Evidence That the Nonstructural Protein of *Tomato spotted wilt virus* Is the Avirulence Determinant in the Interaction with Resistant Pepper Carrying the *Tsw* Gene

P. Margaria, M. Ciuffo, D. Pacifico, and M. Turina

Istituto di Virologia Vegetale, Sez. di Torino, CNR, Strada delle Cacce 73, Torino 10135, Italy

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All known pepper cultivars resistant to *Tomato spotted wilt virus* (TSWV) possess a single dominant resistance gene, *Tsw*. Recently, naturally occurring resistance-breaking (RB) TSWV strains have been identified, causing major concerns. We used a collection of such strains to identify the specific genetic determinant that allows the virus to overcome the *Tsw* gene in *Capsicum* spp. A reverse genetic approach is still not feasible for TSWV; therefore, we analyzed reassortants between wild-type (WT) and RB strains. Our results confirmed that the S RNA, which encodes both the nucleocapsid protein (N) and a nonstructural protein (NSs), carries the genetic determinant responsible for *Tsw* resistance breakdown. We then used full-length S RNA segments or the proteins they encode to compare the sequences of WT and related RB strains, and obtained indirect evidence that the NSs protein is the avirulence factor in question. Transient expression of NSs protein from WT and RB strains showed that they both can equally suppress post-transcriptional gene silencing (PTGS). Moreover, biological characterization of two RB strains carrying deletions in the NSs protein showed that NSs is important in maintaining TSWV infection in newly emerging leaves over time, preventing recovery. Analysis of another RB strain phenotype allowed us to conclude that local necrotic response is not sufficient for resistance in *Capsicum* spp. carrying the *Tsw* gene.

Tomato spotted wilt virus (TSWV), genus *Tospovirus*, family *Bunyaviridae*, causes an important disease of pepper (*Capsicum* spp.) occurring worldwide (Adkins 2003), and the most effective and environmentally sound control strategy relies on the availability of resistant cultivars. Unfortunately all such cultivars possess the same single dominant resistance gene, called *Tsw* (Jahn et al. 2000). Plants carrying this gene usually show necrotic local lesions on the inoculated leaf, followed by premature abscission (Boiteaux 1995). The plant does not become systemically infected.

Boiteaux and Nagata (1993) first reported a TSWV resistance-breaking (RB) strain in *Capsicum chinense*. Several RB strains have now been reported from Italy in *Capsicum* spp. carrying the *Tsw* gene (Roggero et al. 1999, 2002) and, recently, new isolates have been reported in Spain (Margaria et al. 2004) and Australia (Thomas-Carrol et al. 2003).

TSWV has a genome of three RNA segments: small (S) and medium (M) RNAs have ambisense coding strategies, whereas the large (L) RNA is of negative polarity. Jahn and associates (2000) have shown that the S RNA carries the genetic determinant for breakdown of *Tsw* resistance, but the specific avirulence factor has not been identified so far. The small segment is known to encode a nonstructural protein (NSs) in the positive strand and the nucleoprotein (N) in the negative strand (de Haan et al. 1990).

A reverse genetic approach developed for viruses of the family *Bunyaviridae* has been described for the genera *Nairovirus* (Flick et al. 2003) and *Bunyavirus* (Bridgen and Elliot 1996) but, thus far, is not feasible for members of *Tospovirus*. However, reassortment experiments can generate progeny with different properties (Qiu et al. 1998) and, through this approach, plus sequencing of M segments from thrips-transmissible and nontransmissible isolates, Sin and associates (2005) demonstrated the role of the M segment and in particular of the glycoprotein-carboxyl terminal (Gc) in transmissibility by thrips. Since the first reassortment study by Best during cross protection experiments between TSWV strains in 1961 (Best 1961), a few features have been unambiguously assigned to the different genome segments through this approach (Hoffmann et al. 2001; Jahn et al. 2000; Okuda et al. 2003). In this article, we show evidence that the NSs open reading frame (ORF) or its product is the source of *Tsw* resistance breakdown. We then discuss some of the molecular features associated with the TSWV-resistant pepper interaction that could be derived from a closer examination of some of the RB isolates from our collection.

RESULTS

Phenotype of RB strains.

Each strain used in our study (Table 1) was evaluated for its symptom phenotype on a set of host species generally used in TSWV studies, and the more relevant are reported in Table 2. All wild-type (WT) isolates produced the same classical symptoms (only necrotic lesion on inoculated leaves) on *C. chinense*-resistant accession number PI152225 (Fig. 1A and B). Symptoms of the various RB isolates varied somewhat. RB strains p105^{RB}, p105/1^{RB}, p105/43.14^{RB}, p105/44.7^{RB}, p267^{RB}, p272^{RB}, Br20^{RB}, and Ve427^{RB} established systemic infections causing leaf chlorosis and yellow mottle, but no necrotic local lesion on the inoculated leaf (Fig. 1C and D). In contrast, infection by RB strain p166^{RB} produced a necrotic lesion on the inoculated leaf, like the wild type, followed by systemic necrotic lesions and leaf crumple but no yellowing (Fig. 1E and F).

Corresponding author: M. Turina; Fax: +39 011 343809; E-mail: m.turina@ivv.cnr.it

With the Sicilian isolates, we observed that p202^{RB} caused rapid lethal necrosis on *Nicotiana benthamiana*, whereas p170^{WT} and p170^{RB} gave a delayed and less aggressive response of leaf crumple and mild mosaic without necrosis. Interestingly, p170^{RB} and p202^{RB} did systemically infect resistant *C. chinense*, but the new leaves emerging after the onset of systemic infection had no symptoms and were not infected, as confirmed by Western blot analysis (data not shown), thus displaying a consistent recovery phenotype (Fig. 1G through I). Systemic infection followed by recovery also was observed for both p170^{RB} and p202^{RB} in *C. annuum*, *Datura stramonium*, and *Solanum lycopersicum*, whereas p170^{WT} severely infected all these hosts permanently without recovery. Isolates p170^{RB} and p202^{RB} caused systemic necrosis on *N. tabacum*, whereas p170^{WT} was limited to local infection. Moreover, isolates p170^{RB} and p202^{RB} were not able to infect *Emilia sonchifolia* and *Ocimum basilicum*, in contrast to the WT strain p170^{WT}.

