Phytopathol. Mediterr. (2010) 49, 342–351

Characterization of *Tomato spotted wilt virus* isolates that overcome the Sw-5 resistance gene in tomato and fitness assays

JOSE ARAMBURU[1], LUIS GALIPIENSO[1], SALVADOR SOLER[2] and CARMELO LÓPEZ[2]

[1]Institut de Recerca i Tecnología Agroalimentaries (IRTA) Ctra. de Cabrils Km 2, 08348, Cabrils, Barcelona, Spain
[2]Centro de Conservación y Mejora de la Agrodiversidad Valenciana-Universidad Politécnica de Valencia (COMAV-UPV) Camino de Vera, 14, 46022 Valencia, Spain

Summary. Resistance-breaking (RB) isolates of *Tomato spotted wilt virus* (TSWV) that overcome the resistance conferred by the *Sw-5* gene in tomato have had only a limited spread since they were first detected in north-eastern Spain in 2002. Symptom expression, homogeneity, stability and the transmission capacity of RB and non-resistance breaking (NRB) isolates were biologically compared. The fitness of both types of isolates infecting tomato plants was determined in competition assays. All TSWV isolates induced similar systemic symptoms in a wide range of plant species, except RB isolates in tomato carrying the *Sw-5* resistance gene and pepper carrying the *Tsw* resistance gene. The mechanical transmission of RB isolates to tomato plants with the *Sw-5* gene failed in some trials, although NRB isolates did not differ noticeably in transmission efficiency when tested with the thrips *Frankliniella occidentalis*. Biological clones from individual local lesions obtained by mechanically inoculating *Nicotiana glutinosa* in some TSWV field samples showed that they were biologically homogeneous. Mixed infections of RB and wilt-type isolates were not found. The RB isolates were relatively stable because no reversion to NRB isolates was seen after serial passages in susceptible tomato plants. In competition assays between RB and NRB isolates, after serial passages in susceptible tomato plants, the prevalence of a particular isolate was not related to its capacity to overcome *Sw-5* gene resistance. The low spread of the RB isolates in Spain does not seem to be related to a loss of fitness in tomato plants or to differences in transmission capacity by thrips, but it could be related to the reduction of the selection pressure of RB isolates as consequence of the gradual replacement of susceptible tomato plants by resistant tomato plants by growers.

Key words: variability, stability, competition, pepper, *Tsw* gene.

Introduction

Tomato spotted wilt virus (TSWV), genus *Tospovirus*, family Bunyaviridae, is one of the most harmful viral pathogens, and is widespread in many agricultural production areas worldwide (Goldbach and Peters, 1994). Susceptible hosts to TSWV include many important agricultural crops such as tomato (*Solanum lycopersicum)*, pepper (*Capsicum annum*) and lettuce (*Lactuca sativa*) (Goldbach and Peters, 1994). Control is difficult because TSWV infects many plant species and is naturally transmitted by several species of thrips (*Thysanoptera: Thripidae)* (Peters *et al.*, 1996). TSWV is transmitted in a persistent and propagative manner because it is acquired only by larvae (Wetering *et al.*, 1996) and is transmitted by second-instar larvae and adults after a latent period in a vector (Nagata *et al.*, 1999; Whitfield *et al.*, 2005). The most effective vector is *Frankliniella occidentalis* Pergande (German *et al.*, 1992), which first introduced TSWV to Spain (Lacasa, 1990). In tomato and pepper, the best strategy to control the disease has been to use the natural host resistance

Corresponding author: J. Aramburu
Fax: +34 937533954
E-mail: josemaria.aramburu@irta.es

found in wild *Solanum* and *Capsicum* species. A hypersensitive response to TSWV occurs in some accessions of *Capsicum chinense* due to the occurrence of a single dominant resistance *Tsw* gene that has been introgressed in the pepper hybrids, but several factors can alter the expression of this resistance (Moury *et al*., 1998). In tomato, most breeding programs have tried to exploit the resistance found in *S. peruvianum* and *S. pimpinillefolium* (Stevens *et al*., 1994). Some tomato cv. that Stevens *et al*. (1994) obtained by crossing *S. lycopersicum* and *S. peruvianum* showed broad resistance to various TSWV isolates (Van Zijl *et al*., 1986). This resistance, conferred by a single dominant gene, *Sw-5* (Stevens *et al*., 1992; Cho *et al*., 1996), has been introgressed into several Spanish commercial tomato hybrids (Aramburu and Rodríguez, 1999). The use of tomato hybrids carrying the *Sw-5* gene has reduced the losses from TSWV in tomato. However, a low percentage of plants still become systemically infected (Roselló *et al*., 1997; Aramburu and Rodríguez, 1999), possibly due to the partial penetration of the dominant *Sw-5* gene, which has been estimated to be about 98.7% (Roselló *et al*., 1996).

