

ABSTRACT

SIN, SANG-HOON Genetic determinants for thrips transmission of *Tomato spotted wilt virus*.
(Under the direction of Dr. James W. Moyer)

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* in the family *Bunyaviridae* and is exclusively transmitted by thrips in nature. To determine the viral RNA segment(s) responsible for thrips transmissibility, a viral genetic system that included reassortment and segment-specific RFLP analysis was developed. A complete set of reassortants between TSWV-RG2, a thrips transmissible isolate, and TSWV-D, a thrips nontransmissible isolate was generated. Thrips transmission assays of the reassortants showed that reassortants containing the M RNA from TSWV-D, a thrips nontransmissible isolate, were not transmissible, 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. This is compelling evidence that thrips transmissibility of TSWV resides on the M RNA.

Initial attempts to delineate specific regions of the M RNA associated with thrips transmission and characterize genetic changes on the M RNA of the TSWV following repeated mechanical or thrips transmission were made by comparing the nucleotide sequence of the M RNAs of the 3 poorly-transmissible TSWV isolates and transmissibility-restored isolates. After 24 transmission cycles by mechanical means or by thrips, whole M RNA sequences were determined by RT-PCR and sequencing. The mean mutation frequency of the NSm open reading frame (ORF) for the three isolates per passage was 9.2×10^{-5} nucleotide substitutions per base per mechanical or thrips passage. A similar pattern was observed in G_N/G_C ORF with 1.1×10^{-4} and 1.3×10^{-4} nucleotide substitutions per base per mechanical or thrips passage, respectively. Interestingly, two NS mutations and one synonymous S mutation in the G_N/G_C ORF were conserved in the three isolates after the series of mechanical transmission. Two NS mutations consist of a substitution of C⁸⁶ to U

(C86U) changing amino acid T²⁹ to I (T29I) and U1373C (M458T), while one synonymous mutation was a substitution of G492A. In addition, two NS substitutions in the G_N/G_C ORF of thrips-nontransmissible TSWV-GT (TSWV-GTN) were found to be responsible for the loss of thrips transmissibility. Two NS substitutions were changes of U1976C (V659A) and A2150G (Y717C).

An alternative strategy was employed to accomplish the fine mapping of determinants for thrips transmissibility on the M RNA. To increase the probability of identifying specific regions of the genome linked to transmissibility, thirty single lesion isolates (SLIs) were generated from TSWV-RG2P24, which was derived from TSWV-RG2 following 24 serial, mechanical passages in *N. benthamiana*. Three SLIs were transmissible, while 27 SLIs were nontransmissible by WFT. Sequence analysis of the M RNA, thrips transmission assays, Western blot analysis, and transmission electron microscopic studies revealed that a specific NS 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. In addition, G_C was only detectable in the transmissible isolates and C1375A mutants but not in the frameshift or nonsense mutants. This is the first report of a specific viral determinant associated with thrips transmission. These results also indicate that the glycoproteins may be dispensable for TSWV infection of plant hosts, but not for transmissibility by thrips.

**GENETIC DETERMINANTS FOR THRIPS TRANSMISSION OF
*TOMATO SPOTTED WILT VIRUS***

BY

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He married Yeon Yee Oh on October 21, 1995, and has two daughters, Soo Min and Young Min.

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**Biology of *Tomato spotted wilt virus* (*Bunyaviridae*: *Tospovirus*):
Molecular Characterization, Vector transmission, and Its Diversity**

Literature Review

Biology of *Tomato spotted wilt virus* (Bunyaviridae: *Tospovirus*): Molecular Characterization, Vector transmission, and Its Diversity

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I. Historical Background

A tomato disease termed “spotted wilt” was first reported in 1919 in Australia (Brittlebank, 1919). Its etiological agent was identified as a virus named “tomato spotted wilt virus” in 1930 (Samuel *et al.*, 1930). Since then, *Tomato spotted wilt virus* (TSWV) has caused severe economic losses in a wide range of hosts. The host range of TSWV includes over 925 species spanning both monocotyledons and dicotyledons in many countries worldwide (Peters, 1998). Economically important hosts include lettuce, peanut, pepper, tobacco, and tomato across temperate, subtropical, and tropical regions (Rosello *et al.*, 1996).

Thrips were discovered as the vector for TSWV around 1930 (Linford, 1932). Among thrips species transmitting TSWV, *Frankliniella occidentalis* (Pergande) is one of the efficient vectors. Introduction of this thrips species into new regions is considered one of the main reasons for a remarkable increase of TSWV outbreaks in a wide variety of crops in the last two decades (van de Wetering, 1999). This thrips were originally reported in California, USA (Moulton, 1931) but it is widespread throughout the world. In addition to its accidental importation, global occurrence of *F. occidentalis* is enhanced by its high level of insecticide resistance and wide host range (van de Wetering, 1999).

Virion morphology of TSWV was revealed in the mid 1960s and genome organization and biochemical properties of its proteins were characterized in the 1990s (Moyer, 1999). With the advancement of molecular biology techniques, other biological and molecular characteristics of TSWV such as virus-vector relationships, cytopathology of the plant hosts, and the virus genetics have been elucidated rapidly since 1990. However, the lack of a reverse genetic system has hampered the increase in understanding of the molecular pathogenesis of TSWV.

II. Biological and Molecular Properties of TSWV

1. Classification

TSWV is the type species of the genus *Tospovirus* in the family *Bunyaviridae* (Fauquet, 1999). There are five genera in the *Bunyaviridae*: *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus* and *Tospovirus*. The *Tospovirus* genus is the only plant-infecting species and the four other genera include important human and animal viral pathogens. Most of these viruses are arboviruses (*arthropod-borne animal viruses*) which are transmitted by hematophagous arthropods, such as mosquitoes, biting flies, and ticks,

except Hantaan virus which is maintained in nature by rodents (Schmaljohn and Hooper, 2001).

TSWV was considered a unique member of a monotypic group of plant viruses until the second member, *Impatiens necrotic spot virus* (INSV) was described in the early 1990s (Law and Moyer, 1990; Law *et al.*, 1992). To date, fifteen species have been assigned to the *Tospovirus* genus (Table 2). However, intensive studies are needed for full characterization of newly isolated viruses like Zucchini lethal chlorotic virus (Resende *et al.*, 1996), Chrysanthemum stem necrosis virus (Resende *et al.*, 1996), Peanut yellow spot virus (Reddy *et al.*, 1990), Physalis severe mottle virus (Cortes *et al.*, 1998b), and Potato stem necrosis virus (Khurana *et al.*, 1998). Initially, each virus was placed under a serogroup in the order of discovery and named numerically (van de Wetering, 1999). But it was later suggested that viruses serologically related with the type member can be placed within the same serogroup (Moyer, 2000). According to the new serogroup system, these are two serogroups, tomato spotted wilt and watermelon silver mottle with eight ungrouped (serologically unrelated) species (Table 2).

The current classification of *Tospovirus* depends on serological relatedness based on nucleocapsid protein encoded by the S RNA (Moyer, 1999). As for classification of the other genera in *Bunyaviridae*, the complement fixation test which can define antigenic determinants on the nucleocapsid gene has also been used to distinguish between different viruses. In addition, the neutralization and hemagglutination inhibition assays to define epitopes on the glycoproteins can be used to classify members of the genera *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, and *Hantavirus* (Nathanson and Gonzalez-Scarano, 1999).

2. Organization of the TSWV

(1) Virus structure

Electron microscopic studies revealed that TSWV virions are quasi-spherical or pleomorphic particles ranging from 80 to 120 nm in diameter (Best and Palk, 1964; Van Kammen *et al.*, 1966). The lipid membrane envelope, which is derived from the host cell, surrounds a core containing three single-stranded RNA (ssRNA) molecules (Fig. 1). The nucleocapsid protein (N) is tightly bound to each RNA and these nucleocapsids are enveloped by lipid membrane with glycoprotein spikes (De Haan *et al.*, 1990). Two glycoproteins, labeled G_N and G_C, are embedded within this membrane and protrude from its surface (Mohamed, 1981; Tas *et al.*, 1977).

The TSWV virion contains three nucleocapsids each of which forms a pseudocircular structure with noncovalently closed circular RNA (De Haan *et al.*, 1991; De Haan *et al.*, 1989; Law *et al.*, 1991; Law *et al.*, 1992). Base-pairing of the terminal nucleotides (nt) accounts for the formation of stable panhandle structures. Terminal sequences of each of the RNAs are inverted complementary repeats. Among those inverted repeats, eight nts (3' UCUCGUUA... 5') are strictly conserved between the three genomic segments in all tospoviruses. This sequence is likely a cis-acting genome replication and transcription element (Moyer, 1999). Viruses, which belong to the other *Bunyaviridae* genera, share this property, but the conserved sequences differ between genera (Schmaljohn and Hooper, 2001).

