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Title: A resistance-breaking strain of *Tomato spotted wilt virus* (TSWV) of pepper in the Central Valley of California: survey, screening for resistance, and genetic variability

Project Leaders: Dr. Robert L. Gilbertson (Primary leader for correspondence)
Plant Pathology Department, UC Davis. Davis CA 95616
Phone: 530-219-7497 FAX: 530-752-5674 e-mail: rlgilbertson@ucdavis.edu

Dr. Mônica Alves de Macedo, Plant Pathology Department, UC Davis, (alvesdemacedo@ucdavis.edu)

Mr. Minor Maliano, Plant Pathology Department, UC Davis (mmaliano@ucdavis.edu)

Dr. Maria R. Rojas, Plant Pathology Department, UC Davis, (mjrojas@ucdavis.edu)

Background

Spotted wilt disease of pepper caused by *Tomato spotted wilt virus* (TSWV) can cause substantial economic losses to pepper production worldwide, including California. In nature, this virus is transmitted by various species of thrips, but mostly the western flower thrips, (*Frankliniella occidentalis*) in California. Although thrips feeding can cause direct damage to pepper, transmission of TSWV and other tospoviruses to pepper results in the greatest economic loss. In conventional production, thrips can be managed with insecticides, but this is often ineffective due to lack of effective insecticides and difficulties targeting locations where thrips feed and reproduce. Furthermore, the most effective use of insecticides for thrips management requires accurate timing of sprays, usually in advance of peak populations, in order to limit feeding damage and virus transmission.

A very effective and desirable means of managing tomato spotted wilt in pepper (and tomato) is through the planting of resistant varieties. In the case of pepper, this involves planting varieties with the dominant *Tsw* gene. Pepper plants carrying this gene usually show necrotic local lesions on inoculated leaves, followed by premature abscission and the inability of the virus to systemically infect and cause symptoms. Studies have revealed that the nonstructural protein NSs, encoded by the small (S) RNA segment, is the TSWV effector that is recognized and triggers the resistant response in peppers mediated by the *Tsw* gene. However, resistance-breaking (RB) strains of TSWV that break or overcome the resistance conferred by the *Tsw* gene have been

reported from a number of geographical locations, including Europe (Hungary, Italy and Spain), the Middle East (Israel), Australia and South America (Argentina). Furthermore, evidence has been presented that the capacity of TSWV to overcome Tsw-mediated resistance is due to specific amino acid (aa) changes in the NSs protein. For example, in Hungary, a single point mutation in NSs gene results in an amino acid change at position at 104 (T for A) was sufficient to overcome *Tsw*-gene-mediated resistance in pepper. However, in other geographic locations, different aa differences in the NSs of TSWV have been reported to be involved in overcoming the *Tsw* resistance gene.

Overall Objectives:

The overall objective of this project is to determine the potential for the emergence of pepper RB-TSWV strains in California, and whether such strains/variants have the potential to cause economic losses. This information will be used in ongoing efforts to develop an effective integrated pest management (IPM) program for TSWV in peppers in California. As part of the latter goal, a grower-focused flyer on spotted wilt disease of pepper was generated as part of this project.

Objective 1. Survey for resistant and susceptible pepper plants and the TSW genes for symptoms of TSWV in Yolo, San Joaquin and Fresno Counties in early and late-planted pepper crops in 2018.

In 2018, we received or collected samples of pepper plants of resistant varieties with spotted wilt-like symptoms from fields early (May-June) and late (September-October). Most of these samples were from varieties thought to possess the *Tsw* gene for resistance to TSWV. These samples were initially tested for TSWV infection with the AgDia TSWV immunostrip and then for capacity to infect a resistant variety (cv. Huntington). The results for the isolates received/collected are shown in Table 1.

Early season samples were obtained only from Yolo County and these were infected with *Alfalfa mosaic virus* or non-RB isolates of TSWV. However, numerous samples were received or collected from late season fields. Most of these were fruits showing discoloration, i.e., light colored rings and blotches. In most cases, tospovirus (TSWV) infection was confirmed by a positive immunostrip test result.

