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Short title: Transmission of different TSWV isolates by *F. occidentalis*

Transmission of Tomato spotted wilt virus isolates able and unable to overcome tomato or pepper resistance by its vector *Frankliniella occidentalis*

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Abstract

Tomato spotted wilt virus (TSWV) causes serious diseases of many economically important crops. Disease control has been achieved by breeding tomato and pepper cultivars with the resistance genes *Sw-5* and *Tsw*, respectively. However, TSWV isolates overcoming these genetic resistances have appeared in several countries. To evaluate the risk of spread of the resistance-breaking isolates, we tested their ability of transmission by the main vector of TSWV, the thrips *Frankliniella occidentalis*. We compared the transmission rate by thrips of several TSWV isolates with different biotypes, able or unable of overcoming tomato or pepper resistance, and divergent genotypes. Our results indicate that TSWV transmission rate was affected by the viral accumulation in thrips but not by its accumulation in the source plants from which thrips acquired the virus. No correlation was found between transmission efficiency and the ability of overcoming both resistances or between transmission efficiency and the genotype. This suggests that resistance-breaking isolates have the same potential of spread than the isolates unable to infect resistant tomato and pepper cultivars.

Key words: genetic resistance, resistance-breaking isolates, thrips, TSWV, Tospovirus

Introduction

Tomato spotted wilt virus (TSWV), the member type of the genus *Tospovirus*, is one of the most destructive plant viruses causing serious economical losses in many agricultural crops worldwide (Adkins, 2000). TSWV has a wide host range including more than 1000 plant species (Hanssen *et al.*, 2010) and is transmitted by several species of thrips (Thysanoptera: Thripidae), among which *Frankliniella occidentalis* (Pergande) is the main vector. TSWV can only be transmitted by adults and to a lesser extent by second instar larvae, after a latent period during which the virus circulates in the vector and replicates in the vector's salivary glands (Ullman *et al.*, 1992; Wijkamp & Peters., 1993; Wijkamp *et al.*, 1993; Nagata *et al.*, 1999; Kritzman *et al.*, 2002). The triangular interactions between virus, plant host and thrips can be very complex affecting not only the transmission efficiency of TSWV, but can also have detrimental or beneficial effects in the reproduction and survival of the thrips (Belluire *et al.*, 2005; Inoue & Sakurai., 2006; Belluire *et al.*, 2008). TSWV genome consists of three negative-sense or ambisense RNA segments: segment L encodes a putative RNA-dependent RNA polymerase; segment M encodes the cell-to-cell movement protein NSm, and the precursor of surface glycoproteins G_N/G_C, which are thought to be involved in TSWV transmission by thrips; and segment S encodes a silencing suppressor NSs and the nucleocapsid N (Plyusnin *et al.*, 2012).

The wide host range and efficient spread of TSWV by thrips makes very difficult to apply prophylactic measures. A method of disease control is breeding resistant cultivars, but so far, only two resistance genes: *Sw-5* in tomato and *Tsw* in pepper, have been found to be effective against a wide spectrum of TSWV isolates (Adkins, 2000; Pappu *et al.*, 2009). However, the virus is able to evolve and overcome resistance in both tomato and pepper (López *et al.*, 2011; Tentchev *et al.*, 2011).

Resistance-breaking isolates have been reported in Australia, South Africa, USA, Italy and Spain (Aramburu & Marti., 2003; Margaria *et al.*, 2007).

Understanding the factors involved in TSWV dispersion is crucial to assess the risk of spread of the resistance-breaking isolates, and to develop integrated management strategies for disease control based on combining breeding resistant cultivars, prophylactic measures and agronomical practices. In this work, we estimated the efficiency of transmission of TSWV by its main vector *F. occidentalis*, taking into account several factors, such as the viral accumulation in source plants and in the thrips vector, the developmental stage of thrips (second instar larvae and adult), TSWV genetic variability and the ability to overcome resistance.

Materials and Methods

Virus isolates

About 70 samples from crops with resistant and non-resistant tomato and pepper cultivars from Spain were collected. These samples were mechanically inoculated to *Nicotiana glutinosa* plants to obtain biological clones from local lesions and they were mechanically inoculated in *Datura stramonium* plants to produce a systemic infection (Aramburu *et al.*, 2010). The purpose was obtaining TSWV isolates from these biological clones, with a homogeneous intra-isolate population of genetic variants, avoiding isolates with a mix of TSWV genomic variants able and unable to overcoming resistance.