The genome of TSWV consists of three negative-sense or ambisense RNA segments, L, M and S. Segment L encodes a putative RNA-dependent RNA polymerase (de Haan *et al*., 1991); segment M encodes the cell-to-cell movement protein, NSm (Li *et al.,* 2009) and the precursor of surface glycoproteins, Gn/Gc, involved in TSWV transmission by thrips (Sin *et al*., 2005); and segment S encodes a silencing suppressor, NSs (Takeda *et al*., 2002) and the nucleocapsid N (de Haan *et al*., 1990). Several resistance-breaking (RB) isolates of TSWV have been reported from tomato cultivars containing the *Sw-5* gene (Cho *et al*., 1996; Thompson and Van Zijl, 1996; Latham and Jones, 1998; Hoffmann *et al*., 2001), and there are indications that the M segment has a major role in overcoming the *Sw*-5 gene (Hoffmann *et al*., 2001).

In Spain, the first RB isolate was detected in 2002 (Aramburu and Martí, 2003) and since then more than 30 TSWV isolates infecting tomato plants carrying the *Sw-5* gene have been collected from different tomato fields in the Catalonia region. However, these isolates are still present in the same localized area where they were first detected seven years ago. The objective of this work was to study the infectivity of RB isolates in several hosts, their stability over time, and their fitness in mixtures, or transmissibility by *F. occidentalis*, as compared with non-resistance breaking (NRB) isolates in order to determine the possible causes of this limited spread of RB isolates.

Materials and methods

Virus isolates and ELISA detection

Samples of individual tomato plants showing bronzing caused by TSWV were collected during three consecutive growing seasons, from commercial crops in north-eastern Spain. The samples were examined for the most frequent viruses in tomato crops of that growing area, to detect any mixtures with other viruses. *Cucumber mosaic virus* (CMV), *Pepino mosaic virus* (PepMV), *Potato virus* Y (PVY) and TSWV were tested by ELISA as described by Clark and Adams (1977) using specific polyclonal antiserum at the recommended dilutions (Loewe Biochemica Gmbh, Sauerlach, Germany). *Parietaria mottle virus* (PMoV-T) was tested or by molecular hybridization or indirect ELISA (Aparicio *et al*., 2009). Only tomato samples infected with TSWV were studied. These field-collected tomato samples, and the TSWV isolates obtained from them, after three serial passages of a single local-lesion in *Nicotiana glutinosa* with subsequent subculture in *Datura stramonium,* were stored at -80ºC until use. A total of seven TSWV-infected samples were selected for the comparative study, separated into two groups depending on their ability to overcome tomato resistance provided by *Sw-5*. Isolates GRAU, GA1L, OK6J and VI were grouped as RB isolates. Isolates GRAU, GA1L and VI were collected from the tomato hybrids Bodar, Bond (Monsanto, Barcelona, Spain) and Verdi (Fitó, Barcelona, Spain) respectively, all carrying *Sw-5* and isolate OK6J was collected from the tomato hybrid Nikita (Syngenta Seeds, Almería, Spain) which does not carry *Sw-5*. Isolates ST-30, ZO and LL-N.05 were grouped as NRB isolates. Isolates ST-30 and ZO were collected from the tomato hybrids Durinta (Western Seed, Las Palmas de Gran Canaria, Spain) and Nikita respectively, and isolate LL-N.05 was obtained from an infected fruit of the tomato cultivar Gordal (Gautier S.A., Alicante, Spain) carry-

ing *Sw-5*, which is possible because NRB isolates sometimes cause concentric ring spots on tomato fruits as a result of an inefficient hypersensitivity response to TSWV infection by direct feeding of viruliferous thrips (Aramburu *et al*., 2000).

Biological characterization of TSWV isolates

TSWV isolates were mechanically inoculated on plant seedlings at the 2–4 leaf stage (cotyledons in the case of cucumber), by rubbing TSWV-infected extracts diluted (1:20, w:v) in 0.05 M phosphate buffer, pH 7.2, containing 0.2% 2-mercaptoethanol, 1% polyvinyl pyrrolidone, molecular weight 40,000, and activated charcoal. Individual TSWV isolates were inoculated in lots of a minimum of five plants of each assayed host. Inoculated plants were kept at least 30 days under controlled conditions in an insect-proof glasshouse at 20–27°C (night/day). The hosts inoculated with TSWV were: Cucumber (*Cucumis sativus*); *Datura stramonium*, lettuce cv. Rubia de Paris, lettuce cv. Maravilla (Western Seed), *Nicotiana glutinosa, N. tabacum* cv. Xanthi; pepper cv. Spiro, pepper cv. Divino (Seminis), both carrying the *Tsw* resistance gene and the susceptible pepper cv. C 804 (Fitó); *Petunia* × *hybrida;* the tomato cv. Bodar (Seminis), the tomato cv. Verdi (Fitó), both carrying the *Sw-5* and the susceptible tomato cv. Marmande (Fitó). Inoculated plants were tested by ELISA 15 days and/or 30 days after inoculation (DAI).