To confirm whether any of these isolates were bona fide RB strains/variants, we next mechanically inoculated seedlings of the resistant cultivar Huntington with sap prepared from *Nicotiana benthamiana* plants infected with these isolates. As is shown in Table 1, the positive control, RB-TSWV-CA-P-1 collected from Yolo County in 2017, induced typical spotted wilt symptoms in the resistant variety, as expected. Importantly, additional RB-TSWV variants were detected in samples of resistant pepper varieties with spotted wilt symptoms from three additional counties in 2018: Fresno, San Joaquin, and Santa Clara (RB strains are highlighted in yellow). Furthermore, RB-TSWV strains/variants also were detected in samples of resistant varieties with spotted wilt symptoms from the same field that RB-TSWV-CA-P-1 was recovered in 2017

(Table 1). This results suggested that this RB-TSWV has the capacity to overwinter in Yolo County.

Together, these results indicate that pepper RB-TSWV strains are appearing in multiple pepper-growing regions of California. These results are not surprising given previous reports of the appearance of RB-TSWV strains/variants infecting peppers with the *Tsw* gene in other regions of the world. Indeed, this has led to the conclusion that 'the *Tsw* gene is not sufficient for tospovirus control in the long-term (Turina et al., 2016). In California, these infections are appearing late in the growing season and at relatively low incidences, and are not currently causing significant economic losses. Thus, it is important to continue to monitor fields planted with resistant varieties in different pepper-growing regions in order to determine if these RB TSWV strains/variants are becoming more prevalent and infecting plants at earlier stages of development.

Table 1. Pepper samples received for testing for possible resistance-breaking (RB) strains/variants of *Tomato spotted wilt virus* (TSWV): Source, location, detection with TSWV immunostrip and capacity to break Tsw resistance

Sample/year	Source	County	Immunostrip[a]	RB[b]
RB-TSWV-CA-P-1-2017	Fruit	Yolo	(+)	(+)
RB-TSWV-CA-P-1-2017	Fruit	Yolo	(+)	(+)
325-2018	Fruit	Santa Clara	(+)	(+)
CG204-2018	Leaf	San Joaquin	(+)	(+)
P3-136-2018	Leaf	Yolo	(+)	(-)
H1-2018	Fruit	San Joaquin	(+)	(+)
H2-2018	Fruit	San Joaquin	(+)	(+)
H6-2018	Fruit	San Joaquin	(+)	(+)
B6-2018	Fruit	San Joaquin	(+)	(+)
CG-214-2018	Leaf	Fresno	Weak (+)	(-)
CG242-2018	Fruit	Monterrey	Very Weak (+)	(-)
CG-60-2018	Leaf	Yolo	(+)	(-)
CG-99-2018	Leaf	Yolo	(+)	(-)
CG134-2018	Leaf	Yolo	(+)	(-)
CG-248-2018	Fruit	Fresno	(+)	(-)
CG-249-2018	Fruit	Fresno	(+)	(+)
CG252-2018	Fruit	Yolo	(+)	(+)
CG253-2018	Fruit	Yolo	(+)	(+)

[a]Results for AgDia TSWV immunostrip test

[b]Indicates the development of spotted wilt symptoms in plants of the resistant variety Huntington

Objective 2. Evaluate the response of a range of sweet-pepper cultivars to the NSs-RB TSWV strain with sap inoculation to test as potential resistance source.

We next asked the question of whether the RB-TSWV strain/variant recovered from pepper plants in Yolo County in 2017 (RB-TSWV-CA-P-1) has the capacity to infect a range of resistant pepper varieties. Here, we conducted an experiment in which we mechanically inoculated pepper seedlings of six resistant and three susceptible cultivars with sap prepared from leaves of *N. benthamiana* plants (a laboratory host that we use to increase the amount of virus inoculum). Controls included a wild-type (WT) TSWV strain recovered from tomato with spotted wilt symptoms in Yolo County, a tomato RB-TSWV strain from Fresno County (RB-TSWV-CA-T-1) and buffer only mock-inoculated plants (negative control).

As shown in Table 2, the WT TSWV strain did not infect plants of the resistant varieties (with the exception of a single plant of cv. Cutlass), whereas it infected and caused typical spotted wilt symptoms in two of the three susceptible varieties (Baron Bell and Jupiter) (Table 2). Interestingly, this WT TSWV strain did not cause symptoms in the mechanically inoculated plants of the other susceptible variety, cv. BP Double Up. Similar results were observed for the tomato RB-TSWV strain, in that the virus did not cause spotted wilt symptoms in any of the resistant pepper varieties, infected and caused spotted wilt symptoms in all inoculated plants of the susceptible cultivars Baron Bell and Jupiter, and it only infected and caused symptoms in 2/7 cv. BP Double Up plants. This was not due to the cv. BP Double Up being resistant to mechanical transmission, as high rates of infection occurred following mechanical inoculation was observed for the pepper RB-TSWV strain/variant (Table 2). Thus, this may indicate the presence of an additional gene(s) for resistance to TSWV in cv. BP Double Up.

