial passage of the R_A-Line through R hosts were subjected to 6 cycles of serial passage through S hosts to establish a new S-line (R_AS_B-Line). In each serial passage of the R_A, S_A, and R_AS_B-Lines, R and S host plants were inoculated from the same source to compare infection rates, symptom expression, and leaf area between genotypes across passages.

Part A of the experiment contained four replicates, conducted at different times, starting with the same isolate that was stored in different samples of *E. sonchifolia* in the freezer (A1, A2, A3, A4). The first three replicates were conducted in the greenhouse between 2010 and 2012. Replicate 4 was conducted in NCSU's Phytotron climate controlled chamber in 2012.

In Part B, there were 6 replicates. B1a, B1b, and B1c were run simultaneously in the Phytotron using a starting virus population from the same R plant in the 6th passage of the

R_A-Line of replicate A1. Isolates B2, B3a, and B3b were run in the greenhouse using isolates originating from the same parent isolate as the other replicates, but were subjected to selection through four serial passages in R hosts run independently of the replicates reported in this study. All isolates exhibited 100% infectivity in R hosts prior to starting the R_AS_B-Line. B3a and B3b were run simultaneously and used the same starting tissue while B2 was run independently from different source tissue.

Mechanical Inoculations. TSWV was transferred between hosts using mechanical inoculations. Seedlings were prepared for inoculation when both cotyledons were present, but true leaves were not expanded (15-20d post-planting). Peppers were maintained in the dark for 24 hours prior to inoculation. Plants were returned to normal conditions at the time of inoculation. Inoculum was prepared by grinding infected leaf tissue in a buffer containing 10 mM Tris·HCl (pH 7.8), 10mM Na₂SO₃, and 0.1% L-cysteine. Cotyledons were coated with 600-mesh Carborundum and gently rubbed using a cotton swab saturated with inoculum. Virus source tissue at each passage consisted of approximately 4 cm² of leaf tissue selected from two infected source plants (ca. 2 cm² from each plant) to be inoculated into a new group (12 plants) of R and S plants. Plants were selected as sources if their issue had the most visible infection (chlorosis or mottle, leaf deformity), but plants showing extreme necrosis were not used due to concerns that titers of viable virus might be low. The R_A-Line used only infected tissue from R plants to infect the next group of R and S plants and the S_A- and S_B-Lines used only infected tissue from S hosts to infect the next group of R

and S plants (Fig. 1). A total of 6 passages between hosts were conducted. The S_A-Line only has 5 passages through S hosts because the first passage was in an R host.

Infectivity. Infectivity was measured after each transmission cycle as the proportion of infected plants out of total number of plants inoculated. Samples of leaf tissue from all plants were collected at 2 weeks and 4 weeks post-inoculation and tested for TSWV infection with double sandwich enzyme linked immunosorbent assay (DAS-ELISA) using antisera to the nucleocapsid protein following the manufacturer's protocol (AGDIA, Elkhart, IN, USA). Samples were classified as TSWV positive when the optical density exceeded the mean plus four standard deviations of the negative controls (4 non-infected samples from sham-inoculated peppers). Measurements were taken at 405 nm using a THERMOmax microtiter plate reader (Molecular Devices Corp., Menlo Park, CA).

Virulence. Visual symptoms were scored by symptom type as present or absent for all plants at 4 weeks post inoculation. Symptoms observed included asymptomatic infection, death, moderate to severe chlorosis and mottle (combined for analysis), small necrotic lesions, leaf deformity, and recovery from infection (showed positive infection at 2 weeks but not 4 weeks using DAS-ELISA). To measure the effect on plant growth, leaf area (cm²) of infected plants was measured and compared to non-infected control plants at 4 weeks post inoculation using a LI-3100 leaf area meter (LI-COR, Biosciences, Lincoln, NE). To observe changes in the R hosts' defense-related responses, the appearance of a necrotic lesion at the site of inoculation on the cotyledons was scored as a positive HR. Since the HR may not be 100%

effective at containing the virus, large necrotic lesions that appeared on non-inoculated leaves were scored as a resistance response as well.

Tissue collection. Leaf tissue samples of approximately 4 cm² were collected from 5 infected plants (when available) from each treatment group and stored in a -80°C freezer in 1.5 ml microcentrifuge tubes. The tissue samples will be analyzed to determine sequence changes so they can be compared to the changes in infectivity and virulence detected in the experiment.

Analysis. The infection rate and symptom data were analyzed with logistic regression (event=1, nonevent=0) using the LOGISTIC procedure (SAS v9.3, Cary, NC) to test for significant effects of host genotype, passage number, and the interaction between host genotype and passage number for each host line (R_A, S_A, or R_AS_B). Analysis of the R_A and S_B-Lines used passage numbers 1-6, but analysis of the S_A-Line used only passage numbers 2-6 because this line began with the first passage into an S host (Fig 1). To analyze relative leaf area of infected plants across passages, each infected plant's leaf area was divided by the average leaf area of the non-infected control plants of the same genotype to express the leaf area of infected plants as a proportion of that of non-infected control plants. This removed any inconsistencies among replicates resulting from changes in the environment over time (i.e. amount of light in greenhouse at different times of year). Leaf area was analyzed with a generalized linear model using the GLM procedure (SASv9.3, Cary, NC) to test for significant effects of host genotype (R or S), passage number (1-6 for R_A and R_AS_B, 2-6 for

S_A), and the interaction between host genotype and passage number for each host line (R_A-Line, S_A-Line, R_AS_B-Line). Data was spliced by host line and host genotype to isolate the effect of passage number.

RESULTS

Infectivity (Table 1, Fig 2). Over the 6 cycles of selection in R hosts, probability of infection of the R_A-line in R host plants increased significantly with the most significant change occurring between passages 1 and 3 (Fig. 2A). In contrast, probability of infection of the R_A-Line in S host plants was initially high and remained high over the 6 passages (Fig. 2A). The passage number by host genotype interaction was highly significant. There was little variation in response to selection among replicate isolates of the R_A-Line (Fig 2D).

