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Characterization of a vector-non-transmissible isolate of *Tomato spotted wilt virus*

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The characteristics of a thrips-non-transmissible isolate of *Tomato spotted wilt virus* (TSWV), designated TSWV-M, were compared with those of a thrips-transmissible isolate, designated TSWV-T. TSWV-M showed a narrower host range than TSWV-T. Adult thrips failed to transmit TSWV-M, although the vector acquired the virus during the larval stages. TSWV-M was detected by RT-PCR in adult thrips bodies, but not in thrips heads, suggesting that loss of thrips transmissibility was the result of the absence of virus in adult thrips salivary glands. Whereas N (nucleoprotein), NSs (non-structural protein) and G_C (the C-terminal portion of the glycoprotein precursor protein) were present in similar amounts in leaf tissue from TSWV-M- or TSWV-T-infected plants, G_N (the N-terminal portion of the glycoprotein precursor protein) was present at much lower amounts in TSWV-M- than in TSWV-T-infected plants. SDS-PAGE and immunoblotting analysis of TSWV-M and TSWV-T virion preparations with G_N- and G_C-specific antibodies revealed similar amounts of the G_N and G_C glycoproteins in TSWV-T virions, but lower amounts of G_N than G_C in TSWV-M virions. This resulted in a statistically significant reduction in the G_N/G_C ratio in TSWV-M virions. In affinoblots, the G_C and G_N glycoproteins of TSWV-M exhibited weak binding with lectins showing affinity for N-linked oligosaccharide structures. Sequence analysis of M RNA (medium segment of the TSMV genome) revealed no deletions or frameshift mutations in the G_N/G_C precursor of TSWV-M. However, five amino acid changes were detected in the G_N/G_C precursor. A single, relatively conservative amino acid substitution (V→I) was observed in the NSm protein. Sequence analysis of S RNA (small portion of the TSMV genome) revealed a large intergenic region with no changes in the N protein and with three amino acid changes in the NSs protein.

Keywords: *Bunyaviridae*, *Frankliniella occidentalis*, glycoproteins, oligosaccharides, RT-PCR, thrips vector transmission, *Tospovirus*

Introduction

The members of the genus *Tospovirus* in the family *Bunyaviridae* are spread from plant to plant by thrips (Thysanoptera: Thripidae) (Sherwood *et al*., 2000; Whitfield *et al*., 2005). The circulative-propagative mode of transmission of tospoviruses, however, is unique among plant viruses in that adult thrips can successfully transmit the virus only when the virus is acquired at the larval stage of the thrip's life cycle (van de Wetering *et al*., 1996). This interdependency between vector life-stage and virus transmission involves multiple infection and dissemination barriers related to the metamorphosis of thrips vectors (Nagata *et al*., 1999; Kritzman *et al*., 2002).

Tomato spotted wilt virus (TSWV), the type member of the genus *Tospovirus*, is by far the most economically important of all the characterized tospoviruses, with a worldwide distribution and a broad host range (Parrella *et al*., 2003). The pleomorphic particles of TSWV contain a tripartite genome (Goldbach & Peters, 1996; Whitfield *et al*., 2005) consisting of three negative sense single-strand RNA segments designated large (L), medium (M) and small (S) RNA. The complementary strand of the negative sense L RNA segment encodes the viral RNA-dependent RNA polymerase. The M and S RNA segments each encode two proteins in an ambisense arrangement. The M RNA codes the precursor to the envelope membrane glycoproteins in the viral complementary (vc) sense and the viral movement protein (NSm) in the viral (v) sense. The precursor of the glycoproteins is probably cleaved co-translationally, presumably by cellular signal peptidase (Goldbach & Peters, 1996; Whitfield *et al*., 2005), to generate two mature glycoproteins termed G_N (the N-terminal portion of the glycoprotein precursor protein) and G_C (the C-terminal portion of the glycoprotein precursor

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protein). The S RNA codes for the nucleoprotein (N) in vc sense and a non-structural protein (NSs) in v sense.

The three genomic RNAs are individually encapsidated by many copies of the N protein and are surrounded by a host-derived lipid envelope in which G_N and G_C are integrated. The two glycoproteins are seen as spike-like projections covering the surface of the virus particle. Recent studies have shown that the glycoproteins in TSWV virions are modified by N-glycosylation. The G_C protein is N-glycosylated with high-mannose- and complex-type oligosaccharides, whereas the G_N protein is glycosylated with high-mannose-type oligosaccharides (Naidu *et al*., 2004). The G_N protein was also reported to be O-glycosylated (Whitfield *et al*., 2004).

As with some other viruses, TSWV loses vector transmissibility when maintained through serial mechanical transmissions under greenhouse conditions. Previous studies demonstrated that serial manual inoculations resulted in two distinct classes of TSWV mutants (Resende *et al*., 1991). One class of mutants, called envelope-deficient mutants, was characterized by the inability to produce enveloped virus particles as a result of accumulation of point mutations or small deletions in the gene encoding the precursor glycoprotein (GP). The envelope-deficient isolates failed to infect thrips midguts after ingestion by larva and hence could not be transmitted by adult vector thrips (Nagata *et al*., 2000). Recent studies using a reassortment-based viral genetic system also identified specific mutations in the precursor-GP-coding region associated with the loss of thrips transmissibility of TSWV (Sin *et al*., 2005). These studies clearly indicate that vector-transmissibility maps to the M RNA and that the envelope-deficient isolates multiply in host plants just as do vector-transmissible isolates. Thus, TSWV GPs are generally considered dispensable for virus replication in plants, but are essential for acquisition and transmission by thrips. The second class of TSWV mutants is characterized by the presence of defective interfering (DI) L RNA, because of the accumulation of truncated L RNA (Resende *et al*., 1992; Inoue-Nagata *et al*., 1998). These isolates were characterized by symptom attenuation by DI L RNA and, although the ingested viruses resulted in limited midgut infections, lack of thrips transmission was explained by the low number of infectious particles in the inoculum ingested by thrips from infected plants (Nagata *et al*., 2000).

This study reports the biological and molecular characterization of a thrips-non-transmissible isolate of TSWV, designated TSWV-M, that differs from thrips-non-transmissible isolates described earlier (Resende *et al*., 1992; Nagata *et al*., 2000; Sin *et al*., 2005), and its comparison to a thrips-transmissible isolate of TSWV, designated TSWV-T.