Reassortant RB isolates.

Inoculum ratios in favor of WT strain Br01^{WT} (10:1 and 5:1) helped to obtain reassorted progeny carrying two genome segments of the WT parental strain and only one of the RB strain. Among the various reassortants strains we concentrated on, three clones—IFA201^{RB} (Br01^{WT}-p267^{RB}), IFA231^{RB} (Br01^{WT}-p105/43.14^{RB}), and IFA225^{RB} (Br01^{WT}-Ve427^{RB})—had the S segment of the RB parental strain and the M and L segments of Br01^{WT} (Fig. 2; data not shown). Among the reassortants able to break resistance conferred by the *Tsw* gene, no WT S segment was detected. Moreover, no reassortant carrying recombinant genomic RNAs was obtained in our study. The reassorted strains gave systemic infection in *C. chinense* PI152225, inducing symptoms similar to those of the RB parental strain.

Sequence analysis of S segments.

The length of the complete S RNA was 2,925 nucleotides (nt) for p166^{RB}; 2,926 nt for p105/44.7^{RB}; 2,927 nt for p105^{WT}, p105/43.14^{RB}, p105/1^{RB}, p105^{RB}, and p267^{RB}; 2,934 nt for p272^{RB}, and 2,922 nt for Ve 430^{WT} and Ve427^{RB} compared with 2,916 nt for Br01^{WT} (de Haan et al. 1990). Br20^{WT} and Br20^{RB} contained an S segment of 2,926 nt. The two Sicilian RB isolates had small deletions in the NSs region when compared with the WT Sicilian isolate p170^{WT} (2,954 nt), giving 2,930 nt for p170^{RB} and 2,961 nt for p202^{RB} (which showed a slightly longer intergenic region).

Alignment of the S segment of RB isolates compared with the WT versions revealed multiple differences for each isolate in the coding and noncoding regions, but no single mutation was common to all the RB strains. However, most did not show any difference from the respective WTs in the 5' and 3' untranslated regions (UTR), leading to the conclusion that mutations in these regions are not involved in overcoming the *Tsw* resistance gene. Although the comparison among the various intergenic regions (IGs) showed a number of mutations, insertions, and deletions, confirming the hypervariable nature of this genomic region, strains p105^{WT}, p105/1^{RB}, and p105^{RB} had the exact same IG sequence. Br20^{WT} and Br20^{RB} also showed the same IG sequence. Taken together, these results imply that mutations in the intergenic region are not necessary to confer the RB phenotype in *Tsw* resistant pepper.

Analysis of the N and NSs proteins encoded by S RNA.

The size of the N protein was 258 amino acids (aa) for all the Italian and Spanish isolates, as described for Br01^{WT} (de Haan et al. 1990). The deduced amino acid sequence of the N protein was identical in all the Italian WT and RB strains. The

Table 1. Wild-type (WT) and resistance-breaking (RB) *Tomato spotted wilt virus* (TSWV) isolates used in this study

TSWV isolate	Source ^a	Date of isolation
Br01 ^{WT}	Brazil	14 August 1996
p105 ^{WT}	Albenga (Italian Riviera)*	7 August 1996
p166 ^{RB}	Albenga (Italian Riviera)*	27 August 1998
p267 ^{RB}	Albenga (Italian Riviera)*	28 May 2002
p272 ^{RB}	Albenga (Italian Riviera)*	4 June 2002
p105/1 ^{RB}	IVV-Torino#	17 June 1999
p105/44.7 ^{RB}	IVV-Torino#	30 March 2000
p105/43.14 ^{RB}	IVV-Torino#	30 March 2000
p105 ^{RB}	IVV-Torino#	5 May 2006
Ve430 ^{WT}	Almeria (Spain)*	2 October 2003
Ve427 ^{RB}	Almeria (Spain)*	19 September 2003
p170 ^{RB}	IVV-Torino ^o	20 September 2005
p170 ^{WT}	Sicily (Italy)*	26 February 1999
p202 ^{RB}	Sicily (Italy)*	5 August 1999
Br20 ^{WT}	Brazil	10 January 2006
Br20 ^{RB}	IVV-Torino ⁺	10 March 2006

^a * = from the same field, # = experimentally derived from p105^{WT}, ^o = experimentally derived from p170^{WT}, and ⁺ = experimentally derived from Br20^{WT}.

Table 2. Symptoms on noninoculated upper leaves of various plant species 2 weeks post inoculation with *Tomato spotted wilt virus* (TSWV) strains^a

Strain ^c	<i>N. benthamiana</i>	<i>N. tabacum</i>		<i>C. chinense</i> ^b		<i>C. annuum</i>		<i>D. stramonium</i>	<i>S. lycopersicum</i>	<i>O. basilicum</i>	<i>E. sonchifolia</i>
		Loc	Syst	Loc	Syst	Loc	Syst				
Br01 ^{WT}	ln	Nil	nm	nll	—	ym	ym	m	nm	ym	ym
p105 ^{WT}	m, lc	M	m	nll	—	ym	ym	m	nm	ym	ym
p170 ^{WT}	m, lc	Nil	—	nll	—	ym	ym	m	nm	ym	ym
p166 ^{RB}	m, lc	Nil	nm	nll	nl	ym	ym	m	nm	ym	ym
p267 ^{RB}	m, lc	Nil	nm	ym	ym	ym	ym	m	nm	ym	ym
p170 ^{RB}	m, lc	Nil	nm	ym	rec	ym	rec	rec	rec	—	—
p202 ^{RB}	ln	Nil	nm	ym	rec	ym	rec	rec	rec	—	—

^a When relevant, a distinction between local (Loc) and systemic (Syst) symptoms was recorded. Species from genera *Nicotiana*, *Capsicum*, *Datura*, *Solanum*, *Ocimum*, and *Emilia*. Legend: ln = lethal necrosis, lc = leaf crumple, nl = necrotic lesions, nll = necrotic local lesion, nm = necrotic mosaic, ym = yellow mosaic, m = mosaic, rec = recovery, and — = not infected as tested by Western blot analysis.