In contrast, probability of infection of the S_A-Line in R hosts in response to 4 passages through S hosts was limited, although variation in response among replicate isolates of this line was considerable (Fig 2B & E). Overall, probability of infection of the S_A-Line in R hosts remained low and decreased in 3 out of 4 replicates, whereas probability of infection in S hosts increased from 83% to 100% (Fig 2B & E). This small but differential response is reflected in the significant passage number by host genotype interaction (Table 1).

In the R_AS_B-Line, which had previously been selected for RB showed no loss of RB after 6 passages through S hosts. Overall, there were no significant differences in probability of infection between R and S hosts or across passage number, and the passage number by host

genotype interaction effect was not significant (Fig. 2C & F; Table 1). However, probability of infection of replicate B1c decreased over the experiment in both R and S hosts, likely representing attenuation of the virus due to fitness costs associated with long-term mechanical inoculations (Resende et al. 1991, 1992, Inoue-Nagata et al. 1997).

2.1 Symptom Severity (Table 2, Fig. 3). In the R_A-Line, there were significant increases in the probability that infection of R hosts resulted in chlorosis/mottle, leaf deformity, and small necrotic lesions over the 6 cycles of selection, whereas the probability that infection of S hosts produced chlorosis/mottle, leaf deformity, or small necrotic lesions decreased, increased, and remained unchanged, respectively (Fig 3A, D & G).

In the S_A-Line, the probability that infected R hosts expressed the various symptom types did not change significantly with increasing passage number but the probability that infected S hosts expressed chlorosis/mottle or leaf deformity increased significantly (Fig 3B & E). The probability of expressing small necrotic lesions was very low in R and S hosts and did not change significantly with passage number (Fig 3H).

In the R_AS_B-Line, the probability that infected R hosts expressed chlorosis /mottling did not change with passage number, whereas the probability of expressing leaf deformity increased and the probability of expressing small necrotic lesions decreased slightly (Fig 3C, F, & I). The probabilities that leaf deformity and small necrotic lesions were expressed by infected S

hosts increased with passage number, whereas there was no change in probability that chlorosis / mottling were expressed (Fig. 3C, F, & I).

Symptoms including death and recovery were too rare to analyze, but occurred occasionally. Asymptomatic plants were rare except in replicate A1 of the R_A-Line, in which symptom expression by infected S hosts became increasingly rare until the final passage when all infected S hosts were asymptomatic. Expression of severe symptoms was still seen in R hosts of the same R_A-Line.

2.2 Leaf area (Table 3, Fig. 4). Relative leaf area of R hosts but not S hosts infected by the R_A-Line declined significantly with increasing passage number (Fig 4A & D). In contrast, leaf area of S hosts infected by the S_A-Line decreased whereas leaf area of infected R hosts remained stable (Fig 4B & E). Leaf area of both R and S hosts infected with the R_AS_B-line did not change with passage number (Fig 4C & F).

2.3. Defense-related Responses (Table 4, Fig. 5). There was a significant decrease with increasing passage number in the probabilities that plants expressed an HR or large necrotic lesions when R hosts were infected by the R_A-Line (Fig 5A & D). In contrast, the probabilities of these responses occurring in R hosts infected by the S_A-Line did not change significantly over the 6 passages (Fig 5B & E). The probabilities of an HR response or large necrotic lesions in R hosts infected by the R_AS_B-Line remained low throughout the trial, although there was a significant increase in probability of an HR (Fig 5C & F).

DISCUSSION

Our study examined the changes in infectivity and virulence of a TSWV isolate with limited RB ability in peppers with the *Tsw* R gene when selected through serial passage in R and S hosts. With increased selection through serial passages in R hosts, it was expected that the isolate would evolve towards higher infectivity in R hosts and consequently, higher virulence, as predicted by the ‘transmission-virulence trade-off hypothesis’ (Anderson and May 1982). However, as this occurred, we expected to see trade-offs manifest as decreased infectivity and virulence in the alternate host (pepper without the *Tsw* R gene). We also expected that selection by serial passages in S hosts would result in a decrease and eventual loss of RB in R hosts, because RB variants would be less fit relative to other variants in the population. To look at the impact of using the *Tsw* R gene frequently in an area in which RB variants are present at low frequency, we tested how serial passages through R hosts affected the stability of RB in the virus population. It was predicted that the population would become more stable, capable of maintaining high infectivity and virulence in R hosts, even after selection by serial passages through S hosts.

After selection by serial passages through resistant and susceptible pepper lines, we expected to see an increase in infectivity in the selecting host and decrease in the alternate host as passage number increased. In both the R_A-Line and S_A-Line, infectivity increased with passage number in the selecting host (Fig 2A & B). However, a significant decrease in infectivity of the R_A-Line was not seen in S hosts, and in fact a slight, but significant increase occurred. This could represent an adaptation of the isolate to peppers as hosts since the

parent isolate was collected from tomato and had no known history in peppers. We also did not see a significant overall decrease in infectivity in R hosts after serial passage of the S_A-Line through S hosts. However, variation was high among replicates (Fig. 2E): infectivity declined appreciably in two replicates and slightly in a third but increased appreciably in the fourth replicate. The loss of RB was expected, because variants with RB mutations are predicted to have lower fitness than NRB variants in environments where the R gene is not present due to trade-offs associated with adapting to a new host. However, variation is also expected to occur because genetic drift is an important evolutionary force in viruses. TSWV experiences genetic bottlenecks during transmission, which can allow less fit variants to propagate in a population (Chao 1990, de la Iglesia and Elena 2007). Even after additional passages of the S_A-Line through S hosts, RB did not decrease in the 4th replicate, suggesting that in this replicate, the RB variants may have experienced a founder's effect and became fixed in the population.

