Viral genetic determinants for thrips transmission of Tomato spotted wilt virus

Sang-Hoon Sin*, Brian C. McNulty†, George G. Kennedy†, and James W. Moyer*‡

Departments of *Plant Pathology and †Entomology, North Carolina State University, Raleigh, NC 27695

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Tomato spotted wilt virus (TSWV) is transmitted exclusively by thrips in nature. A reassortment-based viral genetic system was used to map transmissibility by thrips to the medium (M) RNA of TSWV. To locate determinants of thrips transmission in the M RNA, 30 single-lesion isolates (SLIs) were generated from a single TSWV isolate that was inefficiently transmitted by thrips. Three of the 30 SLIs were transmitted by thrips, and 27 were not. Sequence analysis of the M RNA, thrips transmissibility assays, G_C protein analysis, and transmission electron microscopic studies revealed that a specific nonsynonymous mutation (C1375A) in the G_N/G_C ORF of the M RNA resulted in the loss of thrips transmissibility without inhibition of virion assembly. This was in contrast to other nontransmissible SLIs, which had frameshift and/or nonsense mutations in the G_N/G_C ORF but were defective in virion assembly. The G_C glycoprotein was detectable in the C1375A mutants but not in the frameshift or nonsense mutants. We report a specific viral determinant associated with virus transmission by thrips. In addition, the loss of transmissibility was associated with the accumulation of defective haplotypes in the population, which are not transmissible by thrips, rather than with the presence of a dominant haplotype that is inefficiently transmitted by thrips. These results also indicate that the glycoproteins may not be required for TSWV infection of plant hosts but are required for transmissibility by thrips.

Viruses in the *Bunyaviridae* are among the most medically and agronomically important viruses. Many are frequently listed as emerging or reemerging viruses and are on the select list of viruses considered as potential bioterrorism weapons (1). Four of the five genera in the *Bunyaviridae* are transmitted by arthropods. These viruses have coevolved with both their arthropod vector and their mammalian or plant hosts even though the mechanisms of pathogenesis of these hosts may be quite distinct (2). This virus–host relationship has significant consequences for the epidemiology of the virus and the potential strategies for control. However, our understanding of the viral determinants for arthropod transmissibility is poorly developed. The absence of an efficient system for reverse genetics for any of the members of this family has severely limited the elucidation of the primary elements of this relationship. It is only known that determinants of midgut infection and transmission of La Crosse virus (*Orthobunyavirus*: *Bunyaviridae*) in the mosquito vector cosegregate with the medium (M) RNA and that proteolytic cleavage of G_C is required to infect the midgut epithelium of the mosquito (3–6). In the case of Tomato spotted wilt virus (TSWV) (*Tospovirus*: *Bunyaviridae*), putative thrips receptors have been identified, but there is no specific information regarding viral determinants of the virus–thrips interaction (7–9). It is as yet unclear whether there are indeed separate strategies of pathogenesis in the arthropod and mammalian or plant hosts requiring separate viral genes, or multifunctional viral genes, or if there are in fact similar pathways for pathogenesis in these divergent hosts. In this study, we extend the understanding of the relationship between these viruses and their vectors by using genetic reassortment (10–13) and mutational analysis to identify specific viral determinants of thrips transmissibility and to explain changes in the virus population that quantitatively reduce transmission efficiency by thrips.

TSWV is a plant-infecting *Tospovirus* in the family *Bunyaviridae*. The TSWV virion is a quasispherical particle that ranges from 80 to 110 nm in diameter. A host-derived lipid envelope of TSWV surrounds three single-stranded RNA molecules designated, in order of increasing size, S (small), M (medium), and L (large) RNA (14). The L RNA is of negative polarity and encodes an RNA-dependent RNA polymerase (RdRp) (15, 16). The putative cell-to-cell movement protein (NSm) and the precursor of surface glycoproteins on the envelope (G_N/G_C) are encoded by the M RNA in the viral and viral complementary sense, respectively (17, 18). The glycoprotein precursor is further glycosylated and cleaved into two glycoproteins, G_N and G_C, by an unknown mechanism (19). The S RNA encodes a nonstructural protein (NSs), which was recently reported to suppress gene silencing, and the nucleocapsid protein (20–22).

TSWV is spread in a propagative manner by at least seven thrips species (23–25). Among those, *Frankliniella occidentalis* (western flower thrips) and *Frankliniella fusca* (tobacco thrips) are the most efficient vectors in the southeastern United States (26). Unlike the other arthropod-borne members of the *Bunyaviridae*, old second instar larvae and adults can become transmitters of TSWV only if the virus is acquired by the thrips in the first instar larval stage (27, 28). The midgut epithelial cells of thrips are the initial site of TSWV entry and infection (24, 29). After replication in the midgut, TSWV moves to muscle fibers surrounding the midgut and then migrates to the salivary glands (24, 25, 29–31).