Unlike positive RNA viruses, which are positive sense and thus act directly as template for translation, the genome of negative RNA viruses cannot be translated. The transcription step giving rise to positive sense RNA is required to express viral proteins (Nguyen and Haenni, 2003). The requirement of this mandatory transcription process in the negative RNA viruses forces TSWV to package its RNA-dependent RNA polymerase (RdRp) into its virion (German *et al.*, 1992).

(2) Genome organization and expression strategy

TSWV has three ssRNAs, designated L (large), M (middle), and S (small) in order of decreasing their size (Fig. 2). The L RNA is approximately 8.9 kb and of negative polarity possessing one large open reading frame (ORF) which encodes a 331.5 kDa RdRp in the viral complementary (vc) sense (De Haan *et al.*, 1991). The viral L RNA is the template for replication and the vcRNA serves as the template for translation of the RdRp protein. The virion-associated RdRp is used to transcribe the L RNA into a segment-length mRNA, which encodes the L protein (De Haan *et al.*, 1991; Kormelink *et al.*, 1992b). The L protein has been identified as the putative RdRp through sequence homology with other *Bunyaviridae* L proteins and sequence motifs characteristic for polymerase of negative-strand RNA viruses (De Haan *et al.*, 1991; Tordo *et al.*, 1992).

The direct evidence for the association of the RdRp activity with TSWV virion was first demonstrated by using an *in vitro* assay for purified virions. An RdRp activity in purified virions was shown to synthesize each of the viral RNAs of about 3.0 kb and smaller heterogeneous products. The generation of small products indicates that one or more of viral or host factors might be required (Adkins *et al.*, 1995). Inhibition of TSWV RNA

synthesis with antibody against partial L protein was recently shown as an evidence for direct association of the L protein with RdRp (Chapman *et al.*, 2003).

The ambisense organization of TSWV M RNA was first revealed in *Impatiens necrotic spot virus* (INSV) (Law *et al.*, 1992) and confirmed in TSWV (Kormelink *et al.*, 1992a). This ambisense polarity of the genus *Tospovirus* is a unique feature among *Bunyaviridae* as other genera in this family have the same negative strategy for the M RNA expression (Nathanson and Gonzalez-Scarano, 1999). The 5.0 kb M RNA of TSWV has two ORFs. The smaller ORF located adjacent to the 5' end of the viral (v) sense RNA encodes a nonstructural protein (NSm) of 33.6 kDa whose function is linked to cell-to-cell movement of TSWV. Typical properties of viral movement protein such as synthesis only at early stage of infection, association with nucleocapsids, presence near plasmodesmata, and formation of tubular structure were found with TSWV NSm (Kormelink *et al.*, 1994; Storms *et al.*, 1995). In addition, it was shown that the NSm has binding activity to ssRNA and interacts with the N protein (Soellick *et al.*, 2000). Further indirect evidence that NSm is a movement protein was shown by multiple alignment of the NSm protein sequence of tospoviruses. The 'D-motif', which is a considered highly conserved element in '30K superfamily' of virus movement proteins (Melcher, 2000), was identified in the NSm of TSWV and the other tospoviruses (Silva *et al.*, 2001).

The larger ORF in the viral complementary-sense RNA encodes a 127.4 kDa polyprotein precursor for the two glycoproteins (G_N and G_C), which can be processed into G_C (78 kDa) and G_N (58 kDa) without the involvement of other viral proteins (Adkins *et al.*, 1996; Law *et al.*, 1992). Based on the similarity between TSWV glycoprotein precursor and G_C of *Bunyamwera virus* (*Orthobunyavirus*), and hydropathy plot of the precursor, it was assumed that the smaller glycoprotein (G_N) is located at the N-terminus and the larger one at the C-terminus (Kormelink *et al.*, 1992a; Law *et al.*, 1992). However, precise mapping studies for the functional domains of the G_N/G_C such as the cleavage site and glycosylation site of the G_N/G_C are required to further understand the biological functions of the glycoproteins in thrips vectors and plant hosts. Both ORFs in the M RNA are expressed from subgenomic mRNAs transcribed from opposite sense RNAs. The synthesis of subgenomic mRNAs may terminate at a stable hairpin structure, located at the A-U rich intergenic region (IGR) between the two ORFs (Kormelink *et al.*, 1992a; Law *et al.*, 1992). The glycoprotein precursor has a sequence motif of RGD (Arg-Gly-Asp) which is associated with cellular attachment of animal pathogens such as *Foot and mouth virus*, *Human coxsackievirus A9* and the spirochaete *Borrelia burgdorferi* (Berinstein *et al.*, 1995; Coburn *et al.*, 1998;

Kormelink *et al.*, 1992a; Roivainen *et al.*, 1991). The ambisense strategy of the M RNA of TSWV is a unique feature as other members of the *Bunyaviridae* has a negative polarity of the M RNA (Nathanson and Gonzalez-Scarano, 1999).

The S RNA is approximately 3 kb with an ambisense coding strategy similar to the M RNA. The sequence contains two ORFs, one in the viral sense which encodes a 52.4 kDa nonstructural protein (NSs) and one in viral complementary sense which encodes the 28.8 kDa viral nucleocapsid protein (N) (De Haan *et al.*, 1990; Kormelink *et al.*, 1991). Both proteins are expressed by translation from separate subgenomic mRNAs that possibly terminate at the IGR of S RNA (De Haan *et al.*, 1990). The function of NSs was unknown until recent reports indicated that it has suppressor activity for post-transcriptional gene silencing (Bucher *et al.*, 2003; Takeda *et al.*, 2002). The NSs protein also accumulates in cytoplasm as inclusions like loose aggregate or paracrystalline rods of filaments (Kormelink *et al.*, 1991).

In addition to their primary function of encapsidation of viral nucleic acids, coat proteins of ssRNA viruses have been associated with several functions such as viral movement in their hosts, vector transmission, genome activation, and symptom expression (Callaway *et al.*, 2001). Richmond *et al.* showed that TSWV N protein binds ssRNA in a sequence-nonspecific manner and demonstrated multiple RNA binding domains (Richmond *et al.*, 1998). The molecular analysis of homotypic interaction of TSWV N protein using a yeast two-hybrid system and surface plasmon resonance identified two interaction domains and conserved amino acids crucial for the interaction (Uhrig *et al.*, 1999).

Cap snatching is a characteristic mechanism for transcription initiation of mRNAs in segmented, negative-strand RNA viruses. In this process, cap structures comprising between 12 and 20 5' nts are cleaved from host mRNAs by a virus-encoded endonuclease and are then used to prime viral transcription (Braam *et al.*, 1983; Caton and Robertson, 1980). Non-viral sequences of 12 -21 nts are located at the 5' ends of TSWV N and NSs mRNAs (Kormelink *et al.*, 1992c; van Poelwijk *et al.*, 1996). It was also demonstrated that capped sg mRNAs of *Alfalfa mosaic virus*, a positive strand RNA virus can serve as a cap donor for TSWV (Duijsings *et al.*, 1999). The combined analyses of mutated AMV RNAs and host mRNAs have led to improved insight into the requirements for the length and specific nt composition of cap donors during TSWV transcription initiation. Duijsings *et al.* showed that cleaved leader lengths could vary between 13 and 18 nts and a single base complementarity between the snatched leader and the TSWV RNA template may be sufficient for priming (Duijsings *et al.*, 2001).

(3) Morphogenesis of the TSWV

Early studies showed the involvement of aggregates of nucleocapsid aggregates (viroplasm), and paired parallel membranes, which are found in infected leaf tissues, with virus budding events (Ie, 1971; Milne, 1970). Kitajima *et al.* suggested three models for the morphogenesis of TSWV particles based on morphology. In the first model, nucleocapsids formed by viral RNAs and nucleocapsid protein bud through the endoplasmic reticulum and become wrapped with the glycoprotein-spiked membrane. In the second pathway, the glycoproteins may accumulate in the Golgi complex before the nucleocapsids are enveloped. Finally, in the third model, the glycoproteins could be reinserted into the endoplasmic reticulum after glycosylation in the Golgi system is finished (Kitajima *et al.*, 1992).