Virus transmission by *Frankliniella occidentalis*

The NRB isolates LL-N.05 and ZO, and the RB isolates GRAU and GA1L were selected to compare the efficiency of virus transmission to susceptible and resistant tomato plants by *F. occidentalis*. Tomato plants cv. Marmande were used to test transmission of all TSWV isolates, and tomato plants cv. Verdi were only used to test transmission of RB isolates. A virus-free colony of *F. occidentalis* was grown and maintained on green bean pods in a growth chamber at 26ºC with a 16-hour day. Ten male and female adult thrips were maintained for reproduction on TSWV-infected *D. stramonium* plants; the thrips larvae acquired the virus as previously described (Aramburu *et al*., 2000). Transmission rates by thrips are habitually tested using a local lesion assay on *Petunia* × *hybrid* leaf-disks (Wijkamp and Peters, 1993), but tomato plants were infected with thrips to test for systemic infection. As the feeding by individual adult thrips was very difficult to control under these conditions, second-instar larvae emerging from TSWV-infected plants were selected for this assay. The transmission efficiency of each TSWV isolate was determined using 40 tomato plants, and placing only one larva on each plant. Only tomato plants showing signs of thrips feeding 24 hours later were considered for the assay. Tomato plants were inspected after 10–20 days to test for TSWV, and leaf extracts were analysed by ELISA. The acquisition or transmission efficiency of each TSWV isolate was determined by examining 200 second-instar larvae that had emerged from infected *D. stramonium* plants after being fed on healthy *D. stramonium* leaves for 48 hours so as to eliminate virus particles from their digestive system. Larvae were sprinkled on the grids of a nitrocellulose membrane and the proportion of virus-containing larvae was indirectly determined by a squash-blot assay as described in Aramburu *et al*. (1996), since in that work there was a good correlation between the biological transmission on *Petunia* × *hybrid* leaf disk by adult thrips and the squash-blot assay. Pearson's chi-square test was used to assess the relationship between the isolates and their transmission or acquisition by the thrips. Yate´s correction was applied when necessary.

Fitness assays

To test the relative fitness of RB and NRB TSWV isolates, three assays were designed using the four isolates mentioned above. (i) Homogeneity assays: extracts prepared from original samples corresponding to each TSWV isolate were mechanically inoculated on *N. glutinosa* plants. Thirty biological clones of each isolate were selected four DAI from individual local lesions and were subcultured in *D. stramonium* plants. Each RB and NRB clone was inoculated on tomato, cv. Verdi plants to test its capacity to overcome resistance. (ii) Stability assays: one isolate obtained by biological cloning from each of the two RB isolates was inoculated on susceptible tomato plants cv. Marmande with 10 serial passages at 15-day intervals. Extracts of infected plants corresponding to each passage were also inoculated on six tomato plants cv. Verdi to determine whether they retained the capacity to overcome resistance. (iii) Competition assays: the four possible combinations of isolate pairs (RB vs.

NRB isolates) were co-inoculated on susceptible tomato plants cv. Marmande with 10 serial passages at 15-day intervals. Inoculum was prepared by mixing equal volumes of individual extracts of each isolate. They were identically prepared (1:10, w:v) using a leaf disk of an infected *D. stramonium* plant 15 DAI. The amount of virus was estimated by ELISA absorbance values and also by counting the number of local lesions produced on *N. glutinosa* following mechanical inoculation with each infectious extract. The same dilutions of each isolate were used in these inoculations. Additionally, extracts of the infected plants of each passage were inoculated on six tomato plants cv. Verdi to that confirm the RB isolate occurred in the infectious mixture.

RFLP analysis

To differentiate RB and NRB isolates in the competition assays, a reverse transcription-PCR (RT-PCR) combined with RFLP analysis was developed. The complete sequence of the M segment of the four isolates was obtained and deposited in the EMBL database (Accession FM163370, FM163371, FM163372, and FM163373 for isolates GRAU, GA1L, ZO and LL-N.05 respectively). RT-PCR products were amplified using the primer pair TSWV-1F 5´-GAATCAAATTTAGCCTGTGAC-3´ and TSWV-1R 5´-GACGTTGTATCCAGAAGG-3´. They were designed from conserved sequences after comparing the full-length sequence of the M segment of the four isolates. Differences in the sequences of these RT-PCR products allowed then to be selectively digested with *Xba*I or *Ava*II (MBI Fermentas, Madrid, Spain). Ten microlitres of PCR products and 5 U of the corresponding restriction enzyme were incubated for 2 h at 37ºC. PCR products or their restriction fragments were separated by electrophoresis in agarose gels, stained with ethidium bromide, and their DNA was visualized under UV lighting.

Results

Biological characterization of TSWV isolates

TSWV isolates were cloned in *N. glutinosa* and the symptoms they produced and their host range were compared with each other. No noticeable differences in infectivity were found between RB and NRB isolates for each host after mechanical inoculation, except in tomato and pepper. All TSWV isolates caused necrotic local lesions on mechanically inoculated leaves of *C. sativus* and *P. hybrida*, and these local lesions progressed to systemic infection in the case of *N. glutinosa, N. tabacum* cv. Xanthi, *G. globosa* and *D. stramonium*. The transmission to lettuce cultivars was not consistent in all replications, although no differences occurred between any RB and NRB isolates.