Materials and methods

Virus isolates

TSWV-T and -M were obtained as single-lesion isolates on *Petunia hybrida* by mechanical inoculation with extracts from peanut (= groundnut, *Arachis hypogaea*) naturally infected with TSWV in the greenhouse. The two isolates were subsequently maintained on *Emilia sonchifolia*. Initial studies showed that TSWV-T, but not TSWV-M, was transmissible by western flower thrips (*Frankliniella occidentalis*). Hence, TSWV-T was maintained by thrips transmission at regular intervals and TSWV-M was maintained by vegetative propagation of stem cuttings from infected plants without recourse to mechanical inoculations. *Emilia sonchifolia* was chosen to maintain these isolates because previous studies showed that TSWV isolates can be maintained free of DI RNAs in this host (Inoue-Nagata *et al*., 1997).

Virus purification and host-range studies

Young seedlings of *E. sonchifolia* were mechanically inoculated separately with TSWV-T and -M isolates and virus was isolated from systemic leaves showing an initial flush of symptoms (Mohamed *et al*., 1973). For host-range studies, the following plant species were mechanically inoculated with each isolate: *Datura stramonium*, *P. hybrida*, *Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum* cv. Samsun NN and Xanthi NC, cowpea (*Vigna unguiculata*) cv. Early Ramshorn, cucumber (*Cucumis sativus*) and French bean (*Phaseolus vulgaris*). Inoculated plants were maintained in environmentally controlled growth chambers at 25 ± 1°C with 16-h photoperiod for symptom development. All plants were tested for the virus by antigen-coated plate (ACP) ELISA (Converse & Martin, 1990) using monoclonal antibody (MAb) against the NSs protein of TSWV (Bandla *et al*., 1994).

Enzyme-linked immunosorbent assay (ELISA)

The relative amounts of virus in *E. sonchifolia* plants infected with TSWV-M and TSWV-T were determined by ACP-ELISA using monoclonal antibodies (MAbs) to N, NSs, G_N and G_C proteins. Five leaf discs of 1 cm diameter were randomly punched from comparable leaves of each plant and triturated in phosphate-buffered saline (PBS) containing 0·1 M diethyl dithiocarbamic acid in a ratio of 1:1000 (w/v) to minimize interference from host materials and to avoid saturation of the antigen-antibody reaction. Equal volume of plant extracts and coating buffer (2×) were mixed and wells of Nunc-Immuno™ 96 MicroWell™ plates were coated with 100 μL of the sample. The plates were incubated overnight at 4°C and after appropriate washings, the viral antigen was detected using ACP-ELISA (Converse & Martin, 1990). Purified IgGs of MAbs to G_N and N were used at 0·5 μg mL^{-1}, except anti-NSs, which was used at a concentration of 0·1 μg mL^{-1}. For the detection of G_C, culture supernatant was used at 1:2000 dilution. Each assay was repeated four independent times using samples from different plants. Each replication consisted of six plants and six leaf discs per plant were randomly collected and pooled for extraction. Plants mock-inoculated with buffer served as controls.

Thrips rearing and virus transmission

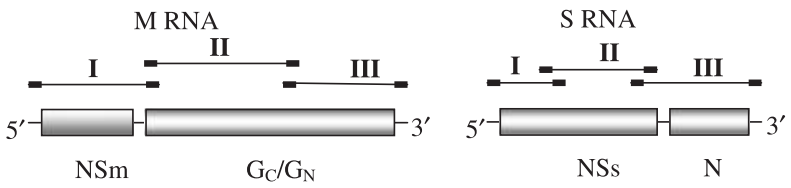
A colony of western flower thrips (WFT) reared on green bean pods in the laboratory (22 ± 2°C under constant fluorescent lights) was used for vector transmission studies as described earlier (Naidu *et al*., 2005). Synchronous-aged, 4-h-old first-instar larvae were given a 24-h acquisition-access period (AAP) by feeding on TSWV-M- or TSWV-T-infected *E. sonchifolia* leaves showing an initial flush of symptoms. After the acquisition access, the larvae were transferred from leaves with symptoms to green bean pods and allowed to pupate and complete their development to the adult stage. As a control, larvae were given acquisition access to non-infected leaves and subsequently the virus-free insects were treated in the same manner as those fed on virus-infected leaves. Transmission of virus by viruliferous adult thrips was evaluated using a modified leaf disc assay (Wijkamp & Peters, 1993). Thrips, either male or female, were given a 48-h inoculation access by placing individual thrips on *E. sonchifolia* leaf discs (1 cm diameter) floating on water in separate wells in 24-well Low Cell Binding Nunc Plates. Each well was sealed with Parafilm™ and the plate was covered with a lid and kept at 25 ± 1°C. After a 24-h inoculation-access period (IAP), each leaf disc was washed and floated on water in individual wells in a 24-well plate and incubated at 25 ± 1°C for 1 week in a growth chamber at 25 ± 1°C with a 16-h photoperiod. Since the inoculated leaf discs of *E. sonchifolia* did not show visible symptoms, infection of these discs, indicative of transmission, was monitored by ACP-ELISA using antibodies specific to the NSs protein as described above.

RT-PCR detection of virus in thrips and plants

Thrips were cut between the head and abdomen, as described by Ullman *et al*. (1990), with a surgical scalpel blade such that the salivary glands remained within the dissected head section of the insect and the abdomen section contained the entire alimentary canal, fat body and all other organs present within the posterior end of the insect. The head and abdomen parts from 100 viruliferous adult thrips were pooled separately and RNA extracted using an RNeasy Plant Minikit (Qiagen Sciences). The head and abdomen sections from an equal number of non-viruliferous adult thrips were included as negative controls. Total RNA was also extracted from healthy and virus-infected *E. sonchifolia* leaves on which thrips larvae were fed for virus acquisition. Total RNA (1 μg) extracted from thrips and plants was used for RT-PCR detection of virus using oligonucleotide primers specific to S-RNA (reverse primer: 5′-AGAGCAATCGTGTCAATTTTGT-GTTC-3′, complementary to nt 2891–2916; and forward primer: 5′-TTACAGCTGCTTTCAAGCAAGTTCTG-3′, identical to nts 1975-2000).

Amplification, cloning and sequence analysis of S and M RNA segments

Total RNA from virus-infected leaves of *E. sonchifolia* was isolated using an RNeasy Plant Minikit. RT-PCR was used to amplify overlapping genomic regions of M and S RNA of TSWV-T and -M isolates using oligonucleotide primers chosen from the published sequence of the TSWV-BR-01 isolate (NC_002050, NC_002051). The location of primer pairs and their sequences used to amplify overlapping segments of M and S RNA are listed in Table 1. The PCR products were gel-purified and cloned into pCR®-blunt II-TOPO® (Invitrogen). The plasmid DNA was isolated from positive colonies and two independent clones were sequenced from both ends. Wherever necessary, direct sequencing of RT-PCR products was carried out to resolve sequence ambiguity.