Most studies of the TSWV–thrips interactions have focused on elucidating cellular and biochemical determinants for acquisition and movement of TSWV in thrips (7–9). Several reports suggest that a receptor-mediated endocytosis governs TSWV entry to thrips midgut cells and that the TSWV G_C protein and a 50-kDa thrips protein may coordinate the TSWV entry process (8, 9). However, the G_N protein was also identified as a viral attachment protein that binds to a 94-kDa thrips protein (7). Additional indirect evidence for the involvement of glycoproteins as determinants for TSWV binding and subsequent infection in thrips cells is that an envelope-deficient isolate of TSWV failed to infect the midgut after ingestion and was not transmitted by thrips (32). It was also shown that thrips transmissibility of TSWV declined after repeated mechanical passages (33). These reports suggest that the M RNA or its translational products may be determinants of thrips transmissibility of TSWV. However, other TSWV RNAs or proteins have also been

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Abbreviations: TSWV, Tomato spotted wilt virus; TSWV-D, TSWV isolate D; TSWV-RG2, TSWV isolate RG2; RdRp, RNA-dependent RNA polymerase; RFLP, restriction fragment length polymorphism; SLI, single-lesion isolate; NS, nonsynonymous; S RNA, small RNA; M RNA, medium RNA; L RNA, large RNA.

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‡To whom correspondence should be addressed. E-mail: james_moyer@ncsu.edu.

implicated as possible control elements for thrips transmission (32). For example, the TSWV RdRp has been implicated in thrips transmission. A TSWV isolate containing defective interfering RNA, which had a truncated L RNA, was not transmitted by thrips (32).

In this investigation, we conducted a viral genetic analysis using reassortment that we previously used to map several viral phenotypes to specific genome segments of TSWV (10–13), combined with sequence analysis of transmission deficient mutants to characterize thrips transmission determinants. Here we present genetic evidence confirming that the M RNA encodes determinants of thrips transmission and identify specific mutations in the M RNA sequence that are associated with the loss of transmissibility by thrips. These investigations characterize specific mutations in the glycoprotein precursor coding region, which are associated with the loss of thrips transmissibility of TSWV. These data also suggest that the decline in thrips transmissibility of TSWV, after repeated mechanical passages, is due to the accumulation of M RNA segments with sequence specific mutations in the viral population. Our results also suggest that G_N and/or G_C may not be required for TSWV replication in plants, but are required for thrips transmissibility.

Materials and Methods

TSWV Isolates and Thrips Colony. Two TSWV isolates were used as parental isolates to generate reassortants. TSWV isolate D (TSWV-D) is not transmitted by thrips, whereas TSWV isolate RG2 (TSWV-RG2) is transmissible by *F. occidentalis* and *F. fusca*. Parental isolates and reassortants between them were maintained in *Nicotiana benthamiana* or *Emilia sonchifolia* by mechanical means. Inoculation buffer (10 mM Tris·HCl, pH 7.8/10 mM Na_2SO_3/0.1% cysteine-HCl) was used in all mechanical inoculations in this study. All plants were kept individually in cylindrical, insect-proof cages. *F. occidentalis* was used in all thrips transmission assays. Thrips were maintained as described (26).

Generation of TSWV Reassortants. Reassortants between thrips-transmissible TSWV-RG2 and nontransmissible TSWV-D were isolated from a dual infection in *N. benthamiana* and subsequent serial passages through *Nicotiana tabacum* cv. ''Burley 21,'' a local lesion host for TSWV. Both isolates were amplified separately in *N. benthamiana* by mechanical inoculation. Systemically infected leaves were mixed and ground in inoculation buffer; the expressed sap was reinoculated to *N. benthamiana*. Symptomatic leaves infected with both isolates were then used as an inoculum source for *N. tabacum* cv. "Burley 21." A set of inocula, which had different ratios of the leaves infected with two parental isolates, was used for the initial coinoculation to boost the probability of generating the six theoretically probable reassortants from both isolates. Each local lesion on the inoculated leaf of the infected *N. tabacum* cv. "Burley 21" was serially transferred a minimum of five times to obtain homogeneous reassortants. Each local lesion that developed on *N. tabacum* cv. "Burley 21" after the final transfer was inoculated onto *N. benthamiana*. Systemically infected leaves of *N. benthamiana* infected with putative reassortants were used for total RNA extraction. The origin of each RNA segment of the reassortants was confirmed by segment-specific RT-PCR and restriction fragment length polymorphism (RFLP).