In terms of the pepper RB-TSWV strain/variant (RB-TSWV-CA-P-1), it was highly infectious and induced typical spotted wilt symptoms in all six resistant varieties and all three susceptible varieties, including BP Double Up (Table 2). Furthermore, plants inoculated with this strain/variant began to develop symptoms as early as seven days post-inoculation (dpi), compared with 10 dpi or longer before symptoms developed in pepper plants inoculated with the other TSWV isolates, indicating that this is a very aggressive strain in pepper. Thus, the pepper RB-TSWV strain/variant from California overcame the (Tsw) resistance in all cultivars tested, which is fully consistent with it representing a bona fide pepper RB strain. The finding that the isolate of the tomato RB-TSWV strain/variant from California (RB-TSWV-CA-T-1) did not overcome the resistance conferred by the *Tsw* gene is consistent with fact that the viral effectors recognized by the TSWV *Sw-5* and *Tsw* resistance gene products are different, i.e., NSm for Sw-5 and NSs for Tsw.

Table 2. Response of resistant and susceptible pepper cultivars to inoculation with a pepper resistance-breaking (RB) strain from California (Yolo County) and wild-type and tomato RB strains

		TSWV Isolate			
Pepper Variety	**R/S**	**WT**	**RB Pepper**	**RB Tomato**	**Mock**
Het TSW	R	0/24	24/24	0/12	0/6
R144158	R	0/26	26/28	0/12	0/6
Huntington	R	0/16	13/15	0/9	0/8
FPP1909	R	0/24	22/24	0/8	0/8
Trifecta	R	0/24	21/24	0/12	0/6
Cutlass	R	1/14	15/16	0/4	0/2
BP Double Up	S	0/12	16/16	2/7	0/4
Baron Bell	S	22/24	16/16	7/7	0/4
Jupiter	S	16/16	16/16	8/8	0/8

Objective 3. Sequence of full-length NSs gene of the NSs RB-TSWV and NSs WT-TSWV strains and compare these sequences to determine genetic variability between these strains.