To evaluate the ability to overcome tomato *Sw-5* and pepper *Tsw* resistance, these isolates were mechanically inoculated to tomato cultivars “Verdi” (Fitó) with *Sw-5* and “Marmande” (Fitó) without *Sw-5*, and to pepper cultivars “Spiro” and “Divino” (Seminis) with *Tsw* and “C804” (Fitó) without *Tsw*. TSWV isolates were classified into

three biotypes: wild-type (WT) isolates which were unable to infect both tomato and pepper resistant cultivars, tomato *Sw-5* resistance breaking (SRB) isolates and pepper *Tsw* resistance breaking (TRB) isolates. No isolate was found to be able to infect both resistant tomato and resistant pepper cultivars.

Nucleotide sequence analysis showed that these isolates can be classified into two main groups or genotypes, named A and B (Debreczeni *et al.*, 2011; Lopez *et al.*, 2011). WT and SRB isolates with both genotypes A and B were found, whereas TRB isolates presented only genotype A. Based on this classification, five Spanish TSWV isolates: 1) ALPA (biotype TRB, genotype A, GenBank accession HQ537114), 2) GRAU (biotype SRB, genotype A, GenBank accession FM163370), 3) Mon1NL2 (biotype WT, genotype A, GenBank accession HM015514), 4) Oller1TL3 (biotype SRB, genotype B, GenBank accession HM15519), and 5) Da1NL2 (biotype WT, genotype B, GenBank accession HM015512) were selected and compared with the Brazilian isolate BR01 (biotype WT, genotype B, GenBank accession S58512), which has been widely used in many studies and can be considered as the reference TSWV isolate (De Avila *et al.*, 1990).

These TSWV isolates were inoculated in *D. stramonium* plants by grinding in a mortar TSWV-infected plant material in chilled inoculation buffer (0.01 M phosphate buffer, pH 7.0), and rubbing this sap extract with celite onto plants with four or five true leaves. The *D. stramonium* plants were planted from seeds and maintained in a growth chamber with controlled conditions (25 °C temperature; 60% relative humidity; 16:8 hours light:dark).

Thrips and transmission assays

A stock culture of the thrips *F. occidentalis* was reared in transparent plastic jars covered with a lid where a hole was cut and closed with filter paper to avoid humidity condensation. A sponge was placed on the bottom of the jar to provide places for thrips to pupate. Thrips fed and oviposited on bean pods (*Phaseolus vulgaris* L.) coated with a water solution of 5% sugar and 0.1% amino acids (Isabion, Syngenta) (Espinosa *et al.*, 2002). The plastic jars with the thrips cultures were maintained at 24 °C.

Transmission assays were performed as follows: eight female adult thrips (*F. occidentalis*) were allowed to lay eggs on each TSWV-infected *D. stramonium* plant, to obtain thrips larvae that had fed on infected plant material during the first hours after emerging from the eggs, as first instar larvae, which is required to effectively transmit TSWV (Ullman *et al.*, 1992; van de Wetering *et al.*, 1996). In total, ten plants were used as virus source: two plants for isolates ALPA, Mon1NL3, Oller1TL3 and Da1NL2, and one plant for isolates GRAU and BR01. The thrips larvae emerged from the eggs were let on the infected plants during five days to ensure that they fed on TSWV-infected plant material. The ability to transmit TSWV by second instar larvae was tested using the Petunia leaf disk assay (Wijkamp & Peters., 1993). This test consisted of letting each larva on a leaf disk of Petunia (*Petunia x hybrida*) inside a 1.5 ml eppendorf tube during 48 h. Subsequently, each thrips larva was placed on a new Petunia leaf disk for other 48 h or until they reached adulthood. Adults were also placed on Petunia leaf disks for subsequent 48 h periods until they died. After removing the thrips larvae or adults, the leaf disks were floated individually on water in a Petri dish for two days at 27 °C. Larvae and adults of thrips were scored as transmitters when the leaf disks developed local lesions (annular red rings or red spots) which are produced as a hypersensitive response triggered by the virus multiplication.