Primer pair	Nucleotide sequence	Location
M RNA:		
I	Sense: 5′-AGAGCAATCAGTGCATCAGAAATATACC-3′	1–28
	Antisense: 5′-GATACTATCAGAGTGGTACTGCTTATTG-3′	1521–1548
II	Sense: 5′-CTTCAGACAAGGTGAGAGAAATC-3′	1328–1350
	Antisense: 5′-ATGGCTCAAACAACCCAATGTTTG-3′	3331–3354
III	Sense: 5′-GGACAGCCTGGAACATAAAAGCATCC-3′	3302–3327
	Antisense: 5′-AGAGCAATCAGTGCAAACAAAAACC-3′	4797–4821
S RNA:		
I	Sense: 5′-AGAGCAATTGTGTCAGAATTTTGTTC-3′	1–26
	Antisense: 5′-AGCTGGAATCGGTCTGTAATATCAG-3′	489–513
II	Sense: 5′-TTGATAACTCTGTTCTGGCACTATC-3′	360–384
	Antisense: 5′-TTATTTTGATCCTGAAGCATACG-3′	1464–1483
III	Sense: 5′-CTTTGCCAGAGGAGAAATTAAAATTTC-3′	1351–1377
	Antisense: 5′-AGAGCAATCGTGTCAATTTTGTGTTC-3′	2891–2916

Table 1 Map of M and S RNA and the location of primers used for RT-PCR amplification of specific regions of M and S RNA genome segments of thrips-transmissible and -non-transmissible *Tomato spotted wilt virus* (TSWV-T and TSWV-M, respectively) isolates. The proteins encoded by the two RNA segments are indicated below the diagram. Primer locations are based on DNA sequence data for M RNA (accession no. NC_002050) and S RNA (accession no. NC_002051) available in GenBank. The nucleotide sequences of sense and antisense primers and their locations in M and S RNA genome segments are listed

The nucleotide sequences were edited, assembled and analysed using DNASIS (Hitachi). Multiple alignments of nucleotide sequences and predicted amino acid sequences of the products encoded by different open reading frames (ORFs) were obtained using CLUSTAL W from Network Protein Sequence Analysis (NPSA, http://npsa-pbil.ibcp.fr) with suggested default settings.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and affinoblotting

Proteins from purified viral preparations of TSWV-T and -M were resolved by SDS-PAGE. The resolved protein bands were stained with Coomassie Brilliant Blue R-250, and analysed by Western blotting using G_N- and G_C-specific monoclonal antibodies and affinoblotting using two biotinylated lectins, *Lens culinaris* agglutinin (LCA) and *Solanum tuberosum* lectin (STL) (Vector Laboratories, Inc.) as described earlier (Naidu *et al*., 2004).

The SDS-PAGE and Western blot images were processed using ADOBE PHOTOSHOP (version 7·0, Adobe Systems Inc.) and the densities of G_C and G_N bands were determined using IMAGEQUANT software (version 5·2, Molecular Dynamics). The average density values for G_C and G_N bands from four independent images were used to calculate G_N/G_C ratios for TSWV-M and -T. Statistical analysis was performed on the average values of G_N/G_C ratios for TSWV-T and -M from SDS-PAGE gels and Western blots by one-way analysis of variance (ANOVA) using PROC ANOVA in SAS (version 9·1, SAS Institute). Fisher's protected least significant difference (LSD) test was used to determine differences between G_N/G_C ratio means of TWSV-M and -T.

Transmission electron microscopy (TEM)

Formvar-carbon-coated grids were floated with the membrane side down on droplets of purified virus preparations for 5 min and then transferred to another droplet of 2% glutaraldehyde. Grids were washed with 15 droplets of water and stained with phosphotungstic acid. Grids were examined under a Philips CM12 electron microscope.

Results

Host range of TSWV-M

The symptoms produced by TSWV-M in *E. sonchifolia* were compared with those produced by TSWV-T. TSWV-T produced extensive veinal chlorosis throughout the newly developing leaves and with time, the chlorosis extended into the interveinal areas, leading to a generalized chlorosis of the entire leaf (Fig. 1a–c). Subsequently developing leaves, however, showed large diffuse chlorotic blotches and severe mosaic mottling. In the case of TSWV-M, infected plants showed only chlorotic spots in the newly developing leaves. Neither veinal chlorosis nor mosaic mottling symptoms were observed in subsequently developing leaves. Thus, TSWV-M produced mild symptoms in *E. sonchifolia* when compared to TSWV-T.

To determine if TSWV-M showed any differences in symptoms produced in other host species, a set of plant species susceptible to TSWV were inoculated with TSWV-M