All tomato hybrids without *Sw-5* showed bronzing in the apex 10 to 15 DAI. The NRB isolates ST-30, LL-N.05 and ZO caused only local lesions on directly inoculated leaves of tomato cultivars carrying *Sw-5*, as a consequence of a hypersensitivity reaction. The RB isolates GRAU, GA1L, OK6J and VI sometimes caused local lesions on directly inoculated leaves of these tomato hybrids and overcame their resistance, reaching the apical leaves, but this systemic infection did not occur in all inoculated plants (Table 1). The failure of mechanical transmission of RB isolates to some resistant tomato plants was not dependent on the virus inoculum concentration which was determined by the number of local lesions produced on *N. glutinosa* 4 DAI, but it was dependent on the growth stage of the plant. The proportion of infected plants was higher on younger plants at the two-leaf stage and lower at the four-leaf stage (data not shown). In addition, some plants that were not infected following the first inoculation became infected after the second inoculation.

Pepper plants without the *Tsw* became systemically infected with all TSWV isolates (Table 1). In pepper plants with *Tsw*, all isolates caused local lesions as a consequence of a hypersensitivity reaction, but some plants (mainly inoculated with RB isolates), which showed systemic infection 15 DAI, tested negative by ELISA 30 DAI (Table 1).

Virus transmission by *Frankliniella occidentalis*

The proportion of *F. occidentalis* larvae that acquired the virus after feeding on TSWV-infected *D. stramonium* plants, as determined by squash blot, and the proportion of second-instars larvae that transmitted different TSWV isolates to whole tomato plants are shown in Table 2. The percentage of larvae that acquired the virus from TSWV-infected *D. stramoniun* plants ranged from 9.5% to 24.5% among the four TSWV isolates. Statistical analysis detected that GRAU acquisition by

Table 1. Proportion of tomato and pepper plants infected with different *Tomato spotted wilt* virus (TSWV) isolates determined by enzyme-linked immunosorbent assay, 15 and 30 days after mechanical inoculation.

Isolate	C804[a]		Spiro[b]		Divino[b]		Marmande[a]		Bodar[c]		Verdi[c]	
	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d
GRAU[e]	5/5[d]	-	2/10	7/10	3/10	2/10	5/5	-	2/10	8/10	6/10	9/10
GA1L[e]	5/5	-	5/10	0/10	2/10	1/10	5/5	-	8/10	8/10	5/10	5/10
OK6J[e]	5/5	-	2/10	1/10	4/10	2/10	5/5	-	6/10	8/10	3/10	6/10
VI[e]	5/5	-	2/10	0/10	1/10	1/10	5/5	-	1/10	2/10	1/10	1/10
ST-30	5/5	-	0/10	0/10	5/10	0/10	5/5	-	0/10	0/10	0/10	0/10
ZO	5/5	-	3/10	0/10	0/10	0/10	5/5	-	0/10	0/10	0/10	0/10
LL-N.05	5/5	-	1/10	0/10	0/10	0/10	3/5	5/5	0/10	0/10	0/10	0/10

[a] TSWV susceptible cultivars of pepper (C804) and tomato (Marmande).
[b] Pepper hybrids carrying the *Tsw* gene.
[c] Tomato hybrids carrying the *Sw-5* gene.
[d] No. of infected plants/No. of inoculated plants.
[e] Resistance-breaking isolates.
-, Not determined due to the death of some infected plants.

Table 2. Correlation between *Tomato spotted wilt virus* (TSWV) in larvae after feeding on *Datura stramonium* infected plants determined by squash-blot on nitrocellulose membranes and their capacity to transmit different TSWV isolates to tomato plants with or without *Sw-5*.

TSWV isolates	Squash-blot[a]	Verdi[b]	Marmande[c]
GRAU[d]	9.5	12.5	7.5
GA1L[d]	19	5	2.5
LL-N.05	23.5	-	12.5
ZO	24.5	-	5

[a] Percentage of infectious larvae out of 200 larvae tested.
[b] Percentage of infected tomato plants out of 40 tomato plants carrying the *Sw-5* gene tested.
[c] Percentage of infected tomato plants out of 40 tomato plants tested.
[d] Resistance-breaking isolates.
-, Not determined.

trips was significantly lower than acquisition by the other isolates. In the transmission assays, only about 60% of tomato plants that showed signs of thrips feeding (lesions about 2-4 mm^2 on the leaf tissue) 24 hours after placing the larvae on tomato leaf were considered. These larvae could not be analysed by squash blot as 80% of them could not be recovered after the transmission test. The percentage of successful virus transmission to tomato plants in relation to the percentage of viruliferous larvae decreased for all TSWV isolates and ranged from 2.5 to 12.5%. There was no clear correlation between the type of isolate and the extent to which the thrips transmitted the virus, based on Pearson's chi-square test using the Yates´ correction.

Characterization of TSWV isolates in fitness assays

Homogeneity assays

Extracts of the original tomato samples (isolates GRAU, GA1L, ZO and LL-N.05) were mechanically inoculated on *N. glutinosa* plants, and thirty biological clones of each isolate, from individual local lesions, were selected and amplified by subculturing in *D. stramonium* plants. The thirty clones of each TSWV isolate caused similar symptoms in *D. stramonium* plants, and all clones infected tomato plants cv. Marmande. Some clones from isolates LL-N.05 and ZO caused necrotic spots in tomato plants cv. Verdi, but none of them infected the plants systemically. Eight clones from the GRAU and six from the GA1L isolates were not transmitted by mechanical inoculation to tomato plants cv. Verdi in the first attempt, although they were transmitted in a second or third attempt.