Estimation of TSWV titer in source plants and adult thrips by real-time quantitative RT-PCR

Total RNAs from 0.1 g of fresh leaf tissue from TSWV-infected and non-infected *D. stramonium* plants and from individual adults thrips able or unable to transmit TSWV were extracted using a standard protocol with phenol:chloroform:isoamyl alcohol 25:24:1 (v:v:v) (Debreczeni *et al.*, 2011). Real-time quantitative RT-PCR (RT-qPCR) was performed in a LightCycler[®]480 (Roche Molecular Diagnostics) using 25 µl of a reaction mix that contained 12.5 µl LightCycler[®]480 Probe Master Mix (ROCHE), 4.38 µl of RNase-free water, 15 U RT Multiscribe Reverse Transcriptase (Life Technologies), 2 U of RNase Inhibitor (Applied Biosystems), 5 µM of each forward and reverse primer, 0.25 µM TaqMan[®]MGB probe, and 5 µl of total RNA (~10 ng/µl). Cycling conditions consisted of reverse transcription at 48°C for 30 min, incubation at 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min (Debreczeni *et al.*, 2011).

Statistical analysis

Transmission rate by thrips was compared between the different isolates, and between the different biotypes and genotypes with a General Linear Mix Model (GLM) assuming binomial distribution of the variable transmission. Spearman's correlation test was performed to test the relationship between: a) virus titer in source plants and transmission rate of TSWV by second instar larvae and adult thrips, b) virus titer in source plants and viral accumulation in adult thrips, and c) viral accumulation in adult thrips and transmission rate by adult thrips. Transmission rate was compared between

thrips larvae and adult thrips using Mann-Whitney U test. Accumulation of the different TSWV in adult thrips was compared by using Kruskal-Wallis test.

Results

Performance of thrips on TSWV-infected plants

The total number of larvae obtained from 80 adult female thrips was 464, and 179 of them reached adulthood. Thus, in our experimental conditions the average reproductive rate was 5.8 (number of larvae born from each female adult) and the mean juvenile survival rate (proportion of larvae becoming adults with respect to the number of larvae emerged) was 38.6%. There were no significant differences between reproductive rate of thrips carrying different TSWV isolates (Data not shown, Kruskal-Wallis test, $p = 0.19$).

Comparison of TSWV transmission by second instar larvae and adults

To evaluate the transmissibility of TSWV by the different developmental life stages of thrips, the ability to transmit the virus to *Petunia* leaf disks was tested for each individual second instar larvae every 48 h, subsequentially. Once the larvae reached adulthood, their ability to transmit TSWV was also tested in the same way. The percentage of adult thrips transmitting TSWV was 43.5%, significantly higher (GLM, $p = 0.003$) than the 8.1% transmitting thrips larvae observed (Table 1). From all the non-transmitter second instar larvae, 40.7% of them were able to transmit TSWV when they became adults, whereas most (75.3%) of the transmitter second instar larvae conserved their ability to transmit TSWV.

Effect of TSWV accumulation in source plants and in thrips on its transmissibility

Virus titer in the source plants varied from 2.00×10^8 to 1.17×10^{10} and it was not significantly correlated neither to virus titer in adult thrips (Spearman's test, $p = 0.111$) nor to transmission rate by second instar larvae (Spearman's test, $p = 0.310$) or by adults (Spearman's test, $p = 0.276$, Figure 1).

Virus titer in adult thrips varied from 1.14×10^3 to 3.6×10^7 and it was significantly higher in transmitter adult thrips than in non-transmitter adults (Mann-Whitney U test, $p = 0.00001$, Figure 2). Accordingly, virus titer in thrips was positively correlated to transmission rate of TSWV (Spearman's test, correlation coefficient $\rho = 0.724$; $p = 0.00001$).

Comparison between transmission of different TSWV isolates

The differences in viral accumulation of the different TSWV isolates observed in adult thrips were not statistically significant (Kruskal-Wallis test, $p = 0.717$, Fig. 3). Also, there were not significant differences in the transmission rate between the different TSWV isolates neither by thrips larvae (GLM, $p = 0.676$), nor by adult thrips (GLM, $p = 0.489$, Fig. 4).

The same trend was found when data were grouped according to genotype or biotype. Thus, transmission rate was not significantly different between the two TSWV genotypes, neither when performed by thrips larvae (GLM, $p = 0.712$) nor by adult thrips (GLM, $p = 0.295$). Likewise, transmission rate did not differ significantly between the three biotypes (WT, SRB, TRB) neither by thrips larvae (GLM, $p = 0.511$) nor by adult thrips (GLM, $p = 0.610$).

Discussion

TSWV displays a high level of biological diversity and a great ability of evolution and adaptation with respect to other plant viruses (Qiu *et al.*, 1998; Tsompana *et al.*, 2005). Thus, in many areas, after a few years of using resistant tomato or pepper cultivars, TSWV isolates able to overcome such resistances have emerged. Presently, the mutations leading to pepper resistance-breakdown are unknown (Margaria *et al.*, 2007; Tentchev *et al.*, 2011), but there are evidences that tomato resistance-breaking isolates have been generated several times by only one of two possible specific non-synonymous nucleotide substitutions in the gene encoding the movement protein NSm (López *et al.*, 2011).