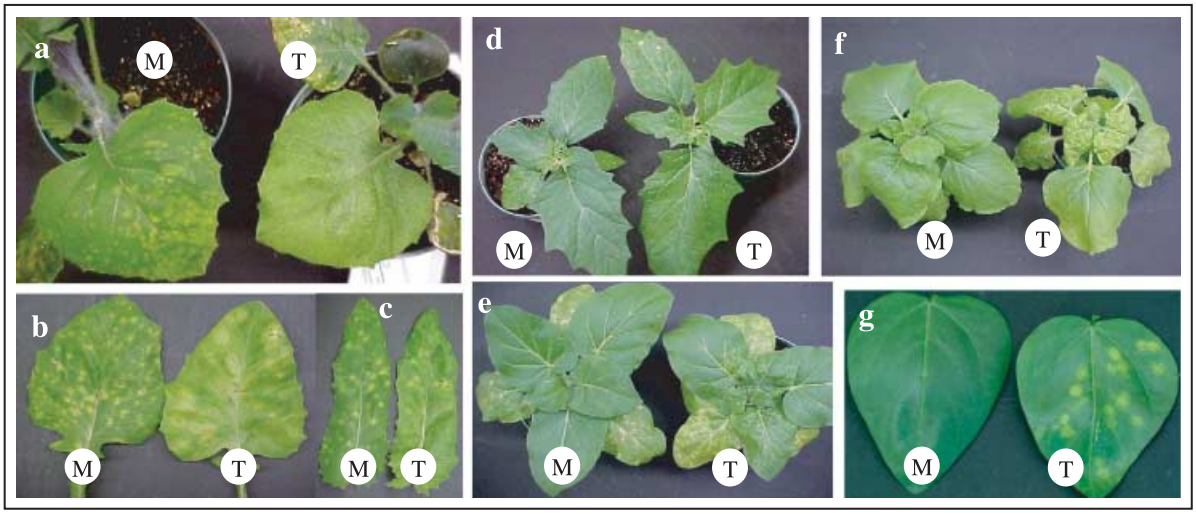


Figure 1 A comparison of systemic symptoms induced by thrips-non-transmissible and thrips-transmissible *Tomato spotted wilt virus* (TSWV-M, labelled M; and TSWV-T, labelled T; respectively). In *Emilia sonchifolia* (a–c) the first flush of symptoms induced by TSWV-M were mild and consisted of chlorotic spots, whereas TSWV-T produced severe symptoms consisting of vein clearing, large diffuse chlorotic spots and severe mottling of leaves. TSWV-M produced chlorotic spots on inoculated leaves of *Datura stramonium* (d) and necrotic lesions on inoculated leaves of *Nicotiana glutinosa* (e). In contrast, TSWV-T infected plants showed both local and systemic symptoms. TSWV-M failed to show infection in *Nicotiana benthamiana* (f) and cowpea (g), whereas TSWV-T produced systemic symptoms in *N. benthamiana* and chlorotic spots on inoculated leaves in cowpea.

and TSWV-T and monitored at regular intervals for symptoms both on inoculated and newly developing leaves. Although both viruses induced necrotic lesions on inoculated leaves of *P. hybrida*, they showed distinct differences in symptoms produced in other host plants (Fig. 1d–g). In *N. glutinosa*, only TSWV-T produced systemic mosaic followed by necrosis, whereas TSWV-M produced only necrotic lesions on inoculated leaves. In *D. stramonium*, both isolates produced chlorotic spots on inoculated leaves, but only TSWV-T showed mosaic mottling on newly developing leaves. In contrast to TSWV-T, TSWV-M failed to produce symptoms in *N. benthamiana*, *N. tabacum* NN, French bean, cucumber and cowpea. To ascertain if the lack of symptoms in TSWV-M-inoculated plants was the result of attenuation or inability of the virus to initiate infection in these host plants, both inoculated and newly developing leaves were tested by ACP-ELISA using antibodies specific for NSs protein. Since NSs protein is indicative of virus replication, absence of NSs protein in leaves would indicate lack of virus replication. All plants that did not show symptoms tested negative in ACP-ELISA. The ELISA results were further confirmed by RT-PCR amplification of the NSs gene using gene-specific primers (data not shown). These results indicated that for plant species commonly infected by TSWV isolates, TSWV-M has a narrower host range than TSWV-T.

Structural and non-structural proteins of TSWV-M in infected plants

To determine if the mild symptoms induced by TSWV-M were the result of a low titre of virus, *E. sonchifolia* leaves showing an initial flush of symptoms were tested for viral proteins by ACP-ELISA using MAbs specific to N, NSs, G_N and G_C and were compared with results obtained from TSWV-T-infected plants. As shown in Fig. 2, the mean absorbance values obtained for the N, NSs and G_C proteins indicated that the amount of TSWV-M present in infected leaves was comparable to that of TSWV-T. In contrast, the mean absorbance values for G_N showed distinct differences in TSWV-M- and TSWV-T-infected leaf tissue (Fig. 2). In the case of TSWV-T-infected plants, the mean absorbance values for G_N and G_C were similar. In TSWV-M-infected plants, the mean absorbance values for G_N were approximately 9-fold lower than those for G_C, indicating that the two GPs were not present in similar amounts.

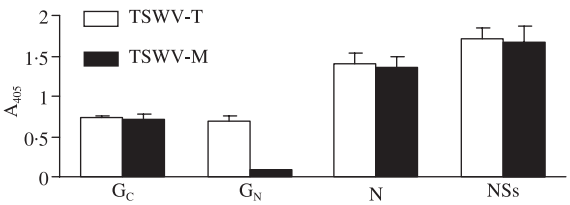


Figure 2 A_{405} values of virion structural (N, G_C and G_N) and non-structural (NSs) proteins in *Emilia sonchifolia* leaves infected with thrips-transmissible and -non-transmissible *Tomato spotted wilt virus* (TSWV-T and TSWV-M, respectively). Bars represent ± SE.

Thrips transmission of TSWV-M

Vector-transmission results showed that TSWV-T was readily transmitted by WFT; males transmitted with an efficiency of 45·95% (34 of a total of 74 viruliferous adults) and females transmitted with an efficiency of 11·88% (12 of a total of 101 viruliferous adults). In contrast, WFT failed to transmit TSWV-M, since leaf discs inoculated with either male ($n = 143$) or female ($n = 178$) thrips tested negative for the virus in ACP-ELISA.

Since ELISA results indicated similar amounts of TSWV-M and -T in leaves used for virus acquisition (Fig. 2), lack of TSWV-M transmission by WFT could be caused either by the failure of WFT larvae to acquire the virus or the inability of viruliferous adult thrips to transmit the virus. To differentiate between these two possibilities, initial experiments were aimed at detecting the virus in the larvae. After a 24-h virus AAP, the larvae were placed on healthy bean pods for a 24-h post-acquisition feeding period to ensure that the virus particles ingested during the AAP would have either passed into the gut epithelium or been degraded in the gut and subsequently eliminated in the voided excretory materials. Total RNA was then extracted from cohorts of 100 larvae and used in RT-PCR to amplify the NSs ORF. Thrips larvae fed on leaves infected with TSWV-T and healthy leaves were used as positive and negative controls, respectively. The entire experiment was repeated twice using thrips larvae that acquired TSWV-T or -M from different plants. The RT-PCR results showed that NSs-specific sequences were amplified from larvae exposed to leaves infected with either virus, but not healthy leaves (results not shown), suggesting that the larvae were competent in acquiring TSWV-M as well as TSWV-T. In order to determine if TSWV-M was present in viruliferous adults, cohorts of larvae from the same batch of insects were reared to adults, and total RNA was extracted from 50 adults and tested by RT-PCR. As shown in Fig. 3 (lanes 5 and 6), NSs-specific sequences were amplified from adult thrips that acquired virus as larvae from TSWV-M- or TSWV-T-infected leaves. This suggested that both viruses replicated in thrips and survived through different developmental stages of the vector.