Virulence was also predicted to increase in the selecting host and decrease in the alternate host as passage number increased. This prediction is based on the transmission-virulence trade-off hypothesis and assumes that the virus evolves a higher fitness in the selecting host as indicated by transmission efficiency. The appearance of severe symptoms increased and relative leaf area decreased in the selecting hosts in both the R_A-Line and S_A-Line, but severe symptoms and relative leaf area remained more stable in the alternate hosts in both lines (Fig. 3 & 4). However, in one trial (A1), asymptomatic infections occurred in all S hosts in the R_A-Line by the 6th passage, even though infectivity of S hosts was 100% and the appearance

of severe symptoms was common in the R hosts. This suggests that a change in the virus population occurred that affected the interaction between the virus and S hosts, resulting in a decrease in virulence, despite maintaining high infectivity. The appearance of defense-related responses was used as a measurement of virulence in R hosts. It was shown to decrease as the virus population evolved higher infectivity in R hosts through selection by the R_A-Line. In contrast, after selection in the S_A-Line, the appearance of defense-related responses remained high in R hosts. The appearance of defense-related responses occurs when the plant identifies avirulence factors in the virus population (Brommonschenkel et al. 2000, Nimchuk et al. 2003). The decrease in appearance of defense-related responses in R hosts after selection in the R_A-Line suggests that the proportion of NRB to RB variants is either decreasing and/or the RB variants are evolving to become more effective at suppressing the plant's TSWV gene silencing response.

The final objective was to determine if RB would remain stable after selection by serial passages through R hosts. Our results indicated that the probability of infection, symptom expression, leaf area, and defense-related responses did not change when the R_AS_B-Line, which was initiated from plants infected by the 6th passage of the R-Line, was subjected to 5 serial passages in S hosts (Fig. 2C, 3C, F, I, 4C & 5C & F). This lack of response to selection indicates that the RB isolate was stable in the absence of the *Tsw* gene. The stability of the RB isolate predicts that durability of the *Tsw* gene may be low in areas where there has been repeated selection for RB.

Conducting selection experiments is challenging with viruses because genetic drift can lead to unexpected outcomes. While most replicates revealed consistent trends, the sudden increase in infection rate seen in R hosts in the S_A-Line of A3 is an exception and may reflect a genetic bottleneck due to a founder effect (French and Stenger 2003). Genetic variability is also likely to decrease during transmission to novel hosts, especially hosts with R genes (Schneider and Roossinck 2001). This could result in fixation of RB variants in a population and also explain why the R_AS_B-Line did not see a loss of RB after five serial passages in S hosts.

Another potentially confounding issue for selection experiments that involve repeated mechanical passage of TSWV isolates is the generation of defective interfering RNAs (DI RNAs), which can cause attenuation of the virus (Resende et al. 1991, 1992, Inoue-Nagata et al. 1997). If this had been a problem in our experiment, we would have seen a decrease in infectivity occur in both R and S hosts, although it may have occurred in one replicate of Part B of our experiment (Fig. 1 F, replicate B1c). We chose to avoid using thrips transmission instead of mechanical transmission, despite its advantage of reducing the risk of DI RNAs, because thrips transmission efficiency can be very variable. Furthermore, we were able to detect changes in infectivity and virulence within a low number of passages, which kept the risk of DI RNAs to a minimum.

Our results are consistent with the findings of Thomas-Carroll and Jones (2003), demonstrating that RB is relatively stable within 4 serial passages in susceptible hosts, but

that some fitness cost appears to exist as evidenced by a decrease in RB in 3 out of 4 replicates. Since their studies were limited to very small sample sizes and replicates and potentially suffered from an attenuating virus, our study provided a more rigorous investigation. In addition, our findings show that selection by serial passages in resistant hosts results in highly stable RB isolates. Our results are also consistent with the hypothesis that virulence increases with transmission during serial passages in a selecting host. However, a longer-term experiment might show that virulence reaches an intermediate level reflecting a trade-off between transmission and virulence. The next step in this project is to sequence the isolates collected at each passage to detect and link specific mutations with changes in infectivity and virulence. Previous sequencing work has revealed that *Tsw* RB mutations occur on the NSs gene, but exact changes are not clear (Margaria et al., 2007). In isolates collected from infected *Sw-5* tomato plants, it was shown that two different single amino acid substitutions conferred RB, demonstrating that it is relatively easy for new variants to be generated that overcome resistance (Lopez et al. 2011). By sequencing samples from this experiment that are highly RB or NRB, we will be able to detect the complexity of mutations that enable RB in this population. Furthermore, we may be able to detect mutations that increase infectivity and virulence in selecting hosts.

The emergence of RB variants of TSWV is a major threat to the long-term durability of resistance genes like *Tsw* and *Sw-5*. A high fitness cost to RB isolates in susceptible hosts is a strong predictor of R gene durability. When fitness costs exist, it can be effective to temporarily rotate resistant crops with susceptible genotypes and have growers rely on other

control tactics like reflective mulches and anti-feedants (Momol et al. 2004, Riley et al. 2012). This strategy worked in Hawaii, where a RB isolate of TSWV became so prevalent that *Sw-5* tomato production ceased completely. When *Sw-5* tomatoes were grown several years later, the RB isolate was no longer found, suggesting that there was a fitness cost to the virus (Gordillo et al. 2008). We saw a fitness cost in our RB isolate after serial passages in S hosts and found that serial passages in resistant hosts leads to a more stable RB isolate, suggesting that rotation of R and S peppers could help increase the durability of the *Tsw* gene.

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Table 1. Logistic regression analyses of the probability of infection in the R_A, S_A, and R_AS_B- Lines to test the effects of host genotype, passage number (PassNum), and their interaction. Additional analyses of infectivity were run individually for R and S genotypes within a line to test for effects of passage number in each host genotype.