Stability assays

The four isolates were mechanically transmitted to susceptible tomato plants cv. Marmande, following 10 serial passages at 15-day intervals. After each passage, extracts of infected plants were inoculated on tomato plants cv. Verdi, but only the extracts of RB isolates infected them systemically.

Competition assays

Infectious mixtures of one RB plus one NRB isolate were mechanically co-inoculated on tomato plants cv. Marmande with 10 serial passages at 15-day intervals. The following isolate pairs were inoculated: GRAU/ZO, GA1L/ZO, GRAU/LL-N.05 and GA1L/LL-N.05. The ratio of both competitors in the progeny was determined using an RT-PCR-RFLP assay. For this purpose, the primer pair TSWV 1F/1R was designed to amplify a specific region of the Gn/Gc precursor glycoprotein gene. The resulting amplicon was 456 bp in length. Restriction analysis facilitated the detection of each type of isolate (NRB and/or RB) in the infected mixture of each passage. In parallel, tomato plants cv. Verdi became systematically infected after being mechanically inoculated with extracts corresponding to each mixture, and this confirmed the presence of RB isolates in the mixture. The RT-PCR fragment from the leaf samples infected with the RB isolates gave two bands of 135 bp and 321 bp after digestion with *Xba*I (Figure 1, lanes 1 and 2), whereas none were obtained with *Ava*II (Figure 1, lanes 5 and 6). In contrast, the RT-PCR fragment from the leaf samples infected with the NRB isolates was not digestible with *Xba*I (Figure 1, lanes 3 and 4), while producing two bands of 132 bp and 323 bp after digestion with *Ava*II (Figure 1, lanes 7 and 8). When the competitors GRAU and ZO were co-inoculated, the ZO isolate predominated, and 15 days after the first inoculation only the ZO isolate was detected by the RT-PCR-RFLP assays (Figure 2, lanes 2 and 12), although the presence of the GRAU isolate in the mixture was confirmed by its mechanical transmission to tomato plants cv. Verdi. The ZO isolate also predominated when co-inoculated with GA1L, although both isolates were detected by the RT-PCR-RFLP assays 15 days after the first inoculation (Figure 2, lanes 3 and 13). No further assays were carried out after the mixtures GRAU/ZO and GA1L/ZO failed to infect the tomato plants cv. Verdi, after the third and fouth passages respectively, and the GRAU and GA1L isolates were not detected by the RT-PCR-RFLP assays (Figure 2, lanes 6 and 16, and lanes 7 and 17, respectively). In contrast, with the competitor pair GRAU/LL-N.05 and GA1L/LL-N.05, the RB isolates GRAU and GA1L predominated over the NRB isolate LL-N.05, and 15 days after the first inoculation only the GRAU and GA1L isolates were detected by RT-PCR-RFLP (Figure 2, lanes 4 and 14 for the competitors GRAU/LL-N.05; and lanes 5 and 15 for GA1L/LL-N.05). As expected, at the tenth passage only the GRAU

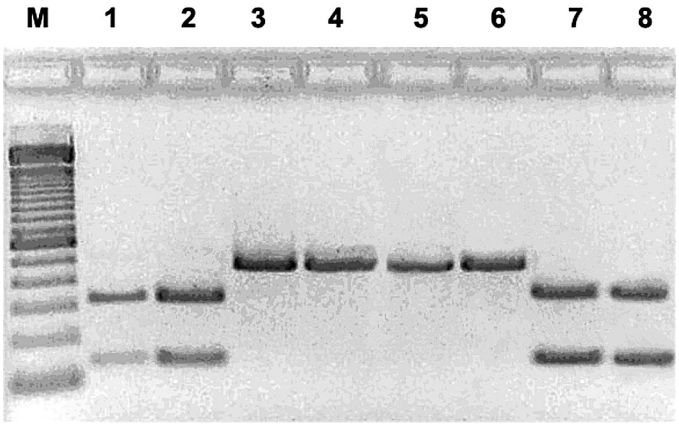

Figure 1. RT-PCR-RFLP pattern of leaf samples infected with different *Tomato spotted wilt virus* isolates, following amplification using primers 1F/1R and digestion with *Xba*I or *Ava*II. Lane M: 100 bp DNA ladder marker; lanes 1–4 were digested with *Xba*I and lanes 5–8 were digested with *Ava*II. Isolate GRAU digestion products are shown in lanes 1 and 5, isolate GA1L digests in lanes 2 and 6, isolate ZO digests in lanes 3 and 7, and isolate LL-N.05 digests in lanes 4 and 8.

(Figure 2, lanes 8 and 18) and GA1L isolates (Figure 2, lanes 9 and 19) were detected. A weak non-digested DNA band in some mixtures (Figure 2, lanes 4, 5, 8, 9, 12, 16 and 17) was a consequence of incomplete digestion and not due to the presence of DNA from the competitor isolate, since it was also seen in the digestion fragments of the control isolates GRAU and ZO (Figure 2, lanes 1 and 20, respectively).