To determine if non-transmissibility of TSWV-M was caused by lack of virus in salivary glands, the head and abdomen parts dissected from 100 adult thrips were pooled separately, total RNA extracted and tested for virus by RT-PCR. As shown in Fig. 3, virus was detected in both abdomen and head sections of adults that acquired TSWV-T during the larval stage (lanes 11 and 12). In contrast, virus was detected in abdomen but not head sections of adults that acquired TSWV-M during the larval stage (lanes 9 and 10). Similar data were obtained in two independent experiments. These results indicated that thrips acquired TSWV-M during the larval stages and that the virus was able to multiply in the abdomen section

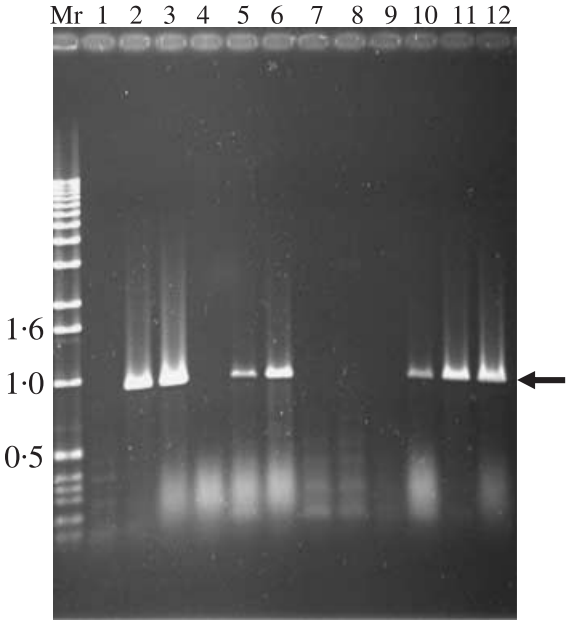


Figure 3 RT-PCR detection of thrips-transmissible and -non-transmissible *Tomato spotted wilt virus* (TSWV-T and -M, respectively) in plants and vector thrips (WFT) using primers specific to the NSs gene. A DNA fragment of about 900 bp (indicated by arrow) was amplified in TSWV-M- (lane 2) and TSWV-T-infected plants (lane 3), but not from uninfected plants (lane 1). The virus-specific DNA fragment was amplified from viruliferous adult thrips that acquired virus from TSWV-M- (lane 5) and TSWV-T-infected plants (lane 6), but not from non-viruliferous thrips (lane 4). Virus was detected only in dissected body parts (lane 10), not in head parts containing salivary glands (lane 9), of viruliferous adult thrips that acquired TSWV-M as larvae, whereas virus was detected in dissected body (lane 12) and head (lane 11) parts of viruliferous adult thrips that acquired TSWV-T as larvae. No virus was detected in body (lane 7) and head (lane 8) parts of non-viruliferous adults. Mr = 1-kbp mol. wt. DNA markers.

of the insects. The lack of virus in dissected head sections that contained salivary glands suggested that TSWV-M did not invade the salivary glands. Based on these results, it was concluded that lack of TSWV-M transmission by viruliferous adult thrips was the result of the inability of the virus to reach and/or establish an infection in the salivary glands.

TEM of TSWV-M particles

TSWV-M was purified from infected plants under conditions used for the purification of TSWV-T and observed by TEM. Virion preparations from two independent purifications were used to observe the particles. Two grids per sample were prepared and 20–30 virus particles in five random fields were observed. As shown in Fig. 4, TSWV-T particles showed a homogeneous, more structured envelope, with spikes clearly seen on the surface of the particles. In contrast, TSWV-M particles appeared smaller, with a more pleomorphic contour, and the envelope membrane was not as distinct as in TSWV-T. Based on these results, it was concluded that TSWV-M formed virus particles in infected plants; however, TSWV-M virions were morphologically less distinct than virions of TSWV-T.

SDS-PAGE and immunoblotting analysis of TSWV-M glycoproteins

To verify whether the defect in viral particles was caused by altered composition of glycoprotein envelope membrane proteins, purified preparations of TSWV-M and -T virions were analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R 250 and by probing in Western blots with G_N- and G_C-specific MAbs. As shown in Fig. 5, TSWV-M and -T preparations showed three distinct bands corresponding to G_C, G_N and N proteins. In all gels, the G_C and N proteins appeared as sharp bands, whereas G_N appeared as a diffuse band. A comparison of the relative densities of G_C and G_N protein bands using IMAGEQUANT software indicated that the G_N protein was less abundant than G_C in TSWV-M (lanes 3, 4 and 6). Consequently, the G_N/G_C ratio was lower ($0{\cdot}87 \pm 0{\cdot}010$) in TSWV-M than in TSWV-T ($0{\cdot}93 \pm 0{\cdot}015$). Since the difference in G_N/G_C ratio between the two isolates was statistically significant ($P = 0{\cdot}0110$), the results indicated unequal amounts of G_C and G_N proteins in TSWV-M particles.

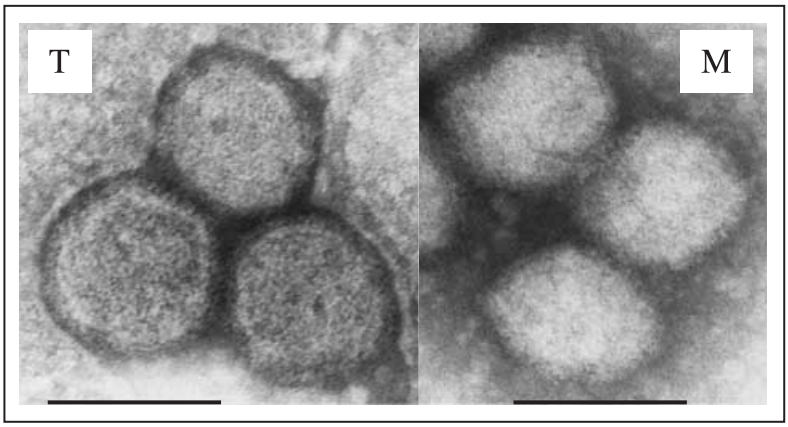


Figure 4 Electron microscopy of virus particles from purified preparations of thrips-transmissible and -non-transmissible *Tomato spotted wilt virus* (TSWV-T and -M, respectively). Bars = 100 nm.