Genetic Markers and Genotyping of Reassortants. Total RNA was extracted from systemically infected leaves of *N. benthamiana* infected with each reassortant by using RNA ISOLATER (Genosys) according to the manufacturer's protocol. The first-strand cDNA was synthesized by using avian myeloblastosis virus reverse transcriptase (Promega) as described (13). The cDNA was used as template for PCR. PCR amplification of specific regions on each viral genomic RNA was carried out with the primer pair L28-L895 for the L RNA, M3050-M4037 for the M RNA, and S70-S890 for the S RNA under the conditions described in ref. 13. The RT-PCR amplified regions were from the RdRp, G_N/G_C, and NSm ORFs of the L, M, and S RNA segment, respectively. The PCR products were cleaned through QIAquick PCR Purification kit (Qiagen, Valencia, CA). Purified PCR products were digested with *Taq*I for the L RNA fragment, *Mse*I for the M RNA fragment, and *Taq*I for the S RNA fragment. The RT-PCR products and restriction fragments were resolved on 0.8% and 1.5% agarose gel, respectively.

Thrips Transmission. Each isolate was maintained in *E. sonchifolia* as an inoculum source for thrips transmission. The transmissibility of each isolate was assayed two or three times. Each time, 600 first-instar *F. occidentalis* <4 h old were individually transferred by a paintbrush onto leaves of infected *E. sonchifolia* for acquisition of the virus. The thrips were allowed to feed on infected leaf material for 48 h at 25 ± 3°C, after which they were transferred to fresh pods of *Phaseolus vulgaris* and reared to adult at 28 ± 3°C for 8–9 days. Potentially viruliferous adult thrips were then confined in groups of 10 per plant to 10 or 30 caged, 2-week-old, noninfected *E. sonchifolia* plants. The plants were then held in a greenhouse under 28°C/20°C (light/dark) and 14 h/10 h (light/dark). After a 3-day inoculation access period, the plants were sprayed with imidacloprid to kill the thrips. Symptomatic leaves of *E. sonchifolia* infected with parental isolates were used as controls. The genotype of each transmitted reassortant isolate was confirmed by RT-PCR and RFLP analysis.

Isolation of Thrips-Nontransmissible Mutants. To rescue thrips-nontransmissible mutants, a TSWV isolate (TSWV-RG2P24), was selected after 24 serial, mechanical passages of TSWV-RG2. Leaves of *N. benthamiana* infected with TSWV-RG2P24 were then inoculated to *Nicotiana tabacum* cv. "Burley 21." Thirty single-lesion isolates (SLIs) were selected after three single lesion transfers and inoculated to *E. sonchifolia* for thrips transmission assay.

Sequence Analysis. The M RNA sequence of TSWV-RG2P24 and SLIs originating from this isolate were determined by RT-PCR and cycle sequencing of overlapping PCR products. The PCR primers were chosen from a published sequence of TSWV isolate BR-01 (18) and are shown in Table 3, which is published as supporting information on the PNAS web site. Amplification of intergenic region was conducted as described (34). Sequencing of the amplified fragments was performed on an ABI3700 automated sequencer with the BigDye Cycle Sequencing kit (version 3.1, Applied Biosystems). Fragments were aligned by using VECTOR NTI software (version 7.0, Informax, Frederick, MD). Numbering system for mutation in G_N/G_C began with the start codon of the ORF.

Transmission Electron Microscopy (TEM). Small pieces of the symptomatic *E. sonchifolia* leaf tissues infected with SLIs obtained from the TSWV-RG2P24 were fixed and processed for TEM as described by Urban *et al.* (35) with modification. Infected leaf tissue was fixed with 1% glutaraldehyde (vol/vol) and 2.5% paraformaldehyde (wt/vol) in 0.1 M potassium phosphate (pH 7.4) overnight at 4°C. After postfixation in 1% OsO_4, the samples were dehydrated and embedded in Spurr's resin. The ultrathin sections were stained with uranyl acetate and lead citrate. The examination was performed by using a JEOL transmission electron microscopy JEM-100S.