The generation of mutations associated to resistance-breakdown is the first step of the process of emergence. This would only become a problem if these mutations are able to disperse and predominate in the viral population. Often, the plant defense system involved in resistance targets amino acid motifs which are functionally important for the virus life cycle causing a strong negative selection against amino acid change (Moffett, 2009). This makes very difficult for the virus to escape from the plant defense and produce resistance-breaking isolates. For this reason, the mutations associated to resistance-breakdown could have fitness costs and be associated to a decrease of the dispersion ability of TSWV (Dieckmann *et al.*, 2002). Therefore, it is essential to evaluate the efficiency of transmission of the resistance-breaking isolates by thrips with respect to wild-type isolates and study the factors determining their transmissibility.

Our results indicated that TSWV accumulation in plants had no effect in the transmission efficiency of TSWV by thrips. A recent study described that *F. occidentalis* needed a threshold amount of TSWV particles in source plants to become

viruliferous (Okazaki *et al.*, 2011). This suggests that the virus titers of the source plants in our experiment were above the concentration threshold needed to allow transmission by thrips. This also means that possible fitness loss of TSWV in the host (eg. replication, cell-to-cell movement) associated to the acquisition of the ability to infect resistant cultivars would have little impact in the transmission of these isolates by thrips at least in similar conditions to those in our experiment.

Viral accumulation in *F. occidentalis* was positively correlated to the transmission rate of TSWV by adults of *F. occidentalis*. This confirms what had been observed in another study on TSWV transmissibility by *F. occidentalis* (Rotenberg *et al.*, 2009). Therefore, viral accumulation in thrips seems to be one of the main factors affecting transmission rate by thrips, although other factors can have a role, e.g. the differences in feeding between males and females, (Nagata *et al.*, 2002; Rotenberg *et al.*, 2009; Stafford *et al.*, 2011).

Adult thrips transmitted TSWV at a higher rate than larvae in our study. This is in agreement with a lower accumulation of TSWV found in thrips larvae, as compared to thrips adults (Inoue *et al.*, 2004) and with previous studies describing lower transmission efficiency of larvae as compared to adult thrips (Moritz *et al.*, 2004). These results, together with the lower mobility of larvae with respect to adults, mean that TSWV dispersion must be performed predominantly by adults. The transmission rate of TSWV by adult thrips obtained in our study ranged from 34 to 69%, which coincides with transmission rates described in previous works (van de Wetering *et al.*, 1999; Belliure *et al.*, 2008).

Finally, comparison of reproductive rate of thrips, virus accumulation in thrips and transmission rates between different TSWV isolates suggested that genotype or biotype (wild-type and tomato and pepper resistance-breaking) did not have an effect on

TSWV dispersion. These results suggest that resistance-breaking TSWV-isolates have the same potential to spread than wild-type isolates.

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Table 1. Percentage of non-transmitter and transmitter thrips larvae and adults.

	Non-transmitter adults	Transmitter adults	Total larvae
Non-transmitter larvae	54.5 (59.3) ^a	37.4 (40.7) ^a	91.9
Transmitter larvae	2.0 (24.7) ^a	6.1 (75.3) ^a	8.1
Total adults	57.5	43.5	100

^aAbsolute percentage (number of individuals of each class with respect to the total number of individuals). Between parentheses is indicated the relative percentage with respect to larvae (absolute percentage divided between the total percentage in each row)

Figure legends

Figure 1. Correlation between accumulation of *Tomato spotted wilt virus* (TSWV) in source plants and transmission rate by thrips A) 2nd instar larvae, B) adults. White squares mark WT TSWV biotypes, black squares mark TRB biotype and grey squares mark SRB biotypes.

Figure 2. Average accumulation (plus standard error of the mean) of *Tomato spotted wilt virus* (TSWV) in thrips adults. A) Non-transmitter thrips excluding those without virus accumulation, B) non-transmitter thrips, including those without virus accumulation, and C) transmitter thrips. Bars accompanied by different letters in the graph were significantly different (Mann-Whitney U test).

Figure 3. Average accumulation (plus standard error of the mean) of different isolates of *Tomato spotted wilt virus* (TSWV) in adult thrips.

Figure 4. Average transmission rate (percentage) of different isolates of *Tomato spotted wilt virus* (TSWV) by adult thrips.