^b *C. chinense* used for symptom evaluation was accession PI152225.

^c WT = wild type and RB = resistance breaking.

Italian N protein coding sequences also were the same, except that p267^{RB} had U instead of C at nucleotide 266, and both p267^{RB} and p105/43.14^{RB} had A instead of U in position 762; both are silent. Alignment of the N coding sequence of the Spanish WT and RB isolates showed A substituted for G (silent) at nucleotide 756.

The size of the NSs protein was predicted to be 467 aa for the northern Italian isolates compared with 464 aa for Br01^{WT} (de Haan et al. 1990). The Sicilian isolate p170^{WT} also had a NSs protein of 467 aa (but differing in sequence from the

northern Italian isolates), whereas p202^{RB} showed a double-base deletion at position 1,171, resulting in a predicted protein of 399 aa; moreover, p170^{RB} showed a deletion of 25 nt at position 684, resulting in a predicted protein of 238 aa. For the two Spanish isolates, we predicted a NSs protein of 468 aa.

Alignment of the deduced amino acid sequences of the NSs ORFs of the RB strains showed one to two amino acid substitutions in the northern Italian, Spanish, and Brazilian strains compared with the NSs of the corresponding WT strain (Fig. 3A, B, and C). No single amino acid substitution was common

Fig. 1. Local and systemic symptoms of resistant pepper plants (*Capsicum chinense* PI152225) inoculated with some *Tomato spotted wilt virus* strains described in this study. **A** and **B**, Inoculated with the wild-type (WT) strain p105^{WT}. **C** and **D**, **E** and **F**, and **G** and **H**, were inoculated with the resistance-breaking (RB) strains p267^{RB}, p166^{RB}, and p170^{RB}, respectively. **A**, **C**, **E**, and **G**, Plants evaluated 7 days post inoculation (dpi); **B**, **D**, **F**, and **H**, evaluated 15 dpi. Different systemic symptom phenotype for strain p166^{RB} is to be noticed. **I**, Recovery of symptoms on newly emerged leaves at 30 dpi for p170^{RB}-inoculated plants.

to all the Italian RB strains. One substitution was common to p267^{RB} and p105/43.14^{RB} strains (I to V in position 437) and to p272^{RB} and p105^{RB} (Y to C in position 78). The alignment of the NSs protein sequences of the Spanish isolates showed two

single substitutions at position 85 and 408 (Fig. 3B). A different substitution but in the same site was observed for p267^{RB} (Italian isolate) and Ve427^{RB} (Spanish isolate) (D to V and D to N, respectively) in comparison with the related WT (Fig. 3A and B). The Brazilian RB isolate had two amino acid changes compared with its WT at positions 174 and 255 (I to M and K to N, respectively) (Fig. 3C). Alignment of the NSs proteins encoded by the Sicilian isolates is shown in Figure 3D. We looked at possible significant structural changes among the different NSs encoded by WT and RB strains with the protein prediction server (Rost 1996), but no obvious changes were noticed.

Transient expression of S RNA-encoded proteins through viral vectors and agroinfiltration.

A number of viral vectors for the expression of N and NSs proteins from WT and RB small genomic segments were derived from the *Tomato bushy stunt virus* (TBSV) vector TBSV-green fluorescent protein (GFP)/p19 (Qiu et al. 2002) and from a *Potato virus X* (PVX) expression vector, pP2C2S (Chapman et al. 1992) (Table 3). In *N. benthamiana* and *N. tabacum* (not shown), the N protein (WT or RB allelic form) could be expressed abundantly by both TBSV (Fig. 4A) and PVX (not shown) vectors, whereas NSs protein expression never could be detected from either TBSV- (Fig. 4B) or PVX- (not shown) derived vectors. Northern blot analysis with RNA extracted from plants infected with TBSV-derived vectors (Fig. 4C) showed that full-length genomic segments containing the NSs coding region always failed to accumulate, whereas full-length genomic segments encoding for N or GFP could be detected. Most of the time, in the NSs-expressing vector, viral replication was shut down altogether; sometimes, a much-delayed accumulation of viral vector without insert could be observed. In both *C. annuum* and *C. chinense* PI152225, N or NSs protein accumulation from both viral vectors could not be detected (not shown).

Fig. 2. Restriction fragment length polymorphism of the reverse-transcriptase polymerase chain reaction-amplified fragments of the large, medium, and small (L, M, and S, respectively) RNAs, used to determine the parental origin of the segments of one of the resistance-breaking (RB) reassortant isolates. **A**, S2 fragment digested by *TaqI* and S1 fragment digested with *HindIII*. **B**, Fragment M1 digested with *NcoI* and fragment M2 digested with *DdeI* and *RsaI*. **C**, Fragment L1 digested with *XbaI* and fragment L2 digested with *HindIII* and *BglII*. In all panels, lanes 1, 2, and 3 correspond to digestion products from RB parental strain p267^{RB}, reassortant strain IFA201^{RB}, and wild-type (WT) strain Br01^{WT}, respectively; M = 1-kbp DNA molecular weight marker (Gibco-Life Technology). Each panel shows that the reassortant isolate has the molecular markers of the M and L segment of the WT strain; whereas, for the S segment, the markers are those of the RB strain.