Line	Source	DF	Estimate	Standard Error	Chi-square	p-value	
R _A	Intercept	1	-0.041	0.3593	0.013	0.9091	
	Host Genotype	1	-1.9212	0.3593	28.5853	<0.0001	***
	Passage Number	1	0.9089	0.1446	39.525	<0.0001	***
	PassNum*HostGeno	1	0.5374	0.1446	13.8193	0.0002	***
	PassNum in R	1	1.4463	0.2212	42.7569	<0.0001	***
	PassNum in S	1	0.3714	0.1862	3.9786	0.0461	*
S _A	Intercept	1	-0.7735	0.7982	0.9389	0.3326	
	Host Genotype	1	1.1746	0.7982	2.1653	0.1412	
	Passage Number	1	0.8548	0.3374	6.4206	0.0113	*
	PassNum*HostGeno	1	-0.9507	0.3374	7.9422	0.0048	**
	PassNum in R	1	-0.0959	0.0936	1.0495	0.3056	
	PassNum in S	1	1.8058	0.6683	7.3011	0.0069	**
R _A S _B	Intercept	1	2.4631	0.2961	69.207	<0.0001	
	Host Genotype	1	-0.2989	0.2961	1.0193	0.3127	
	Passage Number	1	-0.1021	0.0707	2.0874	0.1485	
	PassNum*HostGeno	1	-0.0125	0.0707	0.0311	0.8599	
	PassNum in R	1	-0.1145	0.086	1.7725	0.1831	
	PassNum in S	1	-0.0896	0.1121	0.639	0.4241	

Significance: p<0.05 * p<0.01 ** p<0.0001 ***

Table 2. Logistic regression analyses of the probability of symptom expression for the R_A, S_A, and R_AS_B-Lines to test the effects of passage number (PassNum) within a line, spliced by host genotype.

Symptom	Line	HostGeno	DF	Estimate	Standard Error	Chi-square	p-value	
Moderate to Severe Chlorosis/Mottle								
	R _A	R	1	1.7195	0.2152	63.8449	<0.0001	***
	R _A	S	1	-0.3229	0.1131	8.1577	0.0043	**
	S _A	R	1	0.018	0.0949	0.036	0.8496	
	S _A	S	1	1.8058	0.6683	7.3011	0.0069	**
	R _A S _B	R	1	-0.0951	0.0698	1.8527	0.1735	
	R _A S _B	S	1	-0.1007	0.1139	0.782	0.3765	
Leaf deformity								
	R _A	R	1	0.9923	0.1304	57.926	<0.0001	***
	R _A	S	1	1.0253	0.1552	43.6183	<0.0001	***
	S _A	R	1	0.2193	0.1152	3.6222	0.057	
	S _A	S	1	0.6159	0.1942	10.0562	0.0015	**
	R _A S _B	R	1	0.2309	0.0654	12.4736	0.0004	**
	R _A S _B	S	1	0.5119	0.0774	43.6953	<0.0001	***
Small necrotic lesions								
	R _A	R	1	0.3441	0.0758	20.6277	<0.0001	***
	R _A	S	1	0.182	0.1851	0.9672	0.3254	
	S _A	R	1	0.0226	0.1062	0.0451	0.8318	
	S _A	S	1	1.0791	0.8653	1.5555	0.2123	
	R _A S _B	R	1	-0.1551	0.072	4.6371	0.0313	*
	R _A S _B	S	1	0.9818	0.2331	17.735	<0.0001	***

Significance: p<0.05 * p<0.01 ** p<0.0001 ***

Table 3. ANOVA analyses of infected leaf area proportion relative to uninfected controls in the R_A, S_A, and R_AS_B-Lines to test the effects of host genotype, passage number (PassNum), and their interaction. Additional analyses of infectivity were run individually for R and S genotypes within a line to test for effects of passage number in each host genotype.

Line	Source	Error		F	p-value	
		DF	DF			
R _A	HostGeno	1		25.62	<0.0001	***
	PassNum	1		6.55	0.0109	*
	PassNum*HostGeno	1		2.02	0.1559	
	Model	3	404	11.4	<0.0001	
	PassNum in R	1	189	9.5	0.0024	**
	PassNum in S	1	215	0.91	0.3421	
S _A	HostGeno	1		15.81	<0.0001	***
	PassNum	1		7.53	0.0065	**
	PassNum*HostGeno	1		2.29	0.131	
	Model	3	282	8.54	<0.0001	
	PassNum in R	1	104	0.17	0.6828	
	PassNum in S	1	178	19.86	<0.001	***
R _A S _B	HostGeno	1		150.04	<0.0001	***
	PassNum	1		0.1	0.7476	
	PassNum*HostGeno	1		1.15	0.2842	
	Model	3	696	50.43	<0.0001	
	PassNum in R	1	340	0.32	0.57	
	PassNum in S	1	356	0.89	0.345	

Significance: p<0.05 * p<0.01 ** p<0.0001 ***

Table 4. Logistic regression analyses of the probability of defense-related responses for the R_A, S_A, and R_AS_B-Lines to test the effects of passage number (PassNum) within a line, spliced by host genotype.