By using a protoplast infection system and immunocytochemistry, Kikkert *et al.* reported that TSWV particle morphogenesis occurred in the Golgi system (Kikkert *et al.*, 1999). In their model for TSWV maturation, the glycoproteins accumulate in the Golgi before the envelopment process, during which the nucleocapsids obtain a double membrane and these doubly enveloped particles form singly enveloped particles by fusing to the endoplasmic reticulum (Kikkert *et al.*, 1999). This process is different from the morphogenesis of the animal-infecting *Bunyaviridae* species. Accumulation of the glycoproteins in the Golgi system is also found in *Uukuniemi virus* (*Phlebovirus*) but, rather than producing the doubly enveloped particles as TSWV, animal-infecting bunyaviruses form singly enveloped particles by direct budding of nucleocapsids into the Golgi cisternae with the glycoproteins (Kuismanen *et al.*, 1984; Pettersson and Melin, 1996).

III. Vector Transmission

1. Thrips as a Vector of Tospoviruses

TSWV is transmitted exclusively plant-to-plant by thrips in nature while the virus can be maintained by mechanical means in the laboratory. Like other animal-infecting viruses in the *Bunyaviridae*, TSWV multiplies in its arthropod vector, thrips (Schmaljohn and Hooper, 2001). Thrips are very small insects in the order of *Thysanoptera*. Adults of thrips are only a few millimeters long. It is assumed that more than 8,000 species are distributed worldwide of which approximately 5,000 are now taxonomically identified. Two suborders and eight families are currently recognized in the order, but most of the known species belong to the family *Thripidae* (Mound, 1996). All vectors of tospoviruses are members of the genera *Frankliniella* and *Thrips* (Table 2). Mound suggests that the ability to transmit TSWV is due

to the result of evolution between these polyphagous thrips and the virus rather than morphological similarities among these thrips (Mound, 1996). In addition to transmitting TSWV, thrips cause significant damage by sucking up the exuding sap and inciting injury like a characteristic silvery scar (German *et al.*, 1992).

Thrips have two active feeding stages; 1st and 2nd instar larvae. These are followed by two non-feeding pupal stages and an adult stage. As for western flower thrips (WFT), two and four days are needed for each of the larval stages at 25°C, respectively. After these feeding stages, thrips move to the ground or hide in leaf buds for 3-4 days during which they complete the prepupal and pupal stages. Adult females deposit approximately 150-300 eggs into plant tissue. These eggs hatch in 3 days. Winged adult thrips can survive for 30 – 45 days, during which they can transmit TSWV if they have acquired it by feeding on an infected plants as a 1st instar larva. The long distance transport of TSWV by adult thrips can be done by wind (van de Wetering, 1999).

Thrips was first reported to be associated with transmission of 'spotted wilt' disease of tomatoes in 1927 (Pittman, 1927). At the time of Sakimura's seminal review of the status of thrips-transmitted viruses, four thrips species, *Thrips tabaci*, *Frankliniella occidentalis*, *F. schultzei* and *F. fusca* were linked with TSWV transmission (Sakimura, 1963). However, many populations of *T. tabaci* (onion thrips, OT) do not transmit current tospoviruses such as the Hawaiian TSWV isolate HAW, the Brazilian TSWV isolate BR-01, and *Tomato chlorotic spot virus* (TCSV) (Ullman *et al.*, 1997; Wijkamp *et al.*, 1995). At present, seven species of thrips are confirmed to transmit TSWV (Table 3). While western flower thrips, *Frankliniella occidentalis*, has been an efficient vector globally, the tobacco thrips, *F. fusca*, is the primary vector in eastern and central North Carolina (Groves *et al.*, 2002).

2. Biological Characteristics of Thrips-TSWV Interaction

One of the distinctive properties of TSWV-vector relation is that the only 1st instar larvae can acquire TSWV and adults derived from those can transmit the virus. It was also demonstrated that 2nd instar larvae of WFT can also transmit the virus with 2 – 10% lower efficiency than adults and 2nd instar larvae of *T. tabaci* can acquire the virus, depending on temperature (Chatzivassiliou *et al.*, 2002; Wijkamp *et al.*, 1993). The efficiency of acquiring TSWV declines rapidly as larval development proceeds (Chatzivassiliou *et al.*, 2002; Nagata *et al.*, 1999; van de Wetering *et al.*, 1996). The transmitting ability of 2nd larvae, however, may not be crucial in epidemics of TSWV because their plant-to-plant movement is limited. The primary transmitter is winged adults.

The latent period can be defined as the time interval from the start of the acquisition access period (AAP) to the end of inoculation access period (IAP) in which the first transmission occurred. Mean latent period is the time interval at which 50% of the larvae completed their latent period. Temperature affects the latent period for TSWV transmission by thrips. As for WFT, mean latent periods of 4 and 8 days were reported at 27°C and 20°C for TSWV, respectively, based on the result from a petunia leaf disc assay (Wijkamp and Peters, 1993).

The minimum AAPs for OT and WFT were reported as 15 and 5 minutes, respectively (Sakimura, 1963; Wijkamp *et al.*, 1996b). After five-minute acquisition, WFT transmitted TSWV with an average efficiency of 8.2%, suggesting small amounts of ingested virus would be sufficient for initiating successful inoculation. Optimum and mean AAPs were approximately 21 hours and 90 minutes for TSWV-WFT interaction. Similar to the AAP, approximately one or two hours of mean IAPs were shown by inoculating to petunia (*Petunia x hybrida*) or jimsonweed (*Datura stramonium*) (Wijkamp *et al.*, 1996b).

The viruliferous 1st instar larvae retain the virus during subsequent molting, pupation, and emergence to adults, which means that once the larvae ingest the virus, they can transmit the virus throughout their life span (transtadial passage) (Sakimura, 1963; Wijkamp *et al.*, 1996a). Newly deposited eggs from infected adult thrips, however, do not have TSWV (Wijkamp *et al.*, 1996a). Inability of insect vector, thrips to transfer TSWV to their offspring is quite uncommon in other arboviruses of *Bunyaviridae*, in which transovarial passage was known for genera *Orthobunyavirus*, *Phlebovirus*, and *Nairovirus*. For successful transovarial transmission of virus, the infection of ovaries is thought to be prerequisite, but there has been no report of TSWV presence in thrips ovaries (Nichol, 2001). Replication of plant viruses in their arthropod vector generally results in transovarial transmission of virus to eggs of the vector, although vertical transmission has not been shown for *Maize rayado fino virus* (*Marafivirus*), which is propagated in its vector, *Dalbulus maidis* (Gamez and Leon, 1983; Mandahar, 1990).

TSWV propagation in its thrips vector has raised questions concerning negative effects on viruliferous thrips. It is still controversial whether there are pathological effects on viruliferous thrips due to TSWV multiplication. As for other animal-infecting arboviruses in *Bunyaviridae*, there has been no evidence for detrimental effect on their arthropod vectors. In an early study of the interaction of TSWV with onion thrips (*Thrips tabaci*), there was no difference in longevity between the infected and no infected adult thrips (Sakimura, 1963). However, adverse effects on TSWV-infected thrips such as decreased survival and

reproductive potential, and slower developmental rate were observed when WFT were fed on infected plants with TSWV or INSV (DeAngelis *et al.*, 1993; Robb, 1989). However, Wijkamp *et al.* argued that those effects might be due to a nutritional deficiency of TSWV-infected leaf tissues rather than the virus infection itself and indicated no pathological effects in viruliferous WFT (Wijkamp *et al.*, 1996a). Mc Nulty showed that mortality of *F. fusca* was increased when the thrips were reared on leaves infected with TSWV and there was no difference in mortality of *F. fusca* confined on TSWV-D, a known thrips nontransmissible isolate, and healthy plants. This suggests that a reduction in the nutritional value of TSWV-infected plant may not be responsible for the increased mortality of *F. fusca*. But, the aforementioned author could not find increased mortality of WFT, that acquired thrips transmissible TSWV isolates (Mc Nulty, 2001). More comprehensive studies are needed to understand pathological effects of TSWV on its thrips vector.