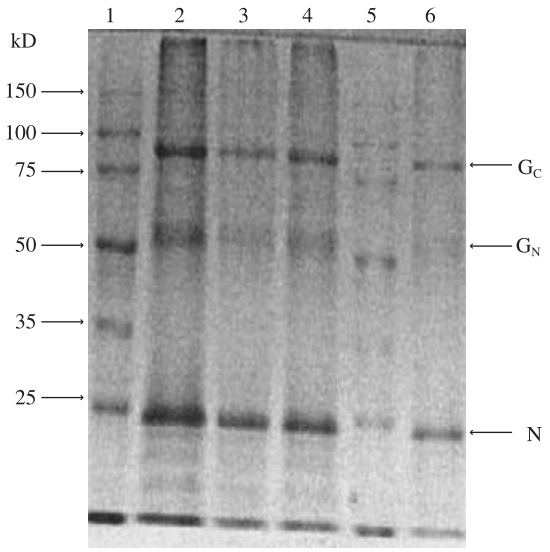


Figure 5 SDS-PAGE analysis of virion preparations isolated from *Emilia sonchifolia* infected with thrips-transmissible and -non-transmissible *Tomato spotted wilt virus* [TSWV-T (lane 2) and TSWV-M (lanes 3, 4 and 6), respectively] isolates. Twenty μg of total protein were loaded in lanes 2 and 4, whereas 10 and 5 μg protein were loaded in lanes 3 and 6, respectively. Prestained proteins with known molecular mass (low range, Bio-Rad Laboratories) loaded in lanes 1 and 5 as size markers are indicated by arrows to the left. The locations of G_C, G_N and N proteins are indicated by arrows to the right.

To substantiate the observation that G_N was less abundant than G_C in TSWV-M, virion preparations were further analysed by Western blotting using MAbs specific to G_C and G_N proteins. As shown in Fig. 6a, the G_C and G_N proteins of TSWV-T reacted with G_C- and G_N-specific MAbs with a single band for each protein. In contrast, the G_C glycoprotein of TSWV-M showed two band sizes, designated G_{C1} and G_{C2}. These two bands were distinctly seen in Fig. 6a, lane M1. The relative electrophoretic mobility of the G_N proteins from TSWV-M and TSWV-T virions did not show noticeable differences. Comparison of signal intensity, measured by IMAGEQUANT, showed that similar amounts of G_N and G_C were present in TSWV-T virions. In contrast, a low signal intensity for G_N, compared with that of G_C, was observed in TSWV-M, indicating less G_N in TSWV-M virions. Consequently, the G_N/G_C ratio was significantly lower in TSWV-M ($0{\cdot}51 \pm 0{\cdot}047$) than in TSWV-T ($0{\cdot}97 \pm 0{\cdot}042$) ($P = 0{\cdot}0110$). These results indicated the presence of unequal amounts of the glycoproteins in TSWV-M particles, in contrast to equal amounts of the glycoproteins in TSWV-T particles, and were consistent with the data from SDS-PAGE (Fig. 5) and ELISA (Fig. 2).

Affinoblotting of TSWV-M glycoproteins

Previous results indicated the presence of high-mannose- and complex-type N-linked oligosaccharides on G_C and high-mannose-type on G_N of TSWV particles (Naidu *et al.*, 2004). In order to determine the N-glycosylation state of the two glycoproteins of TSWV-M, viral proteins separated by SDS-PAGE were probed with two biotin-labelled lectins, namely LCA with specificity to α-mannosyl residues in the tri-mannosyl core portion of N-linked oligosaccharides, and STL with binding affinity to unbranched poly-N-acetyllactosamine-residues in the outer chain structures of oligosaccharides. As shown in Fig. 6b, LCA showed binding with G_N and G_C of TSWV-T, indicating the presence of high-mannose-type oligosaccharides. As expected, only TSWV-T G_C showed specific binding to STL, indicating the presence of complex-type N-linked oligosaccharides, as reported earlier (Naidu *et al.*, 2004). Both G_{C1} and G_{C2} of TSWV-M showed binding with both lectins, indicating the presence of complex-type N-linked oligosaccharides on G_{C1} and G_{C2}. The G_N of TSWV-M showed binding with LCA, but not with STL. The G_N of TSWV-M showed weak binding to LCA, probably because of the lower amounts of G_N in TSWV-M than in TSWV-T. The N protein of TSWV-T and -M showed binding with LCA and not with STL. The binding of N protein to LCA was previously shown as nonspecific binding to high-mannose-type lectins (Naidu *et al.*, 2004).

Comparative sequence analysis of M and S RNA segments of TSWV-M with other tospovirus sequences

The complete nucleotide sequence of S and M RNA of TSWV-M and TSWV-T (AY870389, AY870390, AY870391

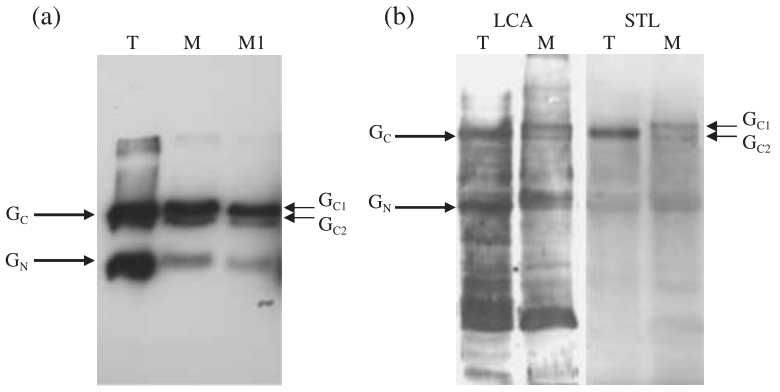


Figure 6 Detection of envelope glycoproteins using G_N- and G_C-specific MAbs in immunoblots (a) and biotinylated lectins in affinoblots (b). Equal amounts (10 μg) of purified viral preparations (5 μg in lane M1) of thrips-non-transmissible and thrips-transmissible *Tomato spotted wilt virus* [TSWV-M (M) and -T (T), respectively] were resolved on 10% polyacrylamide gels by SDS-PAGE and transferred onto nitrocellulose membrane by Western blotting. One blot was incubated with G_N- and G_C-specific MAbs. The other two blots were incubated with lectins LCA and STL. The locations of G_C, G_N, G_{C1} and G_{C2} are indicated.