Detection of G_C. Western blot analysis was performed to detect the presence of G_C in *E. sonchifolia* leaves infected with the TSWV

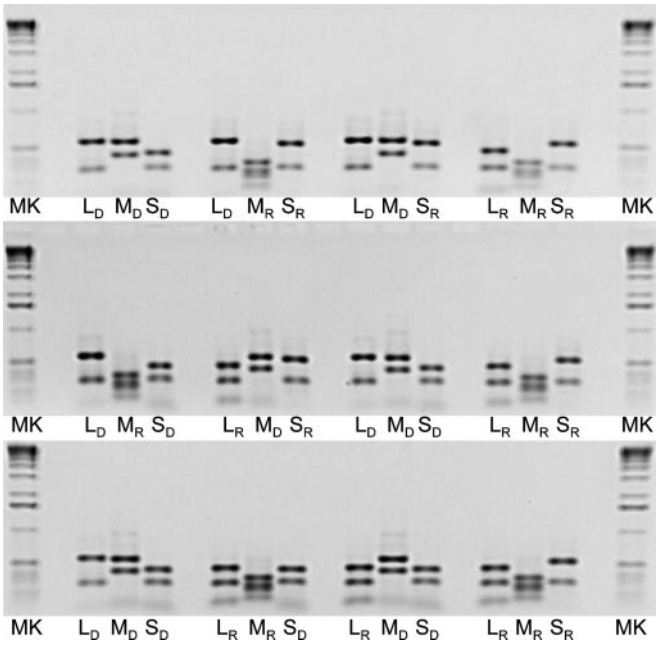

Fig. 1. Genotype analysis of the two parental TSWV isolates and six reassortants. Lane MK contains 1 Kb DNA marker. D and R indicate TSWV-D and RG2, respectively. L, M, and S indicate amplified fragments from nucleotides 28 to 895 of L RNA digested by *Taq*I, amplified fragments from nucleotides 3050 to 4037 of M RNA digested by *Mse*I, and amplified fragments from nucleotides 70 to 890 of S RNA digested by *Taq*I, respectively.

mutants. Sample preparation, electrophoresis, and electrotransfer were conducted as described by Law *et al.* (36). Monoclonal antibody to G_C was a kind gift from J. L. Sherwood (University of Georgia, Athens). The dilution rate of the monoclonal antibody was 1:1,000, and detection of the band was carried out by using enhanced chemiluminescence (Amersham Pharmacia).

Results

Generation and Confirmation of Reassortants. A molecular marker system was developed to differentiate TSWV-D, a thrips-nontransmissible isolate, and TSWV-RG2, a thrips-transmissible isolate, in a segment-specific manner by using RT-PCR and RFLP. The digested fragments of purified PCR products exhibited distinctive profiles specific for both parental isolates (Fig. 1). A complete set of reassortant genotypes between the TSWV-D genotype ($L_DM_DS_D$) and the TSWV-RG2 genotype ($L_RM_RS_R$) was isolated from a mixed infection of these two isolates. The parental origin of reassortants was confirmed by RT-PCR and RFLP. The RFLP profiles of the RT-PCR-amplified fragments for both parental isolates and six reassortants are shown in Fig. 1. Reassortant genotypes of $L_RM_DS_R$, $L_RM_RS_D$, and $L_RM_DS_D$ were generated when equal amounts of inoculum of two parental isolates were coinoculated, whereas $L_DM_DS_R$, $L_DM_RS_D$, and $L_DM_RS_R$ were generated when TSWV-D inoculum was increased 2-fold to that of TSWV-RG2. The two parental isolates and the six reassortants induced necrotic local lesions followed by systemic mottling in *N. benthamiana*.

Thrips Transmissibility of the Reassortants. Thrips transmissibility assays by *F. occidentalis* of the six reassortants revealed that reassortants $L_RM_RS_D$, $L_DM_RS_D$, and $L_DM_RS_R$ were transmitted by thrips, whereas $L_RM_DS_D$, $L_DM_DS_R$, and $L_RM_DS_R$ were not (Table 1). Reassortant isolates with the M RNA from TSWV-D, a thrips-nontransmissible isolate, were not transmitted by *F. occidentalis*, whereas reassortants with the M RNA from TSWV-RG2, a thrips-transmissible isolate, were transmissible irrespective of the origin of the L and S RNA. The TSWV-RG2 and three transmissible reassortants, $L_RM_RS_D$, $L_DM_RS_D$, and $L_DM_RS_R$ induced concentric, necrotic ringspots followed by systemic chlorosis in *E. sonchifolia* after thrips-mediated inoculation. The viral genotypes of the thrips-transmitted reassortants after inoculation to *E. sonchifolia* were the same as those of the original reassortants. These results provide compelling evidence that the M RNA is responsible for thrips transmission of the TSWV. These studies did not reveal any determinants for transmission on the L or S RNA segments of the TSWV-D.