Thrips tabaci (Onion thrips, OT) was the first recognized vector of TSWV, and has been considered an efficient vector of TSWV for decades (Pittman, 1927; Sakimura, 1963). However, the vector status of OT has been questioned because some populations transmit TSWV with very low efficiency or not at all (Jones, 1959; Paliwal, 1974; Paliwal, 1976; Ullman *et al.*, 1997; Wijkamp *et al.*, 1995). Incompatibilities among thrips populations and TSWV isolates have been considered a main reason for incompetence of OT to vector TSWV (Paliwal, 1976). However, several reports showed that reproduction strategy of OT may be involved in transmission efficiency (Chatzivassiliou *et al.*, 1999; Chatzivassiliou *et al.*, 2002; Tedeschi *et al.*, 2001). Populations of OT differ in their reproductive manner. Only females are found in thelytokous populations in which reproduction is performed by parthenogenesis, whereas both males and females constitute arrhenotokous populations where sexual reproduction is done. In arrhenotokous OT populations, the females are diploid and the males haploid, which means that the females are produced from fertilized eggs and the males from non-inseminated eggs (Lewis, 1973). Recent reports showed that three thelytokous populations of OT from different geographic regions failed to transmit (Wijkamp *et al.*, 1995) or transmitted TSWV poorly (Chatzivassiliou *et al.*, 1999; Chatzivassiliou *et al.*, 2002; Tedeschi *et al.*, 2001), whereas arrhenotokous populations isolated from bean and leek (*Allium porrum*) were also poor transmitters (Chatzivassiliou *et al.*, 1999; Chatzivassiliou *et al.*, 2002; Tedeschi *et al.*, 2001; Wijkamp *et al.*, 1995). Arrhenotokous OT populations obtained from infected tobacco plants transmitted TSWV efficiently and transmission efficiency remained unchanged when reared on tobacco for several generations. Arrhenotokous OT populations from leek plants, however, transmitted

with very low efficiency of average 3.1%, whereas TSWV could not be transmitted by thelytokous populations from leek. In the aforementioned paper, all populations of OT tested could infest leek, however larvae and adults originated from leek died when reared on tobacco irrespective of their reproductive manner. This shows that, in addition to the reproduction strategy of OT, host preference may be linked with inability of OT to transmit TSWV (Chatzivassiliou *et al.*, 2002). Assuming low transmission efficiency of thelytokous OT populations, transmission by OT with arrhenotoky strategy of reproduction might be more important than TSWV spread by thelytokous OT populations in terms of epidemics of TSWV.

3. TSWV Movement in Thrips Vector

Arthropod vectors of viruses in the *Bunyaviridae* can acquire and transmit viruses only at certain developmental stages (Nichol, 2001). The acquisition and transmission of bunyaviruses are limited to adult mosquitoes. Tick-borne viruses in genera *Phlebovirus* and *Nairovirus* can be acquired by larvae and transmitted by instars. TSWV-thrips interaction is more biologically complicated because the virus must be acquired by 1st larvae and transmitted by adults.

In the light of pathogenesis and taxonomy of the virus, it is significant to understand if a virus replicates in its arthropod vector. The multiplication and circulation of bunyaviruses, *Orthobunyavirus*, *Phlebovirus*, and *Nairovirus* genera, in their arthropod vectors has been well documented (Nichol, 2001). Replication of TSWV in its thrips vectors was demonstrated by using immunocytochemical technique and kinetics of viral proteins. A nonstructural protein coded by S RNA (NSs) is not found in purified TSWV but in infected plant cells with TSWV because it is translated via synthesis of sg mRNA transcribed from vc sense S RNA (Kormelink *et al.*, 1991). It was shown that the nucleocapsid (N) protein and NSs of TSWV were localized in epithelial and muscle cells of midgut, and salivary glands of viruliferous adult thrips (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993). The virus titer represented by the amounts of N and NSs proteins was increased after a 2 hour-AAP, which provides evidence for TSWV replication in the thrips vector (Wijkamp *et al.*, 1993). The replication of TSWV in thrips was further supported by multiplication of the virus in primary cell cultures from WFT and OT (Nagata *et al.*, 1997).

TSWV moves through the thrips body in a serial fashion. Briefly, TSWV enters the thrips midgut cells, which is the first site of viral infection. After replication in the midgut, TSWV translocates to salivary glands or other organs. TSWV infection of salivary glands is prerequisite for discharge of TSWV-laden saliva secretion into plant hosts which leads to

transmission of TSWV. For successful transmission, TSWV must break through several cellular barriers in the thrips during this stepwise manner of TSWV movement.

Thrips larvae ingest TSWV through taking up oozing sap of virus-infected plant cells. TSWV moves to midgut through the oesophagus. There has been no evidence for TSWV replication during food uptake. Using electron microscopic and immunohistological study, the midgut is shown to be the site where TSWV replicates for the first time (Tsuda *et al.*, 1996; Ullman *et al.*, 1993; Wijkamp *et al.*, 1993). A midgut infection barrier was suggested as adult thrips could not acquire TSWV by feeding on infected plant tissues (Ullman *et al.*, 1992b).

Several reports suggested that receptor-involved endocytosis may govern entry of TSWV into the thrips midgut. It was suggested that a 50 kDa protein in thrips midgut cells acts as a receptor and G_N of TSWV as counterpart (Bandla *et al.*, 1998; Medeiros *et al.*, 2000). The 50 kDa thrips protein was not found in other nonvector thrips and the protein was detected with high intensity in larvae (Bandla *et al.*, 1998). The G_N envelope glycoprotein was also identified as the viral determinant for the infection process of TSWV in thrips. A thrips protein of 94 kDa was reported to bind G_N. This protein, however, was also present in nonvector thrips and throughout the thrips body, while the 50 kDa protein is found mainly in midgut epithelial cells of viruliferous thrips larvae. Based on its localization of 94 kDa protein, it was hypothesized that this protein may be involved in TSWV circulation in the thrips body rather than in TSWV entry to thrips midgut (Kikkert *et al.*, 1998).

Additional indirect evidence for involvement of the glycoproteins in TSWV entry is that a specific motif of RGD (Arg-Gly-Asp), which is associated with cellular attachment of animal pathogens such as *Foot and mouth virus*, *Human coxsackievirus A9* and the spirochaete *Borrelia burgdorferi*, is present in the G_N ORF (Kormelink *et al.*, 1992a; van den Heuvel *et al.*, 1999). This motif is also present in INSV, which are transmitted by WFT and tobacco thrips (*Frankliniella fusca*) (Law *et al.*, 1992). It was also reported that an envelope-deficient isolate of TSWV failed to infect the midgut after ingestion and could not be transmitted by any thrips stage (Nagata *et al.*, 2000).

Though there is uncertainty regarding the mechanism of TSWV attachment to midgut, at least two hypotheses have been suggested for receptor-mediated entry of TSWV to midgut epithelial cells of thrips larvae (Ullman *et al.*, 2002). The first process is receptor-mediated endocytosis during which viral protein(s) may interact with cellular receptor(s) in the plasmalemma of thrips Mg1 epithelial cells initiating the cells allow processes necessary to engulf TSWV in an endosome. In the case of a virus which replicates in its insect vector,

the fusion between the virus and the endosome can be induced by mildly low pH, followed by an uncoating process during which the nucleocapsids and enzyme like RdRp are deposited into the vector cell (Nagata and Peters, 2001; Ullman *et al.*, 2002). This process was demonstrated to explain the entry of *La Crosse virus* (LAC) virus and *California encephalitis virus* (genus *Orthobunyavirus*) into mosquito cells (Hacker and Hardy, 1997; Ludwig *et al.*, 1989; Ludwig *et al.*, 1991). The second process was reported for human immunodeficiency virus (HIV) (Pierson and Doms, 2003). Instead of forming an endosome, the interaction between viral attachment protein (envelope glycoprotein in HIV) and receptor at target cell makes it possible to fuse the virus with cellular membrane followed by uncoating of the virus in a host cell.

The intestinal food canals of WFT are composed of foregut, midgut, and hindgut like other thrips species. The midgut is further divided with two loops, which forms three separate regions, designated as Mg1, Mg2, and Mg3 (Ullman *et al.*, 1992a; Ullman *et al.*, 1989). Immunohistological studies showed that the signal of infection is first found in Mg1, followed by infection of the muscle cells of Mg1, the other two midgut regions, and foregut (de Assis Filho *et al.*, 2002; Nagata *et al.*, 1999). The process of TSWV transport from Mg1 to Mg2, Mg3, and foregut invites further studies.