Table 3. List of recombinant viral vectors used in this study

Name ^a	Vector ^b	Insert ^c
pTG-P19/wt	TBSV	GFP
pTBSV -Br01 ^{WT} N3	TBSV	N WT
pTBSV -p267 ^{RB} N7	TBSV	N RB
pTBSV -Br01 ^{WT} NSs36	TBSV	NSs WT
pTBSV -p267 ^{RB} NSs4	TBSV	NSs RB
pP2C2S	PVX	–
pP2C2S-Br01 ^{WT} N14	PVX	N WT
pP2C2S-272 ^{RB} N8	PVX	N RB
pP2C2S-Br01 ^{WT} NSs10	PVX	NSs WT
pP2C2S-272 ^{RB} NSs31	PVX	NSs RB

^a WT = wild type and RB = resistance breaking.

^b TBSV = *Tomato bushy stunt virus* and PVX = *Potato virus X*.

^c GFP = green fluorescent protein, N = nucleocapsid protein, NSs = non-structural protein, and – = not infected.

We then proceeded to evaluate the possibility to transiently express NSs (Fig. 4D) and N (not shown) proteins through agroinfiltration. Both proteins in both allelic versions (WT and RB) accumulate abundantly in *N. benthamiana* and *N. tabacum*, confirming that NSs protein is not toxic in itself, but that its coding sequence likely interferes with virus vector replication. Agroinfiltration of pepper species with N- and NSs-expressing vectors failed to show any consistent accumulation of both proteins; in only a few instances, a limited accumulation could

be observed (not shown). A necrotic response was caused by agroinfiltrating the empty vector (pBin61) masking possible elicitation of necrosis by some of the allelic forms of the two TSWV proteins (data not shown).

Silencing suppression activity of NSs allelic forms from WT and RB strains.

Given the possibility to express both full-length NSs^{WT} and NSs^{RB} through agroinfiltration, we first wished to check on the

Fig. 3. Nonstructural protein amino acid sequence comparison among wild-type (WT) and resistance-braking (RB) *Tomato spotted wilt virus* strains. Identical amino acids are indicated by dashes; truncations by points. **A**, Compares p105^{WT} (the WT strain from the Italian Riviera) with closely related field or experimentally-derived RB strains. **B**, Compares a WT and an RB strain collected in the same field in Almeria (Spain). **C**, Compares a WT Brazilian isolate of our collection with an RB isolate directly derived from it by successive mechanical inoculations on *Tsw*-resistant *Capsicum annuum* in our greenhouses. **D**, Compares isolate p170^{WT} (from Sicily) with a field isolate from Sicily (p202^{RB}) and an isolate derived from p170^{WT} in the greenhouse (p170^{RB}) through serial inoculation on *C. chinense* PI152225.

ability of each of the two truncated NSs proteins to suppress silencing, a function previously shown to be performed by WT NSs (Bucher et al. 2003; Takeda et al. 2002). We checked GFP expression of agroinfiltrated 16C *N. benthamiana* plants visually under a UV lamp and we were able to ascertain that both

allelic versions from the WT and RB strains (NSs^{Br01} and NSs^{p272}) were able to equally suppress silencing when coinfiltrated with GFP (Fig. 5A). We then repeated the experiment, comparing a full-length NSs (from p272^{RB}) with the truncated forms derived from Sicilian isolates. GFP expression was ob-

Fig. 4. Western and Northern blot analysis of total extracts from *Nicotiana benthamiana* (Nb) plants infected with transcripts derived in vitro from the *Tomato bushy stunt virus* (TBSV) vector clones or agroinfiltrated with pBin-derived clones. **A**, Primary antibodies were against *Tomato spotted wilt virus* (TSWV) nucleocapsid (N) protein. **B**, Primary antibodies were against nonstructural (NSs) protein. In both panels, each lane is labeled with the exact name of the viral vector from which transcripts were derived in vitro, and used to inoculate *N. benthamiana* plants (compare with Table 3). Lanes labeled with Br01^{WT} and p267^{RB} are loaded with plant extracts infected with the corresponding TSWV strains, and are used as positive control reference; M = protein molecular weight marker. The arrows point to the position of the N and NSs protein bands. **C**, Northern blot hybridization of RNA extracts from *N. benthamiana* plants after inoculation with TBSV vector recombinant transcripts. The membrane was probed with a mix of full-length TSWV S segment probe and a green fluorescent protein probe. Lanes are labeled with the TBSV constructs used to derive transcripts for mechanical inoculation (compare with Table 3); the two lanes labeled with "TranscTBSV-NSs" and "TranscTBSV-N" are loaded with 20 μ l of a 1/1,000 dilution of the transcript reaction from two of the clones representative of an N- and NSs-expressing vector. Arrows point to the position of the full-length transcript. The transcript reaction of the TBSV vectors expressing NSs always also contained a smaller band, possibly from an internal region recognized by the T7 RNA polymerase promoter. **D**, Western blot analysis for NSs protein expressed through agroinfiltration in *N. benthamiana* (Nb) and *N. tabacum* (Nt). Lanes are labeled with the pBin61-derived vectors.

served with the full-length NSs from p272^{RB}, indicating that silencing had been suppressed; however, the clones supposed to express the NSs-truncated forms from p202^{RB} and p170^{RB} did not suppress silencing (Fig. 5B). Given the lack of suppression of silencing from the truncated forms, we checked for their accumulation in agroinfiltrated leaves by Western blot analysis; neither of the NSs-truncated forms could be detected in Western blots, whereas accumulation of NSs^{p272} was abundant (data not shown). We then wanted to check whether the truncated proteins themselves were present at all in virus infected plants. Results for leaf extracts of *D. stramonium* in-

fectected with the WT p170^{WT} and the two mutated RB strains p170^{RB} and p202^{RB} are shown in Figure 6A. A band of the expected size, of approximately 52 kDa, was observed for the control p170^{WT}. A strong band of the expected size of 44.8 kDa also was present in extracts of plants infected with p202^{RB}, but the p170^{RB} expected band of 26.3 kDa could not be observed, indicating possible instability or a limited capability to bind the primary NSs antibodies (a very faint band of similar size is present but also in the negative control). The expected N protein band was observed for all the three isolates (Fig. 6B).