Table 1. Thrips transmissibility of TSWV reassortants generated from transmissible (RG2; $L_RM_RS_R$) and nontransmissible (D; $L_DM_DS_D$) isolates

Reassortant	Transmission, infected/inoculated*	Genotype†
$L_DM_DS_D$	0/10; 0/10; 0/10	NA
$L_RM_RS_R$	6/9; 3/10; 2/10	$L_RM_RS_R$
$L_RM_RS_D$	4/10; 2/10; 2/10	$L_RM_RS_D$
$L_RM_DS_D$	0/10; 0/10; 0/10	NA
$L_RM_DS_R$	0/10; 0/10; 0/10	NA
$L_DM_DS_R$	0/10; 0/10; 0/10	NA
$L_DM_RS_R$	4/10; 3/10; 3/10	$L_DM_RS_R$
$L_DM_RS_D$	4/10; 2/10; 2/10	$L_DM_RS_D$

*Thrips transmissibility was confirmed by symptom development and RT-PCR after inoculation by thrips. Individual experiments are separated by semicolon. The numerator and the denominator represent the number of infected and inoculated plants, respectively. Each plant was exposed to 10 potentially viruliferous thrips.

†Genotype of each symptomatic leaf tissue after thrips transmission was determined by RT-PCR and RFLP.

Isolation and Sequence Analysis of Transmission-Deficient Mutants. To locate the site or region necessary for thrips transmission, SLIs were generated by passaging the TSWV-RG2P24 through *N. tabacum* cv. "Burley 21," a local lesion host of the TSWV. Thirty SLIs were transferred by mechanical transmission to *E. sonchifolia* for thrips transmission assay and sequence analysis. All 30 SLIs produced similar symptom types: concentric, necrotic ringspots followed by systemic chlorotic symptoms in *E. sonchifolia* after the final mechanical transmission. Initially, eight SLIs were randomly chosen for thrips transmission assay; six SLIs (2, 11, 28, 43, 80, and 81) were not transmitted by *F. occidentalis*, whereas two (37 and 41) were transmitted. To identify the viral genomic region responsible for thrips transmission in the M RNA, the complete M RNA sequences of these eight SLIs were compared to the TSWV-RG2P24 M RNA sequence (Table 2). Mutations were found in the G_N/G_C ORF, whereas no mutation was observed in the NSm ORF, the intergenic region between the NSm and the G_N/G_C ORF, or 5′ and 3′ nontranslated regions of the M RNA of the eight SLIs (Table 2). Based on these results, the thrips transmissibility and the G_N/G_C ORF sequence of the other twenty two SLIs were determined (Table 4, which is published as supporting information on the PNAS web site). SLI 4 was transmitted, whereas the other 21 SLIs were not. One nonsynonymous (NS) mutation of C to A at nucleotide 1375 (C1375A) in the G_N/G_C ORF, which converts proline to threonine at amino acid 459 (Pro459Thr), was found in 18 of the nontransmissible SLIs (Tables 2 and 4). The C1375A was detected as the only mutation in three (63, 68, and 81) of the 18 nontransmissible SLIs, which had the C1375A mutation. The C1375A NS mutation was observed with other NS mutations in seven SLIs (13, 28, 43, 65, 73, 75, and 83), and frameshift or nonsense mutations in eight SLIs (2, 3, 11, 33, 39, 54, 58, and 79). Frameshift mutations (deletion or addition) and/or nonsense mutations were found in 16 nontransmissible

Table 2. Mutation analysis of the M RNA of thrips transmissible and nontransmissible SLIs generated from TSWV-RG2P24

		Mutation				
			G_N (1–1452)†		G_c (1453–3408)†	
SLI	Thrips transmissibility*	NSm, IGR, 5′ and 3′ NTR	S	NS	S	NS
2	0/30; 0/30; 0/30	–	–	A deletion at 310, C1375A (P459T)‡	–	–
11	0/30; 0/30; 0/30	–	–	A deletion at 270, C1375A (P459T)	–	–
28	0/30; 0/30; 0/30	–	–	A923G (K308R), C1375A (P459T)	–	–
37	9/30; 8/30; 8/30	–	–	–	U1683C	–
41	7/30; 6/30; 7/30	–	–	–	U1683C	–
43	0/30; 0/30; 0/30	–	–	A776G (H259R), C1375A (P459T)	–	–
80	0/30; 0/30; 0/30	–	–	U144A (stop§) C1054U (L352F)	U1683C	Addition of UUU at 1977 G1975U (V659L)
81	0/30; 0/30; 0/30	–	–	C1375A (P459T)	–	–

IGR, intergenic region; NTR, nontranslated region; S, synonymous; NS, nonsynonymous; –, no mutation found.
*Thrips transmissibility was confirmed by symptom development and RT-PCR after inoculation by thrips. Individual experiments are separated by semicolon. The numerator and the denominator represent the number of infected and inoculated plants, respectively. Each plant was exposed to 10 potentially viruliferous thrips.
†The G_N/G_C annotation was based on refs. 18 and 47. Numbers designate nucleotide number from first nucleotide in ORF.
‡C1375A (P459T) means that the nucleotide at 1375 was substituted from C to A, which changes amino acid 459 from Pro (P) to Thr (T).
§Stop means mutation which converts codon to translation termination codon.