Table 2 Unique amino acid changes in different proteins encoded by S and M RNA of thrips-non-transmissible *Tomato spotted wilt virus* (TSWV-M) in comparison with consensus sequences of corresponding proteins encoded by different TSWV isolates

Segment	Protein	Amino acid position[a]	TSWV-M	Consensus sequence among TSWV isolates[b]
M RNA				
	G_N	3	N	I
		46	G	E
		200	C	Y
	G_C	530	V	D/H/N/R
		960	A	V/I
	NSm	104	I	V
S RNA				
	N	–	–	–
	NSs	224	L	S
		375	E	G/R
		587	K	G/S

[a]Numbering from N-terminus of the protein.
[b]Sequences of different TSWV isolates used for comparisons. GenBank accession numbers: NC_002050/S48091, AF208498, AF208497, AB010996, AY744476, AY744477, AY744491, AY744490, AY74486, AY744487, AY744488, AY744489, AY744475, AY744484, AY744481, AY744482, AY744483, AY744485, AY744492, AY744493, AB190818, AJ418778.

and AY870392) were compared with each other and with sequences from other TSWV isolates available in GenBank.

The M RNA segment of TSWV-M was 4763 nt in length, whereas the published M RNA sequences of other TSWV isolates varied from 4756 nt (AB010996, Japan) to 4827 nt (AY744486, California). Alignment of the M RNA sequence of TSWV-M with respective sequences of other TSWV isolates revealed nucleotide identity between 90% (NC_002050, the Netherlands) and 99% (AY744487, N. Carolina). The deduced amino acid sequence of the NSm protein of TSWV-M showed amino acid identity of 95–99%, whereas the G_N/G_C precursor showed amino acid identity between 94 and 98% with respective sequences in other TSWV isolates. Multiple sequence alignment of the TSWV-M protein sequences with corresponding proteins of other TSWV isolates currently available in GenBank (Table 2) indicated that the NSm of TSWV-M had one unique change at amino acid position 104 (V→I) and the G_N/G_C precursor had five unique changes in the G_N/G_C precursor; one in the N-terminal signal sequence at amino acid position 3 (I→N), two in the ectodomain of G_N at 46 (E→G) and 200 (Y→C), and two in the ectodomain of G_C at 530 (D/H/N/R→V) and 960 (V/I→A). None of these amino acid changes in TSWV-M affected the potential N-glycosylation sites in the G_N/G_C precursor protein. The 5′ and 3′ noncoding regions were identical to those of other TSWV isolates.

The S RNA segment of TSWV-M was 3047 nt in length, whereas published S RNA sequences varied from 3364 (isolate 10HK96, Bulgaria, AJ418778) to 2916 (isolate CPNH9, the Netherlands, NC_002051). Alignment of the TSWV-M S RNA sequence with that of other TSWV isolates revealed nucleotide identity between 85% (isolate 10HK96, Bulgaria, AJ 418778) and 95% (isolate TSWV-T, Georgia (USA), AY 870392; and isolate TSWV-NC-2, N. Carolina, AY744477). The deduced amino acid sequence of the N protein of TSWV-M showed high similarity (97–100%) with all TSWV isolates sequenced so far, whereas the NSs protein showed amino acid identity between 94–98%. Clustal w alignment of TSWV-M NSs protein sequences with corresponding proteins of other TSWV isolates currently available in GenBank indicated that TSWV-M NSs had three changes at amino acid positions 224 (S→L), 375 (G/R→E) and 387 (S/G→K) (Table 2). No amino acid changes were observed in the N protein when compared to the respective proteins in other TSWV isolates. The intragenic region (IGR) of S RNA was 625 nt long, the second largest IGR among the known isolates of TSWV (Heinze *et al*., 2003). Several GTTT repeats were present in the IGR of TSWV-M, but absent in the IGRs of other TSWV S RNA sequences. It is not likely that the GTTT repeats were caused by RT-PCR, cloning, or sequencing artifacts, since direct sequencing of several independent PCR amplicons showed identical sequences. The 5′ and 3′ noncoding regions were identical to other TSWV isolates.

Discussion

The results presented in this study showed that the TSWV-M isolate failed to infect plant hosts that are susceptible to TSWV infection. Even though the molecular mechanism(s) involved in the host-range phenotype of TSWV-M remain(s) largely unresolved in this study, several effects, alone or in combination, may have an influence in modulating the pathogenesis of this isolate. Because of a lack of a reverse genetics system for plant infecting bunyaviruses, further characterization of the genetic determinants relating to the pathogenesis of TSWV-M is difficult when there are several nucleotide/amino-acid changes compared to the sequences of wild-type TSWV isolates. Nevertheless, studies using a reassortment-based viral genetic system (Qiu *et al*., 1998; Sin *et al*., 2005) involving TSWV-M and TSWV-T would help to elucidate the cellular biology of tospoviruses and mechanisms underlying tospovirus pathogenesis. Such an approach has been used (Okuda *et al*., 2003) to map symptom differences on *Tetragonia expansa* between two isolates of *Watermelon silver mottle virus* to the S RNA segment. Because TSWV-T caused symptoms while TSWV-M failed to infect highly susceptible hosts like *N. benthamiana*, reassortants between the two TSWV isolates would help to define the role of each genomic segment in determining the host range and possibly in understanding the genetic basis of pathogenesis.

Because TSWV-M and TSWV-T reached similar titres in infected leaves of *E. sonchifolia* (Fig. 2), it is unlikely that the virus concentration in the leaves had any effect on the amount of TSWV-M ingested by the larvae. This would argue against a quantitative or dose-dependent barrier for midgut infection, where low titres of infectious virus particles in the host were correlated with reduced

infection of larval midguts after virus ingestion (Nagata *et al*., 2000). Since TSWV-M particles showed a defect in the overall morphology (Fig. 4) and reduced levels of G_N in virions (Figs 5, 6), it is possible that the receptor binding sites in TSWV-M glycoproteins were altered such that interactions between the viral envelope and thrips receptor were disrupted. These results, together with recent work by Whitfield *et al*. (2004) and Sin *et al*. (2005), indicate that the G_N protein in TSWV-M plays a critical role in virus acquisition by thrips. In addition, since G_C of TSWV-M is poorly N-glycosylated as revealed by lectin binding (Fig. 6b), the stability of virus particles in the thrips midgut environment could be affected, leading to decreased endocytosis. Either or both of these scenarios would affect the ability of virus particles to bind to the thrips midgut epithelial cell surface, leading to reduced endocytosis of TSWV-M into the midgut epithelial cells. Consequently, its initial replication and subsequent accumulation could be affected. In this context, it should be noted that N-linked oligosaccharides (number and composition of oligosaccharides) of enveloped animal/human viruses have been suggested as being important in numerous interactions with host cell surface molecules for purposes such as attachment and receptor binding (Li *et al*., 1993; Ohuchi *et al*., 1997; Goffard *et al*., 2005; Shi *et al*., 2005) and infectious virus production (Wissink *et al*., 2004). Presumably, the N-linked oligosaccharides on the surface of TSWV particles play a role in modulating virion attachment to the thrips midgut cellular 'receptor' proteins (Bandla *et al*., 1998) leading to virus acquisition by thrips vectors.