Symptom	Line	HostGeno	DF	Estimate	SE	Chi-square	p-value	
HR	R _A	R	1	-1.1351	0.1539	54.4328	<0.0001	***
	S _A	R	1	0.0785	0.0936	0.7044	0.4013	
	R _A S _B	R	1	0.6845	0.2234	9.3872	0.0022	**
Large necrotic lesions	R _A	R	1	-1.1057	0.2363	21.9	<0.0001	***
	S _A	R	1	-0.0384	0.1131	0.1149	0.7346	
	R _A S _B	R	1	0.1261	0.1162	1.1767	0.278	

Significance: p<0.05 * p<0.01 ** p<0.0001 ***

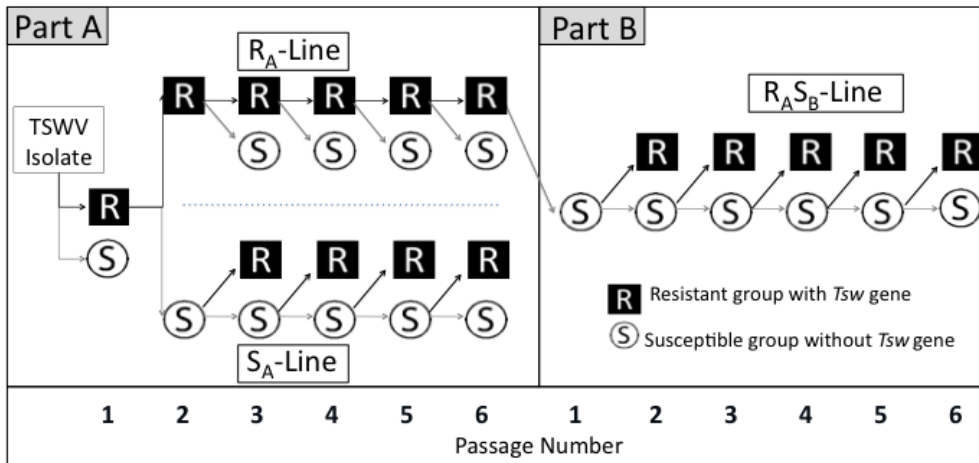


Figure 1. Diagram showing the serial passage sequence of TSWV isolates. Experiment begins at “TSWV Isolate” on left. Origin of arrow is source of inoculum for next passage into R and S host groups, which are designated by direction of the arrow. Part A has two distinct lines (R_A-Line and S_A-Line). Part B uses source inoculum from the end of Part A R_A-Line to form the R_AS_B-Line.

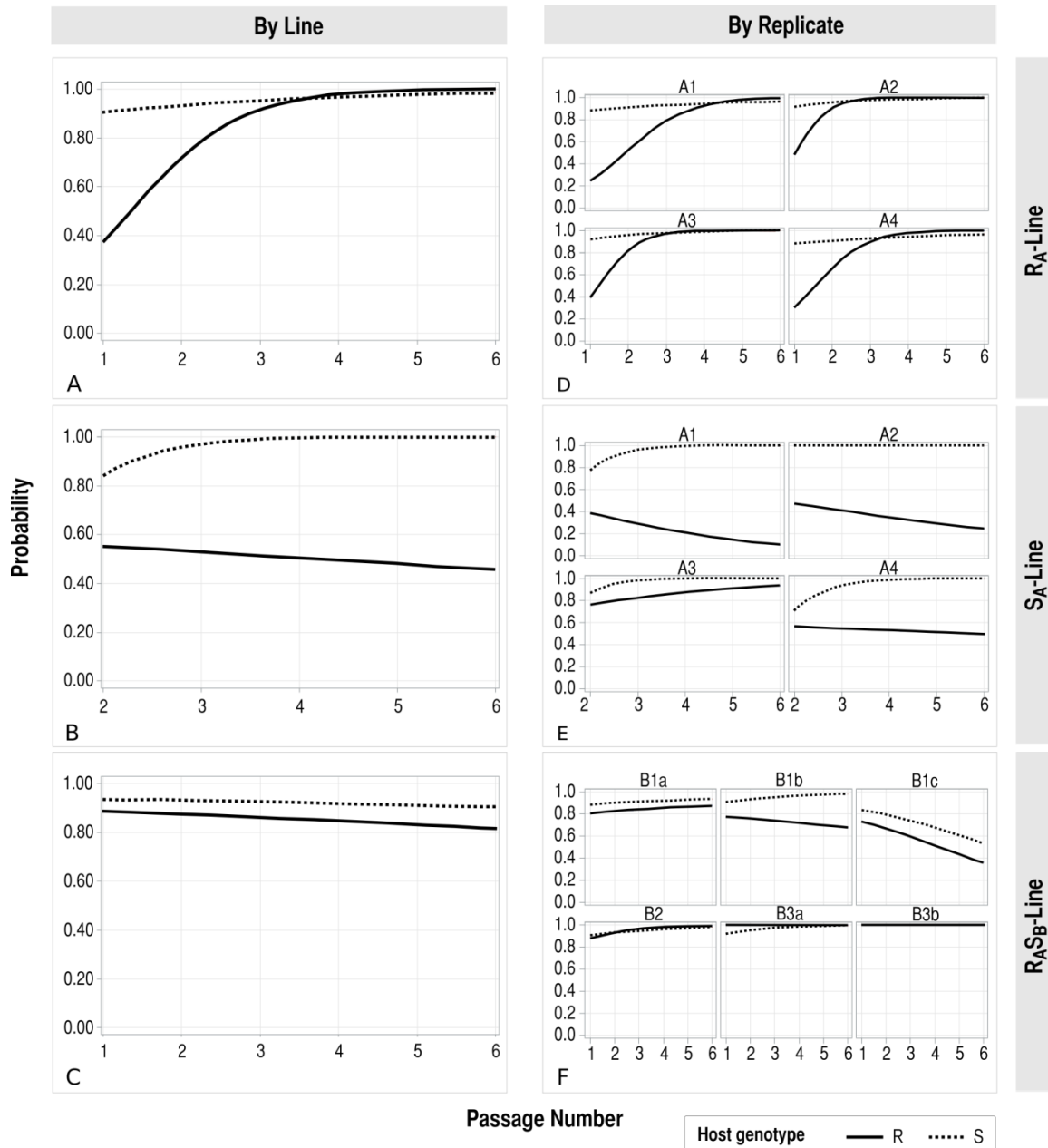


Figure 2. Predicted probabilities for infection rate in the R_A , S_A , and $R_A S_B$ -Lines across passage number. (A-C) shows average probability of infection of all replicates by line for each host genotype (R or S). (D-F) shows infectivity for each host genotype by replicate to show variation.