SLIs. No mutations were observed in the M RNA of transmissible SLI 4, whereas one synonymous mutation of U to C at nucleotide 1683 (U1683C) was observed in the other two transmissible SLIs (37 and 41). A nonsense mutation was observed in nine SLIs (3, 10, 20, 30, 39, 54, 61, 71, and 80). Two NS mutations observed in a nontransmissible SLI 60 were different from the NS mutation of C1375A. These were substitutions of A1202G (Lys401Arg) and G2294U (Gly765Val). Symptom development in *E. sonchifolia* was similar among all 30 SLIs regardless of whether they were transmitted by thrips (SLIs 4, 37, and 41) or only mechanically.

Virion Formation. Symptomatic leaf tissues infected with each of the SLIs from the TSWV-RG2P24 were analyzed by transmission electron microscopy to determine whether the SLIs formed enveloped virions in infected plant tissues. Intact TSWV virions were detected in the leaf tissues of the *E. sonchifolia* plant infected with TSWV-RG2P24, the parental isolate of the SLIs (Fig. 2*A*). Transmissible SLIs (37 and 41) also formed virions in the infected leaf tissues (Fig. 2*B*). The virions were also observed in the infected tissues with nontransmissible SLIs, which had the single C1375A NS substitution as the only mutation in the G_N/G_C ORF (SLIs 68 and 81; Fig. 2*C*). No virions were observed in leaf tissues infected with SLIs (2, 39, 61, 79, and 80), which had frameshift or nonsense mutations or both (Fig. 2*D*). It is well established that cytopathological structures including viroplasms and crystalline filaments are a manifestation of TSWV infection in host plants (35, 37–39). The viroplasms and loose nucleocapsid aggregates characteristic of TSWV infection were observed in all of the symptomatic leaf tissues infected with each of the SLIs, including the TSWV-RG2P24 (Fig. 2). No TSWV virions were observed in the two SLIs (13 and 65) that had other NS mutations in addition to the C1375A mutation, or in SLI 60, which had two NS mutations different from the C1375A mutation.

Detection of G_C. Symptomatic leaf tissues infected with SLIs were analyzed by Western blot for G_C accumulation in plant tissues infected with the SLIs. G_C was detected in *E. sonchifolia* leaf tissues infected with transmissible SLIs (37 and 41) and a SLI that only had the C1375A mutation in G_N/G_C ORF (81), whereas G_C was not found in *E. sonchifolia* leaf tissues infected with SLIs, which had nonsense or frameshift mutations or both (2, 61, and 80) (Fig. 3). In addition, G_C was not found in the leaf tissues infected with SLI 60 which had two NS mutations, A1202G and G2294U, indicating that these mutations may also be linked to the loss of G_C. The Western blot analysis of three nontransmissible SLIs with nonsense or frameshift mutations or both supports the envelope deficiency property of those SLIs revealed by sequence analysis and TEM.

Discussion

Elucidation of the molecular determinants of TSWV-host interactions has been limited by the absence of an efficient system for