Fig. 5. Green fluorescent protein (GFP) expression on agroinfiltrated 16C *Nicotiana benthamiana* plants, photographed under UV light 7 days post agroinfiltration. Each construct was co-agroinfiltrated with a GFP-expressing plasmid. **A**, Nonstructural protein (NSs) alleles from wild-type (WT) (Br01^{WT}), and resistance-breaking (RB) (p272^{RB}) strains are equally capable of suppressing silencing; one-half of a leaf was inoculated with the empty vector itself (pBin61) and the other half with vectors expressing NSs from an RB (NSs^{p272}) or WT (NSs^{Br01}) small *Tomato spotted wilt virus* segment cloned in pBin61. **B**, Lack of silencing suppression from truncated NSs forms derived from Sicilian strains: one half of a leaf was inoculated with a known suppressor of gene silencing (NSs^{p272}) and the other half leaf was agroinfiltrated with pBin61 or with derived plasmids with the potential to express the truncated form of the NSs protein from p202^{RB} and p170^{RB} strains, respectively.

DISCUSSION

Thus far, reassortment experiments have been the only tool available for TSWV, to link phenotypic features to molecular structures in individual genome segments (Hoffmann et al. 2001; Jahn et al. 2000; Qui and Moyer 1999; Qiu et al. 1998; Sin et al. 2005). In our work, reassortment experiments have shown that the S RNA is the carrier of the avirulence factor for the *Tsw* resistance gene, confirming a previous report of an RB strain (strain TSWV-A) from Hawaii (Jahn et al. 2000).

Strain TSWV-A was able to overcome resistance in both pepper and tomato. In contrast, none of our pepper RB isolates could infect tomato plants carrying the *Sw5* resistance gene (Roggero et al. 2002). However, it was not specified whether strain TSWV-A was isolated from a plant infected during an open field epidemic or upon a laboratory experiment. Furthermore, the whole S segment sequence was not published, likely because comparison with strain TSWV-D (used in the reassortment experiment) showed a number of differences in both ORFs, because the two strains are fairly distant phylogenetically (Tsompana et al. 2005). Thus, the significant mutations were not identified.

By contrast, in this study, we had the WT strains available from which the RB equivalents were derived, allowing us to closely compare related isolates. Thus, we can say that mutations in the 5' and 3' UTR, the N ORF, the N protein, and the intergenic region are not necessary for overcoming *Tsw* resistance, because a number of RB strain S segments have the same sequence as the related WT in these regions. In contrast, all the northern Italian, Spanish, and Brazilian RB strains contained one or two mutations in the NSs protein. Even deletions, resulting in truncated NSs proteins, were observed in NSs amino acid sequences from Sicilian RB isolates. Moreover, in three instances (amino acid positions 78, 84, and 437), the same amino acids were targeted by the selective pressure of the *Tsw* resistance gene in two distinct (and in one case phylogenetically distant) RB strains.

Several different mutations were distributed evenly through the RB NSs coding regions, and this may explain the relatively fast occurrence of RB strains in fields of resistant pepper hybrids. It also would suggest that the protein, when acting as an avirulence determinant, operates as a whole and not because of a single active domain. It seems unlikely that the multiple mutations we observed result from selective pressure of more than one resistance gene (or different alleles of *Tsw*) present in the host plant population, because all the RB strains used in this study were isolated in open fields on resistant *C. annuum* hybrids or cultivars that are all well characterized and uniform for presence of a close molecular marker of the *Tsw* resistance gene. All the RB strains obtained under greenhouse conditions also were isolated on well-characterized *C. annuum* resistant cultivars (with the exception of p170RB, which was isolated from *C. chinense* PI52225). Generally, viral avirulence determinants are characterized through studies of chimeras between only two viral infectious clones (WT and RB). This approach is perfectly appropriate for the identification of a putative avirulence factor, but it may misrepresent and simplify the population aspects of avirulence determinants. Our study addresses this aspect by using several RB isolates. Tsompana and associates (2005) characterized a series of amino acid changes in the NSs of TSWV isolates collected from a variety of host plants and from different geographic locations. None of those represented an RB isolate. We did not observe any of those reported mutations in our collection of isolates, reinforcing the hypothesis that the amino acid changes we observed are, indeed, the result of *Tsw* gene selective pressure.

The function of the NSs protein is not completely understood. It can aggregate as fibrous structures in the plant cell cytoplasm (Kormelink et al. 1991), it is expressed in infectious thrips (Wijkamp et al. 1993), and it recently has been shown to be a suppressor of post-transcriptional gene silencing (PTGS) (Bucher et al. 2003; Takeda et al. 2002). Gene-silencing suppressors are often also proteins that interact with the host and determine viral host range; for instance, as shown for p19 of TBSV (Turina et al. 2003). Thus, our finding that NSs is the putative avirulence determinant is not unique. We then could

Fig. 6. Western blot analysis for nonstructural (NSs) and nucleocapsid (N) proteins from *Datura stramonium* plants inoculated with Sicilian wild-type (WT) and resistance-breaking (RB) *Tomato spotted wilt virus* (TSWV) isolates. **A**, Primary antibodies against protein NSs. **B**, Primary antibodies against N protein. Lanes are labeled with the name of the TSWV isolate; H = healthy *D. stramonium* extracts. Total protein extracts of systemically infected symptomatic leaves were prepared as described in Materials and Methods, except that p170^{WT} and p202^{RB} extracts were diluted 1/10 in *D. stramonium* extract. **C**, Evaluation of total protein loadings through Coomassie staining of the gel. Arrows indicate the approximate position of NSs and N proteins; M = protein molecular weight marker.

demonstrate that lack of elicitation of resistance response from NSs of RB strains is not linked to loss of PTGS suppression capability (Fig. 5A), suggesting that NSs has at least two distinct functions, possibly with the involvement of distinct domains. Elicitation of resistance response and silencing suppression capability also were functionally and molecularly distinguished for TBSV p19 and *Cucumber mosaic virus* 2b proteins (Ji and Ding 2001; Lucy et al. 2000; Qiu et al. 2002; Turina et al. 2003). We then showed that a subset of RB strains did not express a full-length NSs protein (Fig. 6) and, indeed, one of the truncated form (NSs from p202^{RB}) could be detected from virus-infected leaves.