In addition to the midgut infection barrier, three more cellular barriers such as midgut escape, midgut to salivary gland transport, and salivary gland infection barriers were suggested (Nagata *et al.*, 1999; Ullman *et al.*, 1992b). A midgut escape barrier in adults was suggested as the TSWV escape from midgut is restricted to larvae (Ohnishi *et al.*, 2001; Ullman *et al.*, 1992b). The basal lamina, which is a thick extracellular matrix present on the basal membrane of the midgut, is thought to be a physical barrier for midgut escape as indicated in mosquito-borne *La Crosse virus* (genus *Orthobunyavirus*) (Grimstad and Walker, 1991; Nagata *et al.*, 2002). There is, however, no conclusive evidence that this thick matrix acts as a controller for TSWV transport from the midgut. In addition, a mechanism governing the virus escape through the basal lamina is not known. As for the dissemination or transport of virus from the midgut to the salivary glands, a 94 kDa thrips protein is thought to be involved in virus circulation through the thrips (Kikkert *et al.*, 1998).

The salivary gland barrier was originally suggested because TSWV is found in the salivary glands and salivary ducts of viruliferous larvae and adults (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993). This barrier was recently demonstrated in a nontransmitting thelytokous population of *T. tabaci* (OT). In a comparative study of vector competence between WFT and OT, accumulation of N protein in midgut of OT larvae was lower than in

one of WFT larvae and no infection in salivary glands of OT larvae was found (Nagata *et al.*, 2002). It would be interesting to do a similar experiment with antibody against NSs.

Little is known about the pathway of TSWV from midgut to salivary gland. Three model processes were hypothesized (Nagata *et al.*, 2002). The first model is a transport of the virus from midgut to salivary gland through the hemocoel as proposed for mosquito-borne arboviruses (Hardy *et al.*, 1983). The second pathway is a translocation of the virus via ligament, which is a thin structure connecting Mg1 and the salivary gland (Nagata *et al.*, 1999; Ullman *et al.*, 1989). The third proposed model may involve TSWV movement to the salivary gland through tubular salivary glands (Ullman *et al.*, 1992a). Recent reports support the second model in which the ligaments are involved in TSWV translocation from midgut to salivary gland (de Assis Filho *et al.*, 2002; Nagata *et al.*, 1999; Nagata *et al.*, 2002). No virions were found in tubular salivary glands of infected larvae and the ligaments were infected earlier than the salivary glands.

Most of studies made on determinants for thrips transmissibility have been focused on cellular and biochemical aspects of the TSWV-thrips interaction. The glycoproteins of TSWV have been involved in TSWV entry to midgut and circulation throughout the thrips body (Bandla *et al.*, 1998; Kikkert *et al.*, 1998; Medeiros *et al.*, 2000). In addition to the association of glycoproteins with TSWV transmission, it was reported that an envelope-deficient TSWV isolate and an isolate containing defective interfering (DI) RNA, which were generated by repeated mechanical transmission, could not be vectored by thrips (Nagata *et al.*, 2000). Point mutations or a very small deletions in the M RNA were suggested to be a cause of the envelope-defective mutant (Resende *et al.*, 1991), but there are no sequence data supporting this hypothesis. Defective interfering RNAs of TSWV have been found only in the L RNA where a single internal deletion resulted in 2.0 – 5.2 kb in length (Inoue-Nagata *et al.*, 1998). It was shown that a TSWV isolate having DI RNA, which suppresses normal L RNA functions and attenuates symptom expression, was not transmitted by WFT. However, when other DI RNA-containing isolates that barely interfered with symptom expression were tested, they were transmitted with same efficiency as wild type TSWV (Nagata *et al.*, 2000). All DI RNAs reported for TSWV are shortened forms of the L RNA which encodes a putative RdRp. This suggested that the L RNA and/or RdRp are also responsible for thrips transmission of TSWV but there is no conclusive evidence for that.

Vector transmission of the plant virus in a propagative manner is the outcome of coordinative interaction between a plant host, the virus, and arthropod vector. However, there is a limited body of knowledge on the molecular genetic basis for thrips transmission.

Except for the involvement of the glycoproteins, which are encoded by the M RNA, in TSWV-thrips interaction, little is known about functions of other viral proteins in TSWV movement through thrips body. The plant host factors responsible for virus life cycle in the plant have been elucidating since early 1990s, especially for virus replication and movement in a host plant (Ahlquist *et al.*, 2003; Haywood *et al.*, 2002; Maule *et al.*, 2002). It would not be unreasonable to speculate that host protein(s) may be associated with vector transmission of plant viruses, at least for a plant-infecting arbovirus like TSWV. Host factor(s) of plant host which are involved in thrips transmission need also to be uncovered in the future.

IV. Variability of TSWV

Genetic variations of virus are the result of errors occurred during viral genome replication. For RNA viruses, these errors are attributable to the error-prone activity of their RdRp, which do not have proofreading capability (Domingo and Holland, 1997). Plant viruses utilize three major driving forces for their evolution: mutation, recombination, and reassortment (Roossinck, 1997). Mutation and genome segment reassortment are believed to be major driving forces for RNA viruses with divided genomes like viruses in the family *Bunyaviridae* (Elliot, 1995). It has been thought that recombination is a rare event in negative-sense viruses. To date, recombination in members of *Bunyaviridae* has been reported only in the genus *Hantavirus* (Klempa *et al.*, 2003; Sibold *et al.*, 1999; Sironen *et al.*, 2001).

The heterogeneity of TSWV has been reported since early studies on biological diversity of TSWV. Norris separated five strains of TSWV from naturally occurring TSWV complexes based on the responses to host plants and suggested that the diverse symptoms on different hosts was caused by the variable ratio of TSWV strains in a viral complex (Norris, 1946). This method of strain separation was improved by using symptom difference on tomato plants with different (Finlay, 1952; Finlay, 1953). Finlay differentiated ten distinct strains according to their responses to five tomato plants whose genotypes are susceptible or resistant to TSWV. *Lycopersicon pimpinellifolium*, one of the tomato plants he used, was found to be resistant to TSWV before his experiment. However, a resistance-breaking virulent strain was isolated from a *L. pimpinellifolium* plant infected with a complex of TSWV. Based on these results, he hypothesized that the synergism of TSWV strains could cause the breakdown of resistance. In his cross protection experiments of TSWV, Best explained the result by postulating that presumably the “character determinants” could exchange

between two strains of TSWV during the virus replication in the mixed infected host cells (Best, 1954). Based on the phenotypic markers, Best was able to isolate three “recombinants”, which are now thought to be reassortants from plants co-inoculated with two parental strains and proposed that the new strains could arise by an exchange of character determinants between the two parental strains (Best, 1961). These early findings imply the exchange of genetic information might exist in a complex population of TSWV in nature and might be a driving force for evolution of TSWV.

The availability of polyclonal and monoclonal antibodies to viral proteins has made it feasible to investigate serological relatedness among TSWV isolates (Adam *et al.*, 1991; De Avila *et al.*, 1990; Law and Moyer, 1990; Sherwood *et al.*, 1989; Wang and Gonsalves, 1990). Polyclonal or monoclonal antibodies to the N proteins have been suitable for serotyping TSWV isolates. The conserved property of the N proteins of TSWV isolates was shown such that isolates from different hosts and geographical regions were serologically related when compared using the monoclonal antibodies to the N protein (Sherwood *et al.*, 1989). The use of antibodies has played an important role in understanding both diversity and molecular pathogenesis of the virus. However, as molecular techniques advance, sequence data of whole or partial virus genome are being used for phylogenetic analysis and classification of virus.

Since the early 1990s study of diversity of TSWV has been focused on the elucidation of molecular diversity with techniques of molecular biology. Based on sequence comparisons of the N gene of tospoviruses, de Avila *et al.* showed that the N gene was divergent between serogroup I (TSWV) and III (INSV). They also proposed serogroup II (*Tomato chlorotic spot virus* and *Groundnut ringspot virus*) which includes two previously described TSWV isolates because the N gene sequences of these two isolates diverged significantly from serogroups I and III (De Avila *et al.*, 1993). This proposal was later supported by a cladistic analysis of 18 tospoviruses with partial RNA sequences of the N gene (Dewey *et al.*, 1997). Unlike divergence in the N genes between serogroups, a high degree of sequence conservation among the N genes of TSWV isolates was reported (Pappu *et al.*, 1998). Sequence variability was also assessed by comparing the intergenic region (IGR) of the M and the S RNA of TSWV strains, and the NSm genes of tospoviruses (Bhat *et al.*, 1999; Pappu *et al.*, 2000; Silva *et al.*, 2001). The phylogenetic tree constructed with the NSm protein sequences of tospoviruses was shown to be similar to the one from the N proteins (Silva *et al.*, 2001).