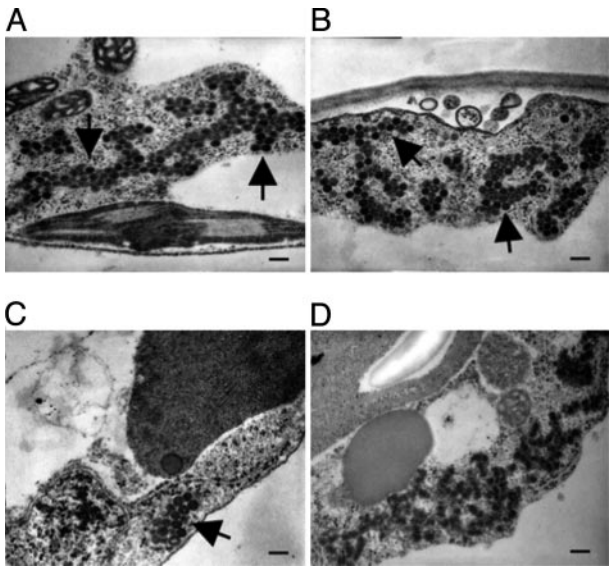

Fig. 2. Transmission electron micrographs of TSWV-infected tissue and mutant SLIs. Arrow indicates an example of groups of enveloped TSWV virions. (Bar, 200 nm.) (*A*) Enveloped TSWV virions in tissue infected with TSWV-RG2P24. (*B*) Enveloped TSWV virions in tissue infected with SLI 37, a thrips transmissible isolate. (*C*) Enveloped TSWV virions in tissue infected with SLI 81, a thrips nontransmissible isolate with a NS mutation C1375A (Pro459Thr). (*D*) No virions were observed in tissue infected with SLI 80, a non-thrips-transmissible isolate with a nonsense mutation and a frameshift mutation in the G_N/G_C ORF.

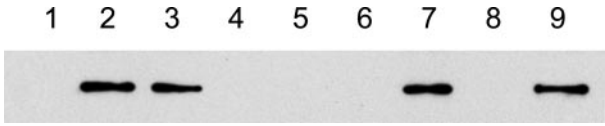

Fig. 3. Detection of G_C in *E. sonchifolia* leaf tissue infected with TSWV SLIs. Samples were analyzed by SDS/PAGE and Western blot. G_C was detected in the plant leaf tissue infected with thrips transmissible SLIs (37 and 41; lanes 2 and 3) and nontransmissible SLI (81; lane 7), which form TSWV virions in *E. sonchifolia* leaf tissue, whereas G_C was not detected in *E. sonchifolia* leaf tissue infected with nontransmissible SLIs (2, 61, and 80; lanes 1, 5, and 6), which have nonsense or frameshift mutations or both in the G_N/G_C ORF. G_C was not detected in *E. sonchifolia* leaf tissue infected with SLI 60 which has mutations of A1202G (K401R) and G2294U (G765V) in G_N/G_C ORF (lane 4). Healthy leaf tissues and leaf tissues infected with the TSWV-RG2P24 were used as negative and positive controls, respectively (lanes 8 and 9).

reverse genetics for the *Tospovirus* genus as well as the entire *Bunyaviridae* family. Replication in mammalian or plant hosts as well as their insect vectors is a characteristic of viruses in four genera in this family (40). Previous investigations have suggested a role for the M RNA and possibly the L RNA as the location of determinants of thrips transmissibility for TSWV (7–9, 32). This association was based on the accumulation of truncated L RNAs and the absence of virion formation in nontransmissible isolates (32). In this study, we developed a system for genetic analysis based on the decline of thrips transmissibility after sequential mechanical passages from plant to plant. We hypothesized that the loss of transmissibility resulted from the accumulation of defective RNAs in the viral isolate and that these could be separated into transmissible and nontransmissible isolates after single lesion transfers. We hypothesized further that it would be possible to map the loss of transmissibility to a segment or segments of the genome by using reassortment analysis. Sequence analysis of the M RNA from transmissible and nontransmissible isolates was used to identify changes at the nucleotide level associated with the loss of transmissibility.

The reassortment analysis between a transmissible and a nontransmissible isolate mapped the determinants of transmissibility to the M RNA. The asymmetric segregation and nonrandom distribution of segment reassortment was similar to that reported for TSWV in our previous studies as well as for Snowshoe hare virus and La Crosse virus (11, 13, 41). Differential replication efficiency of the two corresponding segments of parental isolates and host selective influences on reassortant genotypes have been suggested as possible causes (41, 42). Competitiveness of genome segment exchange of TSWV has been linked to the intergenic region of the S RNA (13).

Previous studies have implicated G_N/G_C, which is coded on the M RNA segment, in viral attachment to the surface of epithelial cells in the thrips midgut (7–9). Point mutations or small deletions in the G_N/G_C ORF have been suggested as the cause of nontransmissibility and loss of envelope formation, although no specific mutations have been identified (32, 43). It has also been reported that a TSWV isolate supporting defective interfering RNA, which suppresses normal L RNA functions and attenuates symptom expression, was not transmitted by *F. occidentalis* (32). However, other defective interfering RNA-containing isolates that minimally interfered with symptom expression were transmitted as efficiently as a wild-type isolate (32). We did not encounter any symptom attenuation in the two parental isolates and six reassortants on *N. benthamiana, N. tabacum* cv. "Burley 21," and *E. sonchifolia*. Whereas that study implicated the L RNA and/or RdRp in thrips transmission of TSWV and was inconclusive regarding deletions, our genetic analysis implicated only the M RNA in the loss of thrips transmissibility of TSWV (Table 1).