Although primary infections were established in the midgut epithelium, as revealed by RT-PCR detection of the virus 24 h after acquisition access, failure of adult thrips to transmit the virus, as revealed by the absence of NSs-specific amplicons in the head segment of thrips (Fig. 3, lane 9), could be the result of the inability of TSWV-M to invade and/or establish an infection in the salivary glands or on the way to the salivary glands. However, these results would indicate that movement of virus through membranes is virus-mediated and not a passive process. The present study did not address whether TSWV-M failed to overcome different membrane barriers (midgut basement membrane, visceral muscle cells, basal membrane of salivary glands) that tospoviruses must cross on their path to the salivary glands. Further studies utilizing a combination of techniques including microdissection and immunohistochemistry of thrips organs (de Assis Filho *et al*., 2002; Kritzman *et al*., 2002) and real-time quantitative RT-PCR should help to gain insight into the dynamics of TSWV-M passage from the midgut and visceral muscles to the primary salivary glands.

Since G_N and G_C are processed from the glycoprotein precursor protein, equal amounts of the two glycoproteins would be expected to be present following completion of translation and processing. Indeed, similar amounts of G_N and G_C were detected in TSWV-T-infected tissue. The most likely explanation for the characteristics of TSWV-M G_N and G_C is the occurrence of mutations in the glycoproteins. The lower amounts of G_N relative to G_C in TSWV-M-infected tissue and virions were probably not caused by mutation(s) in G_N (Table 2) causing a weak antigen-antibody interaction, since the G_N- and G_C-specific MAbs were produced against denatured proteins extracted from SDS-PAGE gels (Bandla & Sherwood, 1995). The most likely explanation would be that mutations in G_N resulted in the glycoprotein being more sensitive to degradation by the ER-associated degradation pathway (Spiro, 2004). While amounts of G_N would be lower than those of G_C, some G_N might escape degradation and be incorporated into virions at lower amounts than G_C. Another possibility is that G_C stabilizes G_N before, during and/or after virion maturation, and mutations in TSWV M G_C result in G_{C1} and/or G_{C2} being less capable of interacting with and stabilizing G_N.

Presently the formation of two bands of G_C (G_{C1} and G_{C2}) reacting to G_C-specific MAb and how each of these bands differs from G_C of TSWV-T is not clear. There were three potential N-linked glycosylation sites at amino acid residues 605, 980 and 1095 in the G_C from both TSWV-M and TSWV-T. So, the difference was probably caused by differential post-translational processing resulting from mutations at amino acid residues 530 and/or 960 (Table 2). The possibility that mutations in G_N could affect subsequent processing of G_C cannot be excluded. The apparent molecular mass difference, between G_{C1} and G_{C2}, although small, could be accounted for by differential glycosylation. Alternatively, G_{C1} and G_{C2} from TSWV-M and G_C from TSWV-T may be similarly glycosylated, but the G_C/G_N precursors of the two isolates may be differentially processed.

As in other bunyaviruses, both G_N and G_C are type-I integral membrane proteins with their own signal sequences. Previous studies suggested that G_N and G_C cleaved from the precursor are transported in a cooperative manner from endoplasmic reticulum (ER) to the Golgi complex, where they mediate particle morphogenesis and intracellular budding (Kikkert *et al*., 1999, 2001). It has been suggested that the formation of noncovalent G_N/G_C complexes is essential for their intracellular transit to the Golgi, as shown with other members of the *Bunyaviridae* (Chen *et al*., 1991; Shi & Elliott, 2002; Spiropoulou *et al*., 2003). However, the differences in the amounts of G_N and G_C in TSWV-M virions would suggest that G_N/G_C interactions are affected, leading to less efficient transportation to the site of virus particle morphogenesis. Therefore, the different amounts of the glycoproteins in TSWV-M virions could explain the altered morphology of the TSWV-M virions.

Since N-linked oligosaccharides perform a broad range of functions in glycoproteins, ranging from protein folding, quality control and entry functions (Varki, 1993; Trombetta & Helenius, 1998; Helenius & Aebi, 2001), an alternative explanation could be that poor N-glycosylation of glycoproteins of TSWV-M (Fig. 6b) could affect their intracellular transport, because only properly folded glycoproteins pass the quality control system of the ER and become transport-competent (Helenius & Aebi,

2001). Terminally misfolded proteins and unassembled subunits of oligomeric complexes are efficiently retained in the ER and are eventually degraded by the ER-associated degradation pathway (Spiro, 2004). Assuming that N-glycosylation and G_N-G_C dimerization are affected, impaired transport of TSWV-M glycoproteins to the Golgi complex could contribute to decreased particle formation in infected cells.

Sequencing analysis indicated that the N proteins from TSWV-M and TSWV-T were identical and therefore not involved in the differences between TSWV-M and TSWV-T. The only difference between the NSm proteins of the two viruses was an I to V amino acid change at residue 104. Since these two amino acids are hydrophobic, it is unlikely that this change was involved with the differences between the two viruses. While NSs was present at similar amounts in TSWV-M- and TSWV-T-infected plants (Fig. 2), it is possible that mutations in NSs could be responsible for the host-range and vector-transmission differences between TSWV-M and TSWV-T. NSs was identified as a suppressor of gene silencing (Takeda *et al*., 2002; Bucher *et al*., 2003). The limited host range of TSWV-M could be caused by a lack of gene silencing suppression activity resulting from mutation(s) in the NSs protein. It is also possible that NSs has a similar function in thrips vectors by counteracting gene silencing. A mutation(s) in this protein could fail to suppress gene silencing in thrips cells to TSWV, resulting in decreased infection and concomitant lack of transmissibility. A critical role of NSs protein in the suppression of gene silencing (Soldan *et al*., 2005), in the antiapoptotic effect (Kohl *et al*., 2003) and in viral replication (Ikegami *et al*., 2005) was also reported for other bunyaviruses.

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