Even though the role of NSs in suppressing PTGS was previously demonstrated using the *Agrobacterium*-mediated transient suppression assay (Bucher et al. 2003; Takeda et al. 2002), here we show for the first time the in planta role of NSs in maintaining the infection in newly emerging leaves, preventing recovery while not affecting initial long-distance movement. The functional link between recovery and silencing is one of the earliest findings with direct relevance to virus biology, which was obtained through research on PTGS (Covey et al. 1997; Ratcliff et al. 1997). To our knowledge, though, it is the first time that mutation in a suppressor of gene silencing is directly linked to a recovery phenotype, because most knock outs of silencing suppressors also affect other aspects of the viral infection cycle (such as severity of symptoms, long- or short-distance movement, or specific virus–host interactions) (Moissiard and Voinnet 2004). Agroinfiltration of clones putatively expressing truncated forms of NSs failed to show suppression of gene silencing, but no conclusion on their PTGS suppression capability can be drawn because NSs from p202^{RB} or p170^{RB} could not be detected in the preparations.

Virus–thrips molecular interaction currently is under close scrutiny (Whitfield et al. 2005), and particularly interesting is the finding that TSWV activates the thrips vector immune system (Madeiros et al. 2004). Given that NSs protein localizes abundantly in salivary gland tissues, and a hypothetical role in vector transmission has been suggested (Goldbach and Peters 1996), our NSs-defective strains provide us with the unprecedented opportunity to study the possible involvement of the NSs protein in the virus–thrips interaction. Reinforcing the hypothesis that the NSs is involved in virus–thrips interaction is the recent finding that TSWV NSs can suppress silencing in tick cells (Garcia et al. 2006).

It is interesting that the recovery phenotype is predicted to decrease virus fitness, because thrips selectively feed in the flower, which would be virus-free. In fact, the two Sicilian RB strains encoding a truncated NSs protein that result in recovery were not isolated during a field epidemic episode; p202^{RB} was found in an isolated susceptible field plant and p170^{RB} was isolated in the greenhouse following mechanical inoculation of resistant plants. On the contrary, all the isolates from field epidemics encoded for a full-length NSs protein, able to suppress gene silencing.

We have not excluded the possibility that the avirulence factor could be the RNA sequence of the NSs ORF. Studies on *Cymbidium ringspot virus* (Szittyá and Burgan 2001) and *Melon necrotic spot virus* (Diaz et al. 2004) point to involvement of a viral RNA sequence in host resistance breaking. However, it must be noted that, in all the RB strains, at least one amino acid mutation was present, suggesting a role of the protein itself.

The symptoms induced by RB strains on resistant *C. chinense* suggest that the TSWV–*Tsw* interaction involves cell-to-cell movement, as suggested previously (Soler et al. 1999). In fact, even though most of the mutant RB strains did not cause necrotic local lesions on resistant *C. chinense*, the RB

strain p166 retained this behavior and also caused necrotic lesions on the upper noninoculated leaves, indicating that local hypersensitivity at the start of infection is not a sufficient cause for resistance, as generally accepted and previously reported for this specific virus–host interaction (Moury et al. 1997). Precedents for separation between the hypersensitive response-like response and resistance response are present in the literature of the last decade (Bendahmane et al. 1999; Clough et al. 2000; Ehrenfeld et al. 2005); particularly relevant for plant viruses is the case study of *Cauliflower mosaic virus* infection of *N. edwardsonii*, where uncoupling of cell death from resistance also was demonstrated recently (Cawly et al. 2005).

Traditionally, a further confirmation that a certain protein is an avirulence determinant comes from studies of its transient expression through viral vectors or agroinfiltration (limiting the latter technique to elicitation of necrotic response, which, as discussed in the previous paragraph, is not always directly linked to resistance). In our case, efforts were inconclusive: on one side, viral vectors could not be used because NSs could not be expressed even in hosts where the viral vectors considered abundantly replicate and move; on the other side, agroinfiltration of the putative avirulence determinant on pepper failed to be consistent and gave an aspecific necrotic response (elicited by the empty vector itself) which masked a possible specific response.

MATERIALS AND METHODS

TSWV isolates and their maintenance.

The WT and related RB isolates we used are shown in Table 1. They consisted of five groups: from Brazil, the Italian Riviera, Sicily, Spain, and the Istituto di Virologia Vegetale (IVV) collection. The Brazilian isolate Br01^{WT} was used as type member of the genus and as the reference WT in the reassortment experiments. The first group included four field isolates (p105^{WT}, p166^{RB}, p267^{RB}, and p272^{RB}) collected from commercial pepper cultivars carrying the *Tsw* gene grown in the same location in the Liguria region (northern Italy) (Roggero et al. 1999, 2002). The second group included four RB strains (p105^{RB}, p105/1^{RB}, p105/44.7^{RB}, and p105/43.14^{RB}) isolated from the WT strain p105^{WT} at the IVV during the final evaluation steps of some breeding work carried out to introgress the *Tsw* gene in some local *Capsicum* cultivars under greenhouse conditions. Two Spanish field isolates (Ve430^{WT} and Ve427^{RB}) collected in the same resistant *C. annuum* field in Almería (Margaria et al. 2004) constituted the third group. Sicilian isolates constituted the fourth group: p170^{WT} and p202^{RB} were open-field isolates, whereas we derived the RB strain p170^{RB} from successive mechanical inoculations on resistant *C. chinense* during maintenance of p170^{WT} in glasshouse condition. We recently obtained the last strain for our study (Br20^{RB}) by successive mechanical inoculations on *Tsw*-resistant *C. annuum* of Br20^{WT}, a strain present in our collection since 1995.