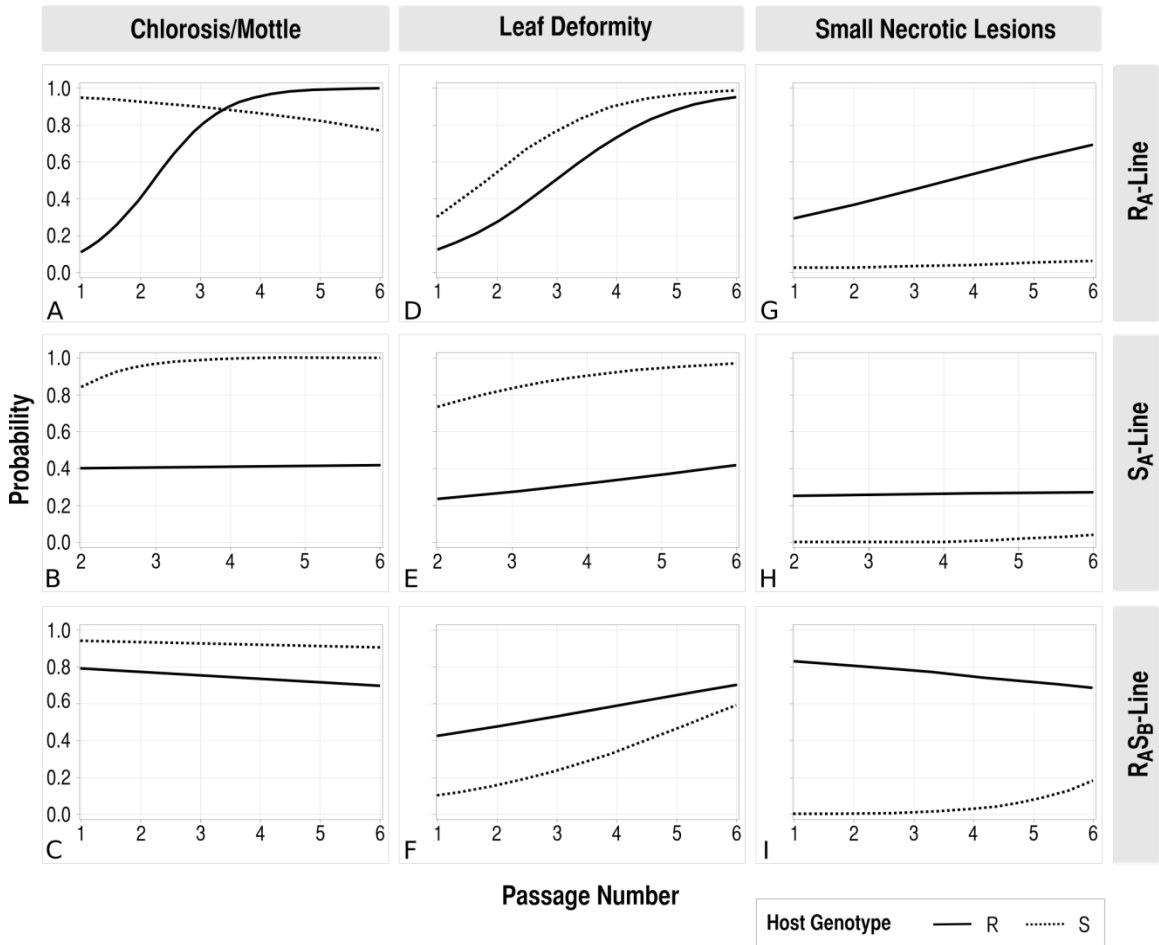


Figure 3. Predicted probabilities for symptom expression in the R_A , S_A , and $R_A S_B$ -Lines across passage number. (A-C) shows average probability of infected R and S plants expressing chlorosis or mottle within a line. (D-F) shows average probability of infected R and S plants expressing leaf deformity within a line. (G-I) shows average probability of infected R and S plants expressing small necrotic lesions within a line.