Although the M RNA has been associated with thrips transmission, knowledge of the molecular determinants controlling thrips transmissibility of TSWV is needed. Sequence analysis of thrips-transmissible and thrips-nontransmissible SLIs from a TSWV isolate, which had low thrips transmissibility, reveals that multiple types of mutations in the G_N/G_C ORF are linked to the loss of thrips transmissibility of TSWV. The absence of mutations in the NSm ORF of 8 SLIs focused our efforts on the G_N/G_C ORF (Table 2). A single NS mutation C1375A (Pro459Thr) was found in three transmission-deficient SLIs. Fifteen additional SLIs were shown to have the C1375A mutation as well as other mutations in the G_N/G_C ORF. This finding suggests strongly that proline 459 is involved as a determinant of thrips transmission of TSWV. The formation of enveloped virions and detectable G_C in host plants infected with each of the nontransmissible SLIs that had only the C1375A mutation in the G_N/G_C ORF suggests that the loss of thrips transmissibility by these mutants may not be due to a change in synthesis of the proteins. The mutation is also outside the RGD motif of G_N/G_C ORF, which has been associated with cellular attachment in some animal pathogens (18, 44–46). The C1375A mutation may not be involved with glycoprotein processing, even though there is a previous report that amino acid 484 of TSWV G_N/G_C may be a cleavage site within the glycoprotein precursor (47). It may be significant that amino acid 459 resides within a putative transmembrane domain of the glycoprotein, which spans from amino acid 428 to 484 (47). The nonsense and frameshift mutations that were also observed in nontransmissible SLIs would prevent G_N/G_C from being translated by terminating translation prematurely or frameshifting of the G_N/G_C ORF, respectively. TEM examination revealed that nontransmissible SLIs, which had frameshift or nonsense mutations in the G_N/G_C ORF of the M RNA, did not form normal TSWV virions in an infected plant host. The absence of detectable G_C in nontransmissible SLIs with nonsense or frameshift mutations is consistent with the absence of enveloped virions. The presence of enveloped virions in SLIs that had the C1375A mutation suggests that the mutation does not inhibit processing of the glycoprotein precursor (G_N/G_C) into two glycoproteins (G_N and G_C). The C1375A mutation apparently is not involved with symptom development in the host plants *N. benthamiana* and *E. sonchifolia*, as no difference in symptom development was observed between the SLIs with mutation C1375A and TSWV-RG2P24.

There are previous reports that serial mechanical transmission generated an envelope-deficient mutant of TSWV, and it was hypothesized that this defect was the result of point mutations or small deletions in the M RNA (43, 48). However, there has been no sequence analysis of that mutant. Our results document that TSWV mutants without an envelope can be generated by repeated mechanical transmission (48, 49). We have characterized specific point mutations or nonsense mutations in the G_N/G_C ORF of the M RNA that are associated with the lack of an envelope. Our report demonstrates the presence of specific mutations in transmission-deficient isolates and indicates that the glycoproteins may not be required for TSWV replication in plants but are necessary for thrips transmission.

The results of our sequence analysis and thrips transmission assays of SLIs originating from TSWV-RG2P24 explain the low transmissibility of TSWV isolates by thrips after repeated, mechanical passages. As mechanical passage proceeded, mutants that are not transmissible by thrips accumulated in the TSWV population. The low proportion of thrips-transmissible SLIs (10%) is consistent with the reduced thrips-transmission efficiency of TSWV isolates after serial mechanical transmission (33). Our results support quantitatively that the TSWV-RG2P24, which was used as a parental isolate for SLIs, was composed of a high proportion of nontransmissible mutants, ≈90%.

In conclusion, we used a reassortment-based viral genetic analysis system to demonstrate the critical importance of the TSWV M RNA in determining thrips transmissibility. Furthermore, we have identified a single NS mutation (C1375A) associated with thrips transmissibility. It was also demonstrated that specific frameshift mutations or nonsense mutations in the G_N/G_C ORF interfere with glycoprotein synthesis and thrips transmissibility. The formation of enveloped TSWV virions by thrips-nontransmissible SLIs containing only the C1375A mutation in the G_N/G_C ORF also suggests that this mutation may alter a specific determinant governing thrips transmissibility of TSWV. Absence of virion formation and the inability to detect G_C indicate that G_C may not be required for TSWV multiplication in its plant host but is required for transmissibility by its thrips vector. Further investigation of the nature of the TSWV glycoproteins and TSWV virion morphogenesis is needed for improved understanding of the mechanisms controlling TSWV movement within the thrips vector. Progress toward this end would be greatly enhanced by the development of reliable reverse genetic system.

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