Taken together the variation in symptom development, serological and molecular diversity, current knowledge of TSWV strongly supports the heterogeneous property of TSWV in nature. However, evolutionary mechanisms driving the variability of TSWV need to be uncovered. To date, most of the molecular phylogenetic studies have been performed with partial nucleotide sequence or only one ORF of TSWV genome. As stated earlier in this introduction, TSWV has three genomic RNA segments, which encode 5 proteins. Comprehensive evolutionary research with at least one whole genome sequence of TSWV awaits further studies. Phylogeographical investigation with TSWV isolates which have different geographical origins would be a good candidate for future research.

V. Genetics of TSWV

There is a limited body of knowledge regarding genetics of the *Tospovirus*. Unavailability of a reverse genetic system has impeded understanding of the viral life cycle including genetics and pathogenesis of TSWV. A reliable reverse genetic system is required to understand the molecular basis of viral pathogenesis. However, viral RNA from negative-strand viruses such as members in the family *Bunyaviridae* is not infectious, which has been a limiting factor for associating biological functions with specific viral proteins. Recent progress toward development of a system for rescue of infectious transcripts from viral cDNA has been made on *Bunyamwera virus* (*Orthobunyavirus*) and *Uukuniemi virus* (*Phlebovirus*), but there is no such system for *Tospovirus* (Bridgen and Elliott, 1996; Flick and Pettersson, 2001).

In the absence of a reverse genetic system, reassortment has been investigated as an alternative tool for linking a viral function to a specific viral genome or genomes in the viruses in the *Bunyaviridae*. During the mixed infection of viruses with segmented genomes, the newly synthesized genome segments from one isolate can reassort with segments from the other in different combinations to generate a genetically distinct isolate. This phenomenon is different from recombination, which results in the exchange of subsegment regions of genome segments and can occur with any virus, whereas reassortment is believed to be unique to viruses with segmented genomes (Pringle, 1996). Evidence of reassortment has been shown for *Arenaviridae*, *Bunyaviridae*, and *Orthomyxoviridae*, all of which have segmented ss RNAs in a single component. In addition, viruses with double-stranded RNAs such as *Birnaviridae*, *Cystoviridae*, and *Reoviridae* employ reassortment as a strategy for increasing their genetic heterogeneity (Pringle, 1996). Plant viruses also use divided genomes as a strategy for their gene expression in host plants (Hull, 2002).

Reassortment has been reported for the genus *Bymovirus* (*Potyviridae*), *Bromoviridae*, *Furovirus*, *Dianthovirus*, *Enamovirus*, *Hordeivirus*, and *Tobravirus* (Pringle, 1996). Except for phytoreoviruses and the genus *Tospovirus*, genomes of the viruses mentioned above are packaged in 2 or 3 components. Reassortants or pseudorecombinants generated artificially have been used as a practical tool for genetic analysis of viral genes (Roossinck, 1997).

The subunit reassortment studies in the genus *Orthobunyavirus* showed that genome segments can exchange only between viruses in the same serogroup or closely related isolates in a single virus (Elliott *et al.*, 1984; Pringle *et al.*, 1984). Elliott *et al.* demonstrated that no evidence of reassortment was found between viruses belonging to different serogroups in the *Orthobunyavirus* after extensive crosses of the viruses in various host cells including both vertebrate and mosquito cell lines (Elliott *et al.*, 1984). This suggests that a barrier in genome segment reassortment exists between the viruses belonging to different serogroups. This incompatibility in reassortment was also indicated even between viruses in the same serogroup as all six viruses of the California serogroup tested could exchange genome segments, whereas only five of the eight Bunyamwera serogroup could reassort (Elliott *et al.*, 1984; Pringle, 1996). Though the mechanisms underlying the restricted segregation are still not fully understood, the serological relatedness or the similarity of the genes and/or gene products were suggested to explain this incompatibility for reassortment of bunyaviruses and influenza viruses, respectively (Hunt and Calisher, 1979; Klenk and Rott, 1988; Pringle, 1996).

In addition to the incompatibility during genome segment exchange, the restricted segregation and non-random distribution of reassortants has been also demonstrated. Gentsch *et al.* could rescue only four reassortants out of the six theoretically possible reassortants between *Snowshoe hare virus* (SSH) and *La Crosse virus* (LAC) which belong to California encephalitis serogroup in *Orthobunyavirus* (Gentsch *et al.*, 1977; Gentsch *et al.*, 1979). In the more critical analysis of reassortment between LAC virus and SSH virus, Urquidi and Bishop could obtain the remaining two reassortants and showed that the eight reassortants including parental isolates did not distribute randomly (Urquidi and Bishop, 1992). This non-random segregation was also demonstrated in subgroup 2 *Human rotavirus* and type 1 Lang and type 3 *Deering reovirus*, both of which are members of the *Reoviridae* (Nibert *et al.*, 1996; Ward *et al.*, 1988). Qiu *et al.* confirmed the non-random distribution of reassortants by comparing expected and observed frequencies of reassortants of TSWV isolates (Qiu *et al.*, 1998).

By using reassortment as a tool for functional mapping in bunyaviruses, several phenotypes such as neutralization, virulence in mice, transmission and dissemination in mosquito vector, and neuroinvasiveness have been assigned to specific viral genomic segment(s) (Beaty *et al.*, 1981; Beaty *et al.*, 1982; Gentsch *et al.*, 1980; Griot *et al.*, 1993). The genetic analysis system including the reassortment and segment-specific restriction fragment length polymorphisms (RFLP) was developed for TSWV, by using the marker system to associate specific genome segments with viral phenotypes (Qiu *et al.*, 1998). The result demonstrated that TSWV isolates readily exchanged genome segments in a nonrandom fashion and the intergenic region (IGR) of the S RNA was correlated with competitiveness of the individual segments in reassortant isolates. As suggested by a survey of *Cucumber mosaic virus* (CMV) in Spain (Fraile *et al.*, 1997), reassortment may not be frequent in nature. However, its impact on the evolution of viruses may be tremendous such as an expansion of host range and an adaptation to suppress the host resistance reaction (Fraile *et al.*, 1997; Qiu and Moyer, 1999). With the same genetic analysis system as Qiu *et al.* used (Qiu *et al.*, 1998), Hoffman *et al.* showed that the M RNA of TSWV might be involved in overcoming host- and pathogen-mediated resistance in tomato and tobacco (Hoffmann *et al.*, 2001). In addition, overcoming host resistance of *Sw-5* in tomato and *Tsw* in pepper mapped to the M and S RNA of TSWV, respectively (Jahn *et al.*, 2000). It was recently reported that the S RNA of *Watermelon silver mottle virus*, a cucurbit-infecting tospovirus, determined symptom expression in *Tetragonia expansa* by reassortment-based genetic analysis system (Okuda *et al.*, 2003).

VI. Scope of the Dissertation

Viral diseases of plants are difficult to control with pesticides unlike diseases caused by fungi, bacteria, and nematodes. Control of insect-borne viral diseases such as TSWV is more complicated than other viral diseases which are not transmitted by vectors because the diseases caused by insect-borne viruses are the outcome of complicated interactions among plant hosts, insect vectors, and the viruses. Moreover, novel control measures are definitely needed to establish a durable control strategy for TSWV, which is transmitted by thrips in a propagative fashion. The knowledge of the viral fate in thrips will be of help to set up strategies for diminishing losses caused by TSWV. In the beginning of this research, most of the information on thrips transmission of TSWV has been focused on elucidating cellular and biochemical interactions. There is a limited body of knowledge on viral genetic basis of TSWV-thrips interactions.

Experiments were carried out in an effort to understand the viral genetic determinant(s) associated with thrips transmission of TSWV. The objectives of this project were, i) to determine the genomic segment(s) of TSWV which are responsible for thrips transmission by using a viral genetic system including reassortment and segment-specific RFLPs. ii) to map the specific viral genomic region(s) in the genome segment(s) involved in thrips transmission. iii) to isolate thrips transmissible and non-transmissible isolates from a TSWV isolate, which has a low transmissibility. iv) to analyze the degree and type of mutations that accumulate during repeated mechanical or thrips transmission of TSWV isolates.

As mentioned earlier, a reliable system for rescuing TSWV from cloned viral cDNA is needed to increase the understanding of the viral life cycle. Unfortunately, there are no reports on a reverse genetic system for TSWV. The viral genetic system used in this study was confirmed to be a successful tool for identifying the viral genomic region associated with thrips transmission. The information obtained in this dissertation will be used for understanding a mechanism governing thrips transmission of TSWV in combination with a reverse genetic system which will be developed in near future.