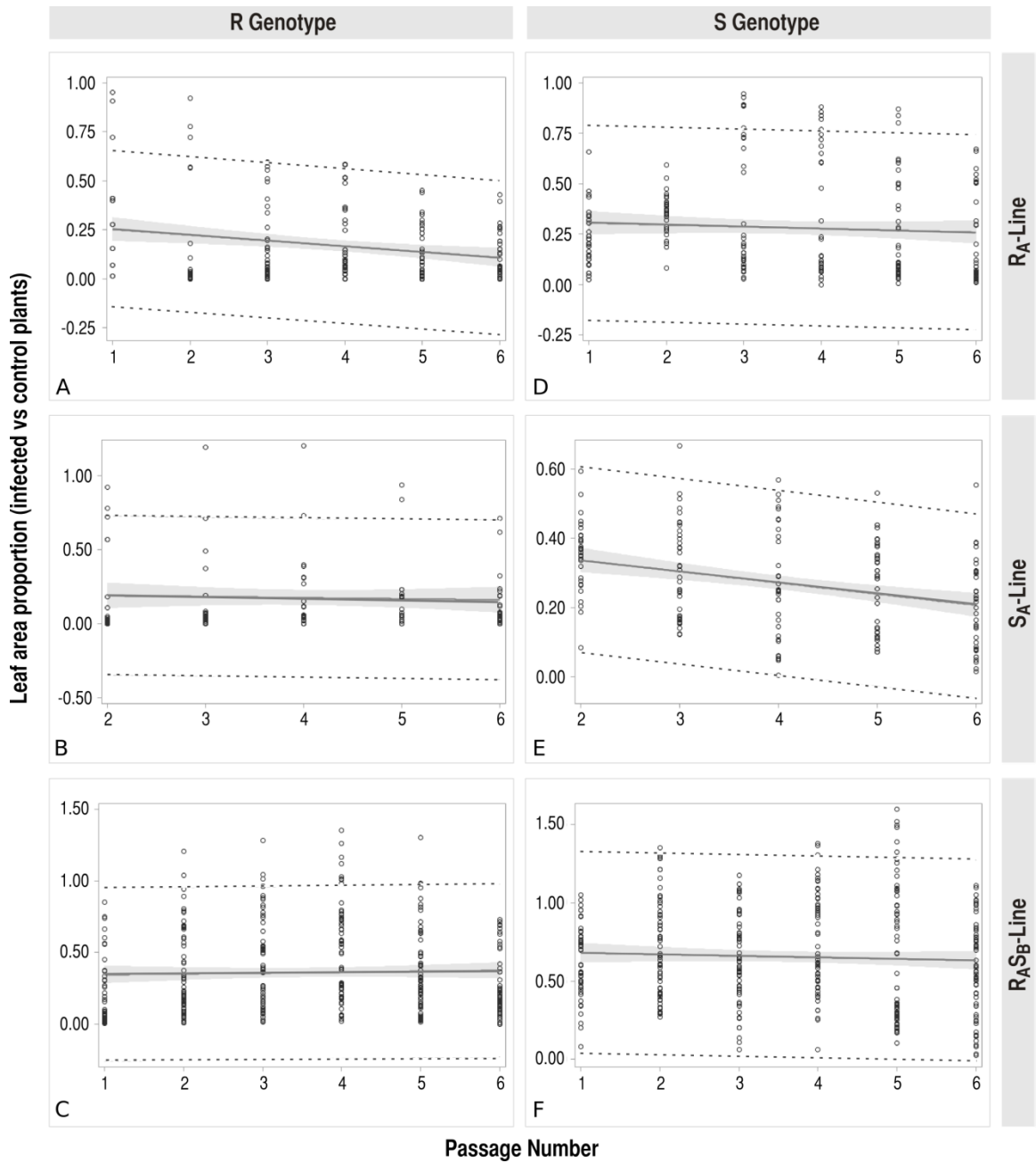


Figure 4. Fit plot for leaf area of infected plants vs uninfected controls by R and S host genotype for R_A, S_A, and R_AS_B-Lines. 95% confidence limits shown in shaded region and 95% prediction limits shown with dotted lines.

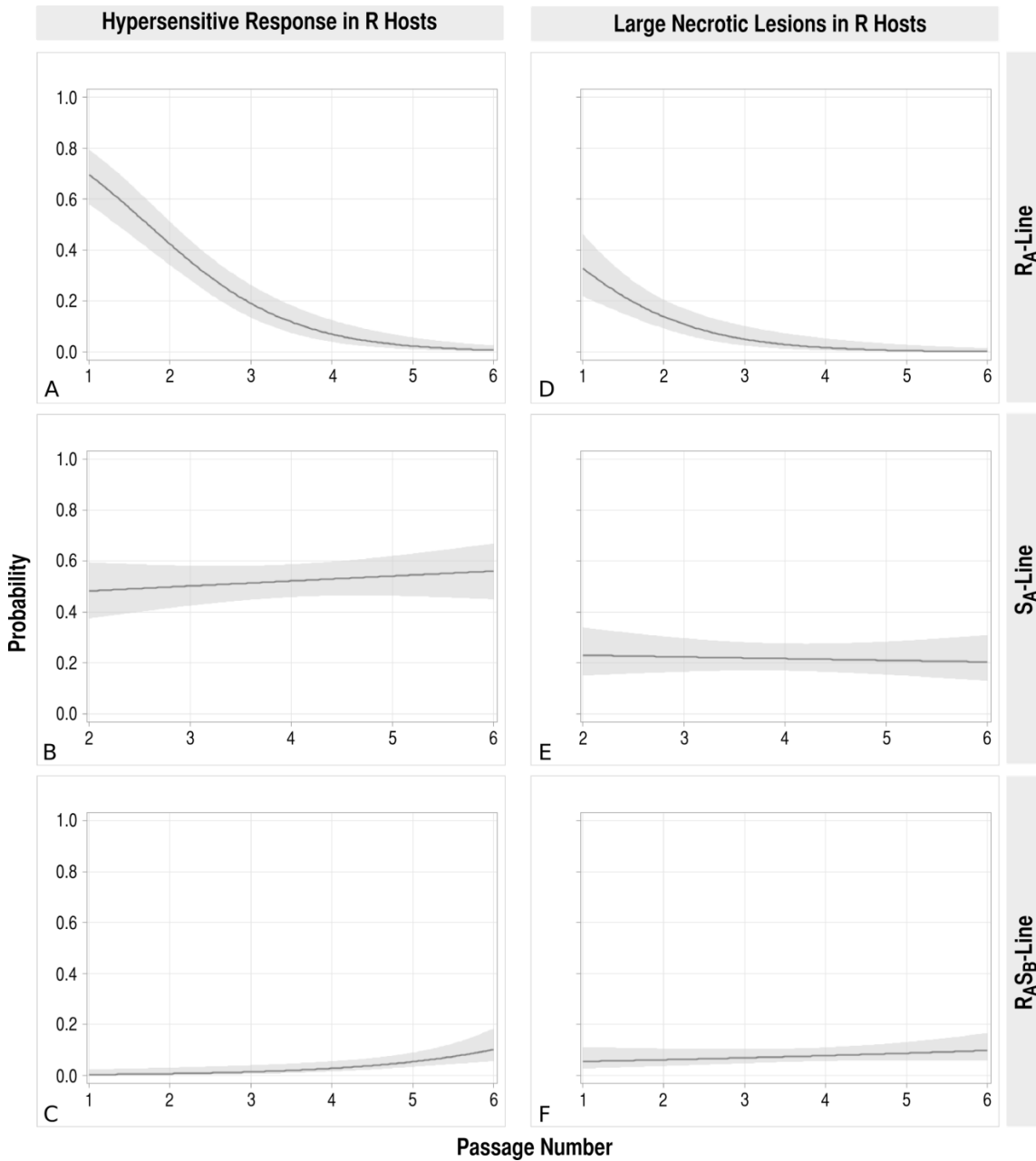


Figure 5. Predicted probabilities for expression of defense-related responses in R hosts in the R_A, S_A, and R_AS_B-Lines across passage number. (A-C) shows average probability of infected R hosts expressing a hypersensitive response within a line. (D-F) shows average probability of infected R hosts expressing large necrotic lesions within a line. Shaded region shows 95% confidence limits.