Discussion

The TSWV isolates that overcome the resistance conferred by *Sw-5* in tomato plants were first detected in north-eastern Spain in 2002. An increasing number of tomato varieties previously resistant to TSWV showed the typical bronzing associated with TSWV infection. The presence of these RB isolates in the field may have increased

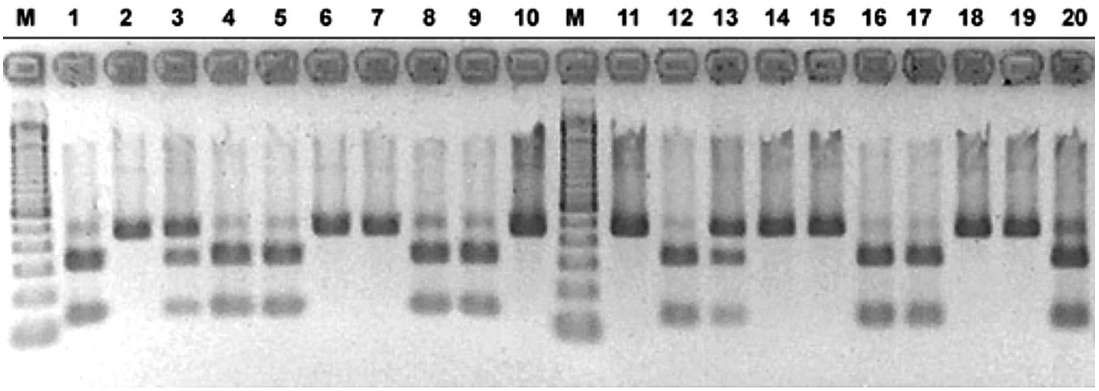

Figure 2. RT-PCR-RFLP pattern of leaf samples infected with different infection mixtures of *Tomato spotted wilt virus* isolates, following amplification using primers 1F/1R and digestion with *Xba*I (lanes 1–10) or *Ava*II (lanes 11–20). M, 100 bp DNA ladder marker; lanes 1 and 10 digestion products of GRAU and ZO isolates respectively; lanes 2, 3, 4 and 5 digestion products of infectious mixtures 15 days after the first inoculation of GRAU vs. ZO, GA1L vs. ZO, GRAU vs. LL-N.05, and GA1L vs. LL-N.05, respectively; lane 6, digestion products of infectious mixtures 15 days after the third passage of GRAU vs. ZO; lane 7, digestion products of infectious mixtures 15 days after the fourth passage of GA1L vs. ZO; lanes 8 and 9, digestion products of infectious mixtures 15 days after the tenth passage of GRAU vs. LL-N.05 and GA1L vs. LL-N.05 respectively. Lanes 11–20, same order for *Ava*II as lanes 1–10 for *Xba*I.

due to the selection exerted by the extensive use of tomato plants carrying *Sw-5* (López *et al*., 2011). However, due to increasing losses in tomato crops planted with cultivars carrying *Sw-5*, growers have gradually returned to cultivating the cheaper and more marketable tomato cultivars without TSWV resistance. Since then, RB isolates have only been detected in a restricted area of about 900 km^2. The occurrence and spread of a particular TSWV isolate in open fields depends on a number of factors including host range, crop management, inoculum pressure, *F. occidentalis* vector preferences and differences in the fitness of the NRB isolates. Some of these factors were investigated to determine their effect on the epidemiology of TSWV isolates.

The RB and NRB isolates selected for the study produced similar symptoms in different hosts, except tomato and pepper. TSWV isolates from Australia overcame, resistance in tomato plants carrying *Sw-5*, but they were not infectious when they were inoculated on *C. chinense* carrying the *Tsw* (Latham and Jones, 1998). However, our RB isolates developed local lesions on inoculated leaves of pepper plants due to a hypersensitivity response, and occasionally developed a systemic infection. Resistance provided by the *Tsw* reduced virus accumulation to levels undetectable by ELISA, but it was partially overcome in some cases and the newly emerged non-inoculated leaves were positive by ELISA, providing optical density values, that were in general, lower than those on susceptible peppers. The low viral accumulation could have been the consequence of a mechanism that restricted the movement of the virus in the tissues of resistant plants, as reported by Soler *et al*. (1998).

The homogeneity assays were carried out using biological clones from individual local lesions obtained by mechanical inoculation of the original samples on *N. glutinosa,* followed by sub-culturing in *D. stramonium* plants to promote viral replication. None of the NRB clones produced systemic infection in tomato plants carrying *Sw*-5, but, all RB clones caused systemic infection. Admittedly, some clones of the two RB isolates were not mechanically transmitted to resistant plants at the first attempt, but they overcame the resistance when a greater number of plants were inoculated at the second attempt. The failure of mechanical transmission of RB isolates to resistant tomatoes seems to be more closely related with the phenological state of the plants than with the quality of the inoculum. This difference in fitness between mechanically inoculated NRB and RB isolates was not well reproduced in the thrips transmission tests, because RB and NRB isolates did not differ statistically in their transmission to tomato plants. On the other hand, in these assays there was no a clear correlation between the efficiency of transmission to tomato plants and the percentage of larvae carrying TSWV, regardless of the TSWV isolate used. The possibility that thrips may replicate TSWV without the capacity to transmit it was reported by Nagata *et al*. (2002). On the other hand, since the transmission assays used second-instar larvae instead of adult thrips, the virus may not have had enough time to reach the salivary glands (Sakimura, 1963; Nagata *et al*., 1999), which is required for the virus transmission (Whitfield *et al*., 2005).