Table 1. Viruses in the family *Bunyaviridae*

Genus	Representative Virus	Genome Organization			Host	Principal Vector
		Large (L)	Medium (M)	Small (S)		
<i>Orthobunyavirus</i>	<i>Bunyamwera virus</i>	- ¹	-	-	Human, Sheep, Cattle, Equine	Mosquitoes
<i>Phlebovirus</i>	<i>Uukuniemi virus</i>	-	-	± ¹	Human, Cattle	Ticks ²
<i>Nairovirus</i>	<i>Dugbe virus</i>	-	-	-	Human, Cattle Seabirds	Ticks
<i>Hantavirus</i>	<i>Hantaan virus</i>	-	-	-	Human	Rodents
<i>Tospovirus</i>	<i>Tomato spotted wilt virus</i>	-	±	±	Plants	Thrips

1. – and ± means negative and ambisense genome organization, respectively.

2. Viruses in the sandfly fever group in *Phlebovirus* genus are transmitted by phlebotomine flies (Calisher, 1996).

Table 2. Classification of the genus *Tospovirus*

Serogroup	Numerical serogroup	Species	Geographical Distribution	Vector
Tomato spotted wilt	I	<i>Tomato Spotted Wilt Virus</i> ¹	World-Wide, except for extreme regions	<i>Frankliniella bispinosa</i> , <i>F. intonsa</i> (FI) <i>F. fusca</i> (FF), <i>F. occidentalis</i> (FO) <i>F. shultzei</i> (FS), <i>Thrips setosus</i> , <i>T. tabaci</i> (?) ¹⁷
	II	<i>Groundnut Ringspot Virus</i> ²	South Africa, Brazil, Argentina	FO, FS
	II	<i>Tomato Chlorotic Spot Virus</i> ³	Argentina, Brazil	FI, FO, FS
	IX	Zucchini Lethal Chlorotic Virus ^{4,5}	Brazil	Not Known
	VIII	Chrysanthemum Stem Necrosis Virus ^{4,6}	Brazil, the Netherlands	FO, FS
Watermelon silver mottle	IV	<i>Watermelon Silver Mottle Virus</i> ⁷	Japan, Taiwan	<i>Thrips palmi</i> (TP), FS
	IV	<i>Watermelon Bud Necrosis Virus</i> ⁸	India	TP
	IV	<i>Groundnut (Peanut) Bud Necrosis Virus</i> ⁹	India, Iran	FO
Serologically unrelated	III	<i>Impatiens Necrotic Spot Virus</i> ¹⁰	USA, the Netherlands, Italy, France, Portugal, Iran	FO, FF
	VI	Iris Yellow Spot Virus ¹¹	Brazil, Israel, the Netherlands, USA	<i>T. tabaci</i>
	X	Peanut Chlorotic Fan-spot Virus ¹²	Taiwan	TP, <i>Scirtothrips dorsalis</i> (SD)
	V	Peanut Yellow Spot Virus ¹³	India, Thailand	TP, SD
	VII	Physalis severe mottle virus ¹⁴	Thailand	<i>T. tabaci</i> (?)
		Potato Stem Necrosis Virus ¹⁵	India	TP (?), <i>T. flavus</i>

		Melon yellow spot virus ¹⁶	Japan	TP
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1. (de Haan, 1991) 2 – 3. (De Avila *et al.*, 1993)

4. These two viruses were grouped into tomato spotted wilt serogroup based on homology of sequence of the amino acid and the nucleotide for nucleocapsid (N) protein (Chu *et al.*, 2001).

5 – 6. (Bezerra *et al.*, 1999) 7. (Yeh and Chang, 1995) 8. (Jain *et al.*, 1998) 9. (Satyanarayana *et al.*, 1996) 10. (Law and Moyer, 1990) 11. (Cortes *et al.*, 1998a) 12. (Chen and Chiu, 1996) 13. (Reddy *et al.*, 1990) 14. (Cortez *et al.*, 2001) 15. (Khurana *et al.*, 1998) 16. (Kato *et al.*, 2000) 17. Vector status is currently questionable or has not been fully demonstrated.

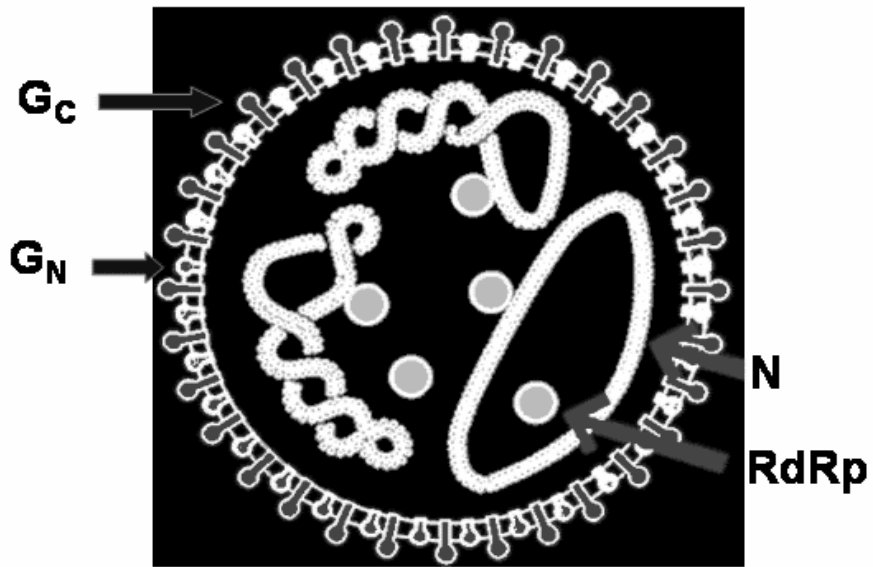


Fig. 1. Morphology of TSWV.

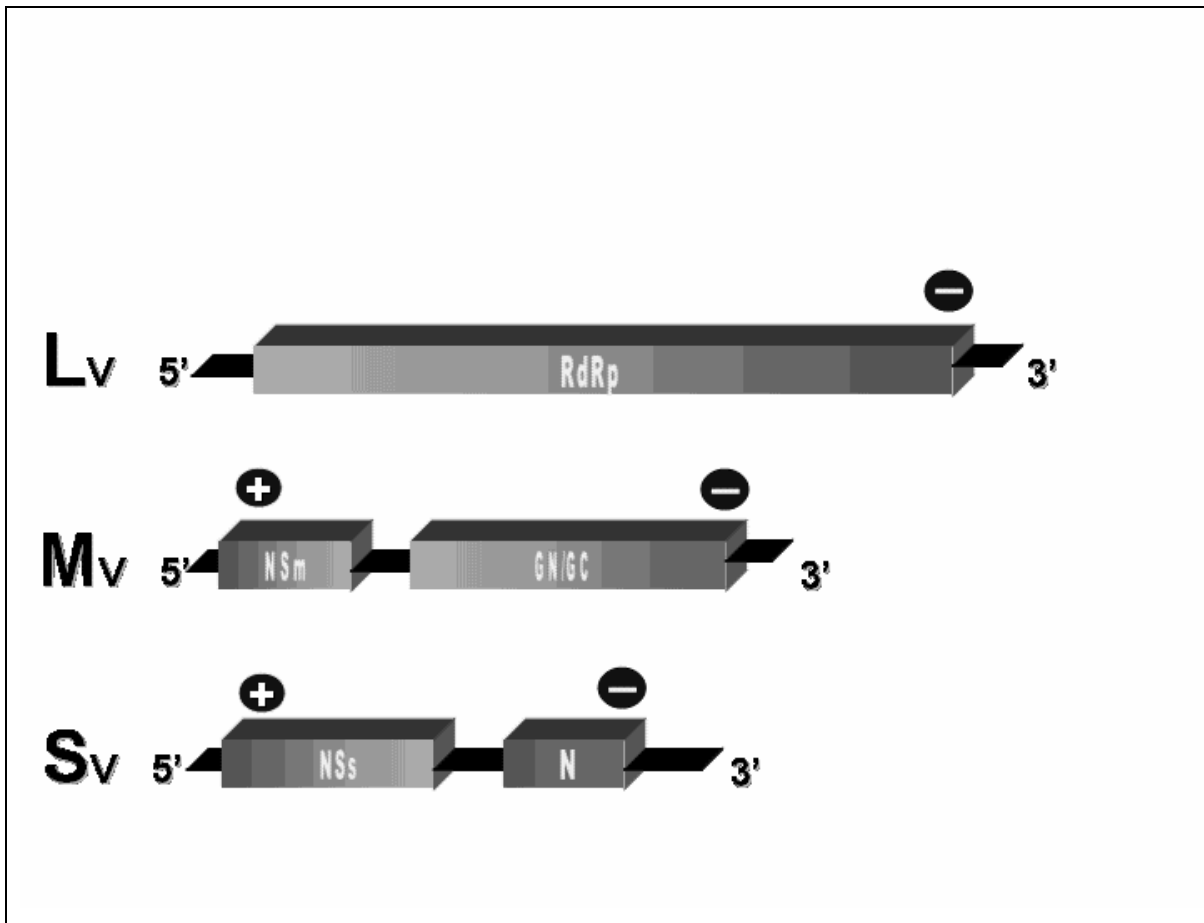


Fig. 2. Genome organization of TSWV.