DISCUSSION

The use of resistance (R) genes to manage TSWV has been met with challenges. A variety of mechanisms have enabled the virus to circumvent host defense responses, including entering the plant through more vulnerable tissue (Aramburu et al. 2000) and mutating to overcome the R gene (Boiteux and Giordano 1993, Hobbs et al. 1994, Cho et al. 1996, Latham and Jones 1998, Roggero et al. 2002, Aramburu and Martí 2003, Margaria et al. 2004, Ciuffo et al. 2005, Sharman and Persley 2006).

It was previously shown that viruliferous thrips feeding on excised green fruit of tomatoes harvested from *Sw-5* plants could infect the fruit, resulting in the development of ringspot symptoms (Aramburu et al. 2000). Given the significant losses in marketable fruit seen in late season infections (Moriones et al. 1998, Culbreath et al. 2003), it is important to understand the factors contributing to these losses. We looked at the younger stages of fruit development to test the vulnerability of blossoms and newly set fruit to infection by non-resistance-breaking (NRB) isolates of TSWV following feeding by viruliferous *F. occidentalis*. We confirmed that the isolates used in our experiments did not break resistance in *Sw-5* tomatoes through mechanical or thrips inoculations into foliage (Table 2). We found that viruliferous thrips feeding on both blossoms and newly developing fruit can transmit TSWV to both resistant (21%) and susceptible (68%) tomato plants (Fig. & 2, Table 3). In 71.4% of these blossom- and fruit-inoculated infections in resistant plants, the virus moved systemically, causing foliage and non-inoculated fruit to develop symptoms (Fig. 1, 2, 3, Table 3). In the field, we saw similar trends, with high numbers of fruit of the *Sw-5* expressing commercial tomato cultivar ‘Redline’ becoming infected (38.9% in 2011 and

12.1% in 2012) and occasional infections found in the foliage surrounding the fruit that were presumably NRB due to their limited systemic movement. When *F. occidentalis* were abundant during the bloom and fruit stage in 2011 (4.25 *F. occidentalis* /blossom), there was a higher percentage of infected fruit on resistant plants than in 2012 when *F. occidentalis* populations were low (1.2 *F. occidentalis* /blossom) (Fig. 4, Table 4). The reason for decreased efficacy of the *Sw-5* gene in blossoms and fruit has not been studied, but in the tobacco-TMV system, it was shown that the N gene was not expressed equally across all types of plant tissue or developmental stages (Kathiria et al. 2013). The *Sw-5* gene appears to provide more protection than susceptible genotypes against inoculation by thrips feeding on blossoms and fruit, but in conditions where *F. occidentalis* are abundant, protection may be inadequate to prevent devastating losses as evidenced by our field observations. Utilizing resistance genes sustainably for crop protection requires understanding their weaknesses and developing strategies to limit risks. Protecting *Sw-5* tomatoes later in the season will mostly likely hinge on effectively managing the *F. occidentalis* populations throughout the season.

Resistance-breaking (RB) isolates of TSWV have emerged throughout the world and threaten the long-term durability of R genes. We examined the effects of serial passages in resistant and susceptible pepper on the infectivity and virulence of a naturally occurring TSWV isolate with limited RB ability in resistant peppers. We expected to see an increase in infectivity and virulence as number of serial passages increased in either resistant or susceptible hosts and found this hypothesis to be supported (Fig 2A & B, 3A-E, 4A & E). When the same isolates that were selected by serial passage in resistant hosts were inoculated into susceptible hosts,

we did not see a decrease in infectivity or virulence, contrary to our hypothesis (Fig. 2A, 3D & G, 4D). We also did not see a significant decrease in infectivity or virulence in R hosts after serial passage through susceptible hosts, but infectivity did decrease sharply in 2 out of 4 replicates, decrease slightly in the 3rd, and increase sharply in the 4th (Fig. 2B & E, 3B, E, & H, 4B, 5B & E). In Part B of the experiment, we correctly predicted that after selection in resistant hosts, RB isolates would remain stable and not revert to the original infectivity and virulence of the parent isolate (Fig. 2C, 3C, F, & I, 4C & F, 5C & F). We showed that loss of RB can decline sooner in isolates that have limited infectivity in resistant hosts, but when in lines that had been selected by resistant hosts through multiple serial passages, RB loss did not occur after 5 passages in susceptible hosts. This finding implies that selection of RB isolates by intense pressure from hosts with the *T_{SW}* R gene can lead to more stable isolates that maintain infectivity and virulence in resistant hosts for extended periods of time, even in the absence of selection for RB. Our findings are consistent with a previous study showing that RB was maintained in an isolate for 4 serial passages before RB was lost (Thomas-Carroll and Jones 2003). Together, our results suggest that RB variants can be relatively fit, but that there is still a fitness cost in susceptible hosts. To maintain durability of the *T_{SW}* gene, it is important to use management strategies that reduce repeated exposure of RB isolates to resistant cultivars. When fitness costs are low in isolates that contain mutations for RB, it is expected that when RB emerges, it will become more easily established in the landscape if selection pressure is high. If fitness costs are high, substitution of resistant cultivars with other control methods can help reduce the establishment of RB. While R

genes are powerful tools in combating plant diseases, we must be aware of their limitations and strive to improve their durability.

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