Resistance breaking isolates did not revert to NRB isolates in the stability assays when they were sub-cultured in susceptible tomato plants, after 10 serial passages, confirming previous studies which found that TSWV isolates that overcame single-gene resistance in plants were relatively stable (Latham and Jones, 1998). In contrast, a partial reversion to NRB TSWV isolates was seen when a *Tsw* RB isolate was sub-cultured by serial passages in susceptible peppers (Thomas-Carroll and Jones, 2003).

The RT-PCR-RFLP assays could not serve to determine the total absence of one isolate in the mixture of isolates in the competitive assays, because no digestion products of RB isolates were obtained after *Xba*I restriction in some passages, although the biological transmission assays demonstrated that they occurred. However, the RT-PCR-RFLP assay was useful in determining the prevalence of a particular isolate in a mixture after serial passages. The ZO isolate was prevalent over isolates GRAU and GA1L, while LL.N-05 was not. Thus the RB or NRB isolates did not show a clear difference of fitness in infecting tomato plants in the competitive assays, although fitness differences may have occurred in other virus-host combinations.

In summary, the TSWV isolates were transmitted as efficiently by thrips as by the NRB isolates. They were relatively stable and did not differ in fitness when infecting tomato. These findings do not

explain the slow spread of these isolates since they were first detected. The difficulty in understanding why fitness was lost could be due to the complexity of the TSWV-vector relationship, in which a high genetic variability of both virus and vector populations was combined with a great diversity of susceptible host species. Nevertheless, the progressive replacement of resistant tomato cultivars by cultivars lacking *Sw-5* may have lessened the positive selection of RB isolates, and favoured the emergence of NRB isolates. Although the spread and survival of the RB isolates could have been minimised by this practise, by the management of integrated control strategies, or by some other undetermined reason, we included these RB isolates in the screening program in order to select new and durable sources of resistance to TSWV in tomato.

Acknowledgements

We thank M. Matas and J. Ariño for locating tomato crops planted with hybrids carrying the *Sw-5* gene infected with TSWV and A. Barba for the excellent technical assistance. This research was supported by grants RTA04-004-C2-1 and RTA2008-00010-C03-00 from the Instituto Nacional de Investigaciones Agrarias (INIA) and ACOMP/2010/050 (COMAV) from the Generalitat Valenciana.

Literature cited

Aparicio F., J. Aramburu, S. Soler, L. Galipienso, F. Nuez, V. Pallas and C. López, 2009. Immunodiagnosis of Parietaria mottle virus in tomato crops using a polyclonal antiserum against its coat protein expressed in a bacterial system. *Journal of Phytopathology* 157, 511–513.

Aramburu J. and M. Martí, 2003. The occurrence in northeast Spain of a variant of *Tomato spotted wilt virus* (TSWV) that breaks resistance in tomato (*Lycopersicon esculentum*) containing the *Sw-5* gene. *Plant Pathology* 52, 407.

Aramburu J., J. Riudavets, J. Arnó, A. Laviña and E. Moriones, 1996. Rapid serological detection of tomato spotted wilt virus in individual thrips by squash-blot assay for use in epidemiological studies. *Plant Pathology* 45, 367–374.

Aramburu J. and M. Rodriguez, 1999. Evaluation of commercial *Lycopersicon esculentum* hybrids for resistance to *tomato spotted wilt tospovirus* (TSWV) in Spain. *Journal of Horticultural Science and Biotechnology* 74, 743–747.

Aramburu J., M. Rodriguez and J. Ariñó, 2000. Effect of *Tomato spotted wilt tospovirus* (TSWV) infection on the fruits of tomato (*Lycopersicon esculentum*) plants of cultivars carrying the *Sw-5* gene. *Journal of Phytopathology* 148, 569–574.

Cho J.J., D.M. Custer, S.H. Brommonschenkel and S.D. Tanksley, 1996. Conventional breeding: host-plant resistance and use of molecular markers to develop resistance to *Tomato spot wilt virus* in vegetables. *Acta Horticulturae* 431, 367–378.

Clark M.F. and A.N. Adams, 1977. Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34, 475–483.

de Haan P., R. Kormelink, R. de Oliveira Resende, F. van Poelwijk, D. Peters and R. Goldbach, 1991. *Tomato spotted wilt virus* L RNA encodes a putative RNA polymerase. *Journal of General Virology* 72, 2207–2216.

de Haan P., L. Wagemakers, G. Peters and R.Golbach, 1990. The S RNA segment of *Tomato spotted wilt virus* has an ambisense character. *Journal of General Virology* 71, 1001–1007.

German T.L., D.E. Ullman and J.W. Moyer, 1992. *Tospoviruses*: diagnosis, molecular biology, phylogeny and vector relationships. *Annual Review of Phytopathology* 30, 315–348.

Golbach R. and D. Peters, 1994. Possible causes of the emergence of tospovirus diseases. *Seminars in Virology* 5, 113–120.