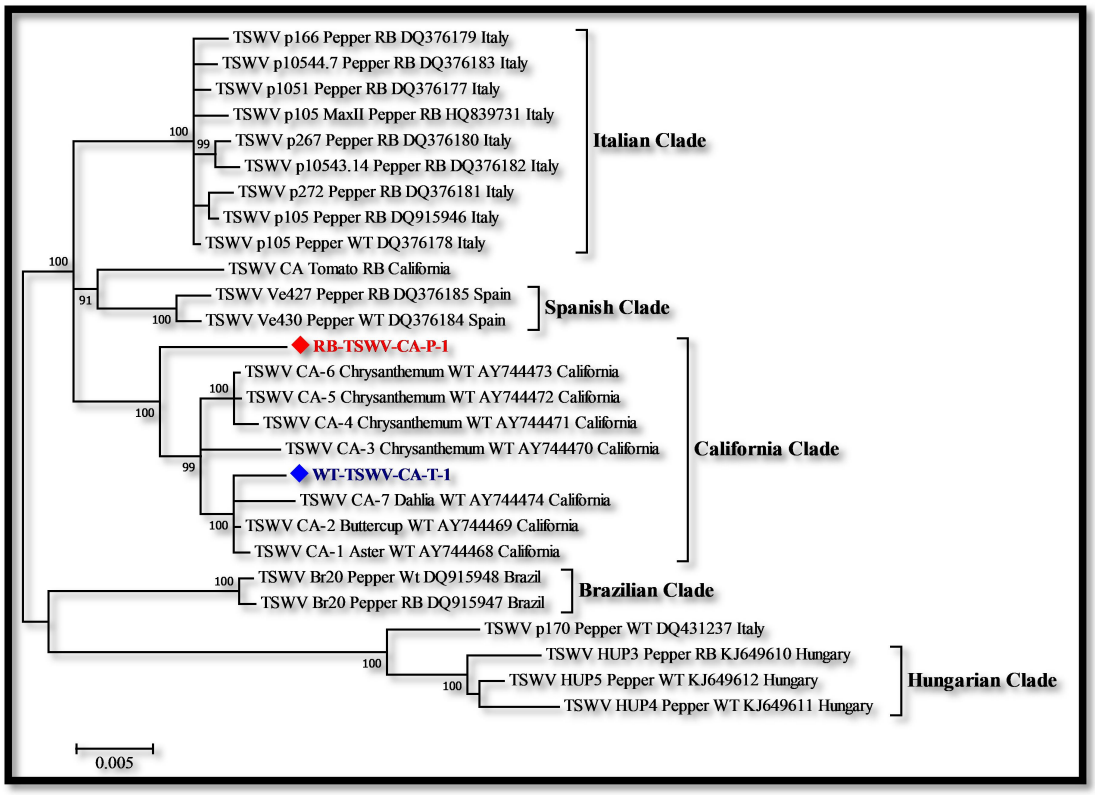
In order to gain insight into the nature of the RB-TSWV strain/variant in California, we determined the complete nucleotide sequences of the NSs genes of the WT-TSWV isolate from Yolo County and the pepper RB-TSWV strain/variant. The NSs nucleotide (nt) sequences were each 1404 nucleotides and encode a predicted NSs protein of 468 aa. The NSs sequences were very similar (~99% nt and aa identity), but there were enough differences to indicate that these were not isolates of the same TSWV strain/variant. Furthermore, the NSs sequences of these California TSWV isolates were also closely related to those of isolates from the states of Washington and California (99% nt and aa identity). Consistent with these results, the phylogenetic tree constructed based on alignment of the complete NSs nt sequence indicated that the RB-TSWV-CA-P-1 strain/variant was placed in a group (clade) with other TSWV isolates from California, and distant from previously characterized RB-TSWV strains from pepper from other geographic locations (Figure 1).

However, there were multiple aa differences (>10) between the NSs aa sequences of the WT-TSWV and the RB-TSWV-CA-P-1 strain/variant. Furthermore, the aas previously reported associated with pepper RB strains from Europe were not present in RB-TSWV-CA-P-1, suggesting it was a new type of RB-TSWV strain/variant infecting pepper.

Taken together, these results indicate that RB-TSWV-CA-P-1 is not an exotic RB-TSWV strain/variant introduced from another geographical region, but rather a variant that evolved locally in California. However, based on the number of differences in the NSs gene and protein sequences, this evolution likely involved multiple events, including mutation and reassortment of RNA segments.

At the present time, the multiple aa differences between the NSs of WT-TSWV and RB-TSWV-CA-P-1 makes it difficult to identify the precise aa(s) that have given rise to the capacity of the RB-TSWV-CA-P-1 to overcome the resistance conferred by the *Tsw* gene. For this, it will be necessary to sequence and compare the aa sequences of multiple pepper RB-TWSV strains/variants and conduct transient expression assays in pepper leaves of resistant and susceptible cultivars.

Figure 1. A phylogenetic consensus tree showing the relationship among resistance-breaking (RB) strains of *Tomato spotted wilt virus* (TSWV) in pepper collected from California, Italy, Spain, Brazil and Hungary and the most closely related sequences of wild type (WT) isolates based on alignments of complete NSs sequences. Phylogenetic analyses were performed with MrBayes 3.2. Branch strengths were evaluated by Bayesian posterior probabilities. Nodes with clade credibility values of ≥70% are shown.



Conclusions

1. Additional pepper RB-TSWV strains/variants were detected in pepper fields planted with resistant (Tsw) varieties in multiple counties in California in 2018.

2. In the field, he infections of resistant pepper plants with RB-TSWV appear later in the season and are most evident based upon development of fruit symptoms.

3. The pepper RB-TSWV strain/variant detected in Yolo County in 2017 infected and caused typical spotted wilt symptoms in all six resistant cultivars tests and was very aggressive in terms of when symptoms appeared.

4. A genetic analysis of the NSs gene/protein sequences indicated that RB-TSWV-CA-P-1 emerged locally, but was not able to reveal the specific amino acid changes that are responsible for overcoming resistance conferred by the *Tsw* gene.