All isolates were passaged three times on *N. tabacum* through single lesions to avoid possible mixed infection and, subsequently, were multiplied systemically in *N. benthamiana*, *E. sonchifolia*, or both in a greenhouse at 22 to 25°C. Tissue from these plants was frozen and stored in liquid nitrogen until further use. We determined the experimental host range for a number of the RB isolates on *C. annuum*, *N. benthamiana*, *N. tabacum*, *N. glutinosa*, *Lycopersicon esculentum*, *O. basilicum*, and *E. sonchifolia* as described by Roggero and associates (2002).

Generation of reassortant isolates.

Reassortant isolates were obtained by coinoculation of parental isolates onto *N. benthamiana*. Br01^{WT} was selected as

Table 4. Restriction enzymes^a

Amplified fragment	Restriction enzyme
S1	<i>HindIII</i> , <i>ClaI</i> , <i>EcoRI</i>
S2	<i>TaqI</i>
M1	<i>NcoI</i>
M2	<i>DdeI</i> , <i>RsaI</i>
L1	<i>XbaI</i> , <i>HpaI</i>
L2 (p267 ^{RB})	<i>HindIII</i> , <i>BglII</i>
L2 (Ve427 ^{RB})	<i>ScaI</i>

^a Enzymes used for restriction fragment length polymorphism analysis of DNA fragments derived from reverse-transcriptase polymerase chain reaction of large, medium, and small (L, M, and S, respectively) RNAs, in order to determine the parental origin of the genomic segments of resistance-breaking (RB) reassortant isolates.

Table 5. Primers used for small RNA reverse-transcriptase polymerase chain reaction amplification and sequencing

Primer	5' to 3' Sequence
p272-1F	agagcaattggccataattttaattc
p105-1100F	tgatgttcataatccc
p105-1470R	catgcttcaggatcaa
Ve427-3000R	agagcaattgtgtcaattttattcaaa
p105-1F	agagcaattgtgtcataattt
Nc-2750R	ggctcgagacacatgtctaagggttaagctc
Nc2000-F	gggagctcttttaagcaagttctg

the WT parent because it was biologically distinct from the other isolates and its genome sequence was sufficiently different from the RB strains to allow for restriction fragment length polymorphism (RFLP) analysis. Regarding the RB parents, p267^{RB} was chosen to represent the Italian RB strains collected in the field; p105/43.14^{RB} to represent the Italian RB strains obtained experimentally in glasshouse conditions, and Ve427^{RB} to embody the Spanish RB parental isolate. Three different ratios of inoculum (1:1, 5:1, and 10:1) of Br01^{WT} and the RB strain in question were mixed and coinoculated.

To select putative reassortants, systemically infected leaves of *N. benthamiana* from each of the different ratios and combinations of isolates were used to inoculate resistant *C. chinense* PI152225. We used systemically infected *C. chinense* tissue serially diluted to inoculate *N. tabacum* in order to obtain single local lesions. Ten single lesions were back-inoculated to *C. chinense*. Of those single lesion isolates able to systemically infect *C. chinense*, we determined the parental origin of the genome segments using the RFLP technique following the protocols described by Qiu and associates (1998). Representative reassortant strains also were passaged three times on *N. tabacum* to ensure clonality of each isolate.

Molecular marker analysis.

For each genome segment, two regions were amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) as suggested by Qiu and associates (1998), using the oligonucleotides described in the same study, in order to obtain two markers for each genome segment, distant enough to check for internal recombination among homologous segments. In order to select restriction enzymes to be used for the RFLP analysis, we compared the complete genome sequence of Br01^{WT} (GenBank accession numbers NC_002050, NC_002051, and NC_002052) (de Haan et al. 1990, 1991; Kormelink et al. 1992) with that of p267^{RB}, selected as representing the Italian isolates and Ve427^{RB}, representing the Spanish isolates. Restriction enzymes for the Italian and Spanish isolates, which enabled us to analyze the reassortants, are shown in Table 4. Restriction fragments were visualized on a 1.2% agarose gel after staining with ethidium bromide.

Cloning and sequencing of the S RNA.

Total RNA was extracted from infected leaves of *N. benthamiana* as described previously (Turina et al. 1998), after three single-lesion passages on *N. tabacum* and a final inoculation on Tsw-resistant *C. annuum* cv. Explorer. Two RT-PCR reactions for synthesis of first-strand cDNA were performed with the "Thermoscript RT-PCR System" (Invitrogen): one reaction employed random primers and the other used the specific reverse primer (Table 5) following the manufacturer's protocol. PCR amplifications were performed by placing 1 µl of cDNA in 50 µl of final reaction solution. Amplification consisted of one cycle at 94°C for 5 min; 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 52°C, and 90 s of extension at 72°C; followed by one cycle of final extension for 10 min at 72°C. As such, two overlapping products of 1.4 and 1.9 kbp from each isolate were obtained to derive the entire S segment nucleotide sequence.

PCR-amplified viral cDNAs were cloned into pGem-T VECTOR (Promega) and competent *Escherichia coli* (D5α strain) was transformed by standard molecular procedures. Recombinant clones were identified by blue-white screening and verified with PCR using the universal primers M13 f/r. Three separate cloning experiments for p105^{WT} and two for the others TSWV strains were performed in order to minimize potential sequence errors introduced by *Taq* polymerase. In the case of the RB strains, the consensus sequence was obtained from comparing the clones of each of their S segments with the corresponding WT strain, considering true only the conserved mutations in the RB sequences through the two cloning events. Each consensus sequence was deposited in GenBank (see accession numbers below).

Selected clones were sequenced from both ends with the universal primers M13 f/r and with internal specific deoxy-oligonucleotides (TSWV-500-F: 5'-catgaacagatctgatat-3'; TSWV-2263R: 5'-tatatcaggatgcaaaa-3'). Sequence data were compiled using Chromas Lite (version 2.0; Technelysium). The BLAST program was used to compare related sequences available from GenBank database. Multiple alignments of the obtained sequences were done using ClustalW (Thompson et al. 1994).