Table 3. Thrips species vectoring TSWV

Scientific name	Common name	Reference
<i>Frankliniella bispinosa</i>	Florida flower thrips	(Webb <i>et al.</i> , 1998)
<i>F. intonsa</i>	European flower thrips	(Wijkamp <i>et al.</i> , 1995)
<i>F. fusca</i>	Tobacco thrips	(Sakimura, 1963)
<i>F. occidentalis</i>	Western flower thrips	(Gardner <i>et al.</i> , 1935)
<i>F. shultzei</i>	No approved common names	(Wijkamp <i>et al.</i> , 1995)
<i>Thrips setosus</i>	No common names	(Fujisawa <i>et al.</i> , 1988; Tsuda <i>et al.</i> , 1996)
<i>T. tabaci</i> ¹	Onion thrips	(Sakimura, 1963)

1. Its status of TSWV vector had become questionable (Wijkamp *et al.*, 1995).

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**Characterization of Genetic Changes after Serial Mechanical or
Thrips Transmission of *Tomato spotted wilt virus*¹**

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Summary. The thrips transmission phenotype was genetically mapped to the medium (M) RNA of *Tomato spotted wilt virus*. In addition, transmissible and nontransmissible TSWV populations derived from single lesions were analyzed by nucleic acid sequencing. The purpose of this investigation was to determine the frequency with which mutations accumulate due to maintenance in plant hosts via serial mechanical transfers and maintenance from plant to plant via thrips transmission. Three TSWV isolates (RG2, CFL, and GT) were divided into two subgroups and passaged 24 times by mechanical or thrips transmission. The M RNA was monitored for the accumulation of mutations by sequence analysis. The mean mutation frequency of the NSm open reading frame (ORF) for the three isolates per passage was 9.2×10^{-5} nucleotide substitutions per base per mechanical or thrips passage. A similar pattern was observed in the G_N/G_C ORF with 1.1×10^{-4} and 1.3×10^{-4} nucleotide substitutions per base per mechanical or thrips passage, respectively. The mean mutation frequency accumulated in the whole M RNA of the 3 isolates was 1.7×10^{-4} or 1.3×10^{-4} nucleotide substitutions per base per mechanical or thrips passage, respectively. Two nonsynonymous (NS) mutations and one synonymous (S) mutation in the G_N/G_C ORF were conserved in the three isolates after 24 mechanical cycles. Two NS mutations were a substitution of C⁸⁶ to U changing amino acid T²⁹ to I and U¹³⁷³ to C changing amino acid M⁴⁵⁸ to T, while one synonymous mutation was a substitution G⁴⁹² to A. Two NS substitutions, changes of U¹⁹⁷⁶ to C converting amino acid V⁶⁵⁹ to A and A²¹⁵⁰ to G converting amino acid Y⁷¹⁷ to C, were found in the G_N/G_C ORF of TSWV-GTN, a nontransmissible isolate. These may be implicated in the loss of thrips transmissibility of TSWV. Our data can be used for modeling TSWV evolution under a major selective force such as extended passaging through plant host or alternating replication of TSWV between thrips vector and plant host.

Introduction

Tomato spotted wilt virus (TSWV) is a thrips-transmitted, plant-infecting virus (*Tospovirus*; *Bunyaviridae*). TSWV and other tospoviruses are serious threats to many agricultural and ornamental crops. TSWV infects more than 900 plants spanning both dicotyledons and monocotyledons [28]. The TSWV virion is a roughly spherical particle ranging from 80 to 110nm in diameter. A host-derived lipid envelope with 2 outward projecting glycoproteins circumscribes the three viral genomic RNA segments of TSWV [16]. The largest (L) RNA encodes a putative RNA-dependent RNA polymerase (RdRp) [1, 7]. The M RNA encodes the glycoprotein precursor for G_N/G_C found in the envelope and a nonstructural protein (NSm), while the small (S) RNA has two ORFs encoding the nucleocapsid (N) protein and a nonstructural protein (NSs) [6, 8, 17, 18].

To date, at least seven thrips species have been shown to vector TSWV [40]. Among those, western flower thrips (*Frankliniella occidentalis* [WFT]), has been considered one of the most efficient vectors worldwide, while the tobacco thrips (*F. fusca* [TT]) is the primary vector in the southeastern USA including North Carolina [11]. TSWV replicates both in its plant host and in its thrips vector [39, 42]. Previous reports indicated that the envelope glycoproteins (G_N/G_C) and/or the L RNA were associated with thrips transmissibility [2, 15, 23, 24]. However, using genetic mapping, we confined a determinant for thrips transmissibility to only the M (See Chapter 2) [36].

The TSWV-thrips interaction is distinctive among 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 [32, 41]. It was also shown that thrips transmissibility of TSWV declined as mechanical passage continued but it was restored to the original level after one thrips transmission [22]. In this investigation, we have documented evolutionary changes that occurred following mechanical or thrips transmission of TSWV. Previous studies have only investigated the diversity of TSWV using isolates at a single point in time [3, 5, 9, 27, 35].

There are previous evolutionary studies after serial mechanical transmission for plus-strand RNA viruses infecting plants. High genetic stability was reported after 20 serial passages in a systemic or local lesion host using RNase T1 fingerprinting analysis for 12% of the *Tobacco mild green mosaic virus* (*Tobamovirus*) genome [31]. The RNase protection analyses of *Satellite tobacco mosaic virus* (STMV, *Satellivirus*) populations after 10 serial passages suggested that there may be hot spots for the genetic change in the STMV genome [20]. It was demonstrated that the Sindbis-like plant viruses of *Tobacco mosaic*

virus (TMV, *Tobamovirus*), *Cucumber mosaic virus* (CMV, *Cucumovirus*: *Bromoviridae*), and *Cowpea chlorotic mottle virus* (CCMV, *Bromovirus*: *Bromoviridae*) have different levels of population diversity after 10 subsequent passages in a common host, even though they have probably a common evolutionary ancestor [33]. The population diversity of RNA virus quasispecies such as TMV, CMV, and CCMV was shown to be governed by host-virus interactions, as the diversity remained constant in a given host but changed in a new host [34]. The effects of host shifts on TMV evolution was investigated by transferring the initial TMV populations 11-12 times in different host species [14]. In *Hibiscus chlorotic ringspot virus* (*Carmovirus*: *Tombusviridae*), it was demonstrated that virulence in its systemic host was lost after repeated mechanical transmission in its local lesion host and 3 NS mutations found in capsid protein were known to be associated with avirulence in a systemic host of the virus [21]. The diversity of TSWV has been determined by analyzing sequence heterogeneity of the N gene from various isolates [5, 9]. Sequence variability was also assessed by comparing the intergenic region (IGR) of the M and the S RNA of TSWV isolates, and the NSm gene from other tospoviruses [3, 27, 35].

There are previous studies on evolution of arboviruses maintained in constant environment (replication in mammalian or insect cells) and changing environments (alternated replication between mammalian and insect cells). It was reported that replicative fitness of *Vesicular stomatitis virus* (*Vesiculovirus*: *Rhabdoviridae*) passaged alternately between mammalian and insect cells was similar to or larger than that observed in a constant environment (in mammalian cells or insect cells) [25, 26]. More mutations accumulated during serial passages of *Dengue 2 virus* (*Flavivirus*: *Flaviviridae*) in mammalian cells than in alternate passages between mammalian and insect cells, while no mutations were found in the virus that was continuously cultured in insect cells [4]. However, little is known about the genetic change over time after repeated mechanical or vector-based