Hoffmann K., W.P. Qiu and M. Moyer, 2001. Overcoming host and pathogen-mediated resistance in tomato and tobacco maps to the M RNA of *Tomato spotted wilt virus*. *Molecular Plant Microbe Interactions* 14, 242–249.

Lacasa A., 1990. Un trienio de *Frankliniella occidentalis* en España. Evolución espacial y temporal de una plaga importada. *Cuadernos Phytoma-España* 6, 3–9.

Latham L.J. and R.A.C. Jones, 1998. Selection of resistance breaking strain of tomato spotted wilt tospovirus. *Annals of Applied Biology* 133, 385–402.

Li W., D.J. Lewandowski, M.E. Hilf and S. Adkins, 2009. Identification of domains of the *Tomato spotted wilt virus* NSm protein involved in tubule formation, movement and symptomatology. *Virology* 390, 110–121.

López C., J. Aramburu, L. Galipienso, S. Soler, F. Nuez and L. Rubio, 2011. Evolutionary analysis of tomato *Sw-5* resistance breaking isolates of *Tomato spotted wilt virus*. *Journal of General Virology* 92, 210–215.

Moury B., K.G. Selassie, G. Marchoux, A.M. Daubeze and A. Palloix, 1998. High temperature effects on hypersensitive resistance to *tomato spotted wilt Tospovirus* (TSWV) in pepper (*Capsicum chinense* Jacq.). *European Journal of Plant Pathology* 104, 489–498.

Nagata T., A.K. Inoue-Nagata, H.M. Smid, R. Goldbach and D. Peters, 1999. Tissue tropism related to vector competence of *Frankliniella occidentalis* for tomato spotted wilt tospovirus. *Journal of General Virology* 80, 507–515.

Nagata T., A.K. Inoue-Nagata, J. van Lent, R. Goldbach R

and D. Peters, 2002. Factors determining vector competence and specificity for transmission of *Tomato spotted wilt virus*. *Journal of General Virology* 83, 663–671.

Peters D., I. Wijkamp, F. van de Wetering and R. Goldbach 1996. Vector relations in the transmission of tospoviruses. *Acta Horticulturae* 431, 29–43.

Roselló S., M.J. Díez, A. Lacasa, C. Jordá and F. Nuez, 1997. Testing resistance to TSWV introgressed from *Lycopersicon peruvianum* by artificial transmission techniques. *Euphytica* 98, 93–98.

Roselló S., M.J. Díez and F. Nuez, 1996. Viral disease causing the greatest economic losses to the tomato crop. I. The tomato spotted wilt virus – a review. *Scientia Horticulturae* 67, 117–150.

Sakimura K., 1963. *Frankliniella fusca,* an additional vector for the tomato spotted wilt virus, with notes on *Thrips tabaci*, another vector. *Phytopathology* 53, 412-415.

Sin S-H., B.C. McNulty, G.G. Kennedy and J.W. Moyer, 2005. Viral genetic determinants for thrips transmisión of *Tomato spotted wilt virus*. Washington, DC, USA, *Proceedings of the National Academy of Sciences of the USA*, 102, 5168–5173.

Soler S., M.J. Diéz and F. Nuez, 1998. Effect of temperature and growth stage interaction on pattern of virus presence in TSWV-resistance accessions of *Capsicum chinense*. *Plant Disease* 82, 1199–1204.

Stevens M.R., S.J. Scott and R.C. Gergerich, 1992. Inheritance of a gene for resistance to tomato spotted wilt virus from *Lycopersicon peruvianum* Mill. *Euphytica* 59, 9–17.

Stevens M.R., S.J. Scott and R.C. Gergerich, 1994. Evaluation of seven *Lycopersicon* species for resistance to *tomato spotted wilt virus* (TSWV). *Euphytica* 80, 79–84.

Takeda A., K. Sugiyama, H. Nagano, M. Mori, M. Kaido, K. Mise, S. Tsuda and T. Okuno, 2002. Identification of a novel RNA silencing supressor, NSs protein of *Tomato spotted wilt virus*. *FEBS Letters* 532, 75–79.

Thompson G.J. and J.J.B. Van Zijl, 1996. Control of tomato spotted wilt virus in tomatoes in South Africa. *Acta Horticulturae* 431, 379–384.

Thomas-Carroll M.L. and R.A.C. Jones, 2003. Selection, biological properties and fitness of resistance-breaking strain of *Tomato spotted wilt virus* in pepper. *Annals of Applied Biology* 142, 235–243.

Van de Wetering F., R. Goldbach and D. Peters, 1996. Tomato spotted wilt virus ingestion by first instar larvae of *Frankliniella occidentalis* is a prerequisite for transmission. *Phytopathology* 86, 900–905.

Van Zijl J.J.B., S.E. Bosch and C.P.J. Coertzee, 1986. Breeding tomatoes for processing in South Africa. *Acta Horticulturae* 194, 69–74.

Whitfield A.E., D.E. Ullman and T.L. German, 2005. Tospovirus-Thrips interaction. *Annual Review of Phytopathology* 43, 459–489.

Wijkamp I. and D. Peters, 1993. Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis,* using a novel leaf disk assay. *Phytopathology* 83, 986–991.

Accepted for publication: October 15, 2010