Recombinant TBSV and PVX vectors for the expression of S RNA-encoded proteins.

TBSV- and PVX-derived clones are schematically represented in Table 3. TBSV clones were derived from digestion of clone pTG-p19/wt (Qiu et al. 2002) with *XhoI* and *SacI* restriction endonucleases to release the GFP insert to be replaced with PCR products corresponding to N and NSs coding region of the two allelic forms (WT and RB), using oligonucleotides described in Table 6. Each clone was sequenced along the whole insert. PVX vector-derived clones were obtained upon digesting the pP2C2S vector (Chapman et al. 1992) with *EcoRV* and *SalI* and inserting the N and NSs coding region amplified by PCR with the oligonucleotides followed by restriction enzyme digestion, as indicated in Table 6. Transcripts were prepared and inoculated on plants as previously described (Turina et al. 1998).

Agroinfiltration and Northern and Western blot analysis.

Agrobacterium tumefaciens competent cells (strain C58C1) were transformed with recombinant plasmids derived from the expression vector pBin61 (Bendahmane et al. 2002; Hoefgen and Willmitzer 1988). First, the full-length S segments were obtained by RT-PCR using the oligonucleotide and reaction conditions previously described (Cortez et al. 2001), exploiting the *Bam*HI site present in pBin61. These were used to obtain clones in both orientations for the S segment of the Br01^{WT} and p272^{RB} isolates. The obtained clones were named pBin61-Br01N,

Table 6. Primers used for polymerase chain reaction amplification of DNA fragments to be cloned into the two viral vectors

Fragment ^a	Vector	Oligonucleotide name	5' to 3' Sequence ^b
N	TBSV	TSWV-N2647- <i>Xho</i> I	ggctcgag acacatgtctaaggttaagctc
		TSWV-N2071- <i>Sac</i> I	gggagctct tttaagcaagttctg
N	pP2C2S	TSWV-N2647- <i>Eco</i> RV-F	gggata tcatcatgtctaagggtta
		TSWV-N2071- <i>Sal</i> I-R	agggtcg actttaagcaagttctgcga
NSs	TBSV	TSWV-Ns-89- <i>Xho</i> I	ggctcgag acacatgtcttcaagtggtt
		TSWV-Ns-1492- <i>Sac</i> I	gggagctct tattttgatcctgaagca
NSs	pP2C2S	TSWV-Ns-89- <i>Eco</i> RV	gggata tcttattttgatcctgaagca
		TSWV-Ns-1492- <i>Sal</i> I	gggtcg acacacatgtcttcaagtggtt

^a N = nucleocapsid protein and NSs = nonstructural protein.

^b Restriction site sequences are indicated with bold type.

pBin61-p272N, pBin61-Br01NSs, and pBin61-p272NSs for the expression of N^{WT}, N^{RB}, NSs^{WT}, and NSs^{RB}, respectively.

To clone the NSs-coding region of strains p272^{RB}, p170^{RB}, and p202^{RB}, respectively, the following primers were used that contain the *Bam*HI restriction site (NSs89F: 5'-ggggatccacacatgtcttcaagtggtt-3'; NSs1492Rev: 5'-ggggatccttattttgatcctgaagca-3'). Each inserted fragment was sequenced. *N. benthamiana* plants were agroinfiltrated as previously reported (Bendahmane et al. 1999). Leaf extracts of virus infected or agroinfiltrated plants were prepared for protein separation and Western blot analysis as previously described (Turina et al. 1998) using 50 mg of tissue. Anti-NSs antibodies (Kormelink et al. 1991) and anti-N antibodies (Roggero et al. 2002) were used as primary antibodies at 1:1000 dilution. Bound antibodies were detected by anti-rabbit immunoglobulin G conjugated with horseradish peroxidase prepared in goat (Sigma), using the Supersignal West Pico Chemiluminescent Substrate (Pierce). Equivalent loading of total proteins in each sample was checked by Coomassie staining of gels in each experiment (Fig. 6C). Total nucleic acids from virus-infected plants were extracted, separated, and transferred to nylon membranes as previously detailed (Turina et al. 1998). Northern hybridization was carried out with digoxigenin-labeled DNA prepared as detailed in the protocols from the manufacturer (ROCHE Diagnostics) following hybridization and detection protocols detailed by the same manufacturer.

Experiments to evaluate silencing suppression capabilities were performed through coagroinfiltration of pBin-GFP and each of the NSs-expressing clones described previously on 16C transgenic *N. benthamiana* (Voinnet et al., 1998). For this purpose, a DNA fragment was amplified by PCR from the viral vector pTG-P19/wt using oligonucleotides GFP-*Eco*RV-F (5'-aaagatatctccggccgatgagtaaaggag-3') and GFP-*Eco*RV-R (5'-ggatatctatccggattgtatagtt-3'). The product was inserted into the *Sma*I site of pBin61 and sequenced with oligo pBinF (5'-tgatgtgacatctcactg-3') to check for its right orientation. GFP expression was evaluated visually with a UV lamp (Blank Ray, Model B 100 AP).

Nucleotide sequence accession numbers.

Consensus sequences of the entire S segment of the different WT and RB strains were deposited in GenBank with the following accession numbers: DQ376177 to DQ376185 correspond to strain p105/1^{RB}, p105^{WT}, p166^{RB}, p267^{RB}, p272^{RB}, p105/43.14^{RB}, p105/44.7^{RB}, Ve430^{WT}, and Ve427^{RB}, respectively; DQ398945 corresponds to strain p202^{RB}; DQ431237 and DQ431238 correspond to strain p170^{WT} and p170^{RB}, respectively; and DQ915946 to DQ915948 correspond to p105^{RB}, Br20^{RB}, and Br20^{WT}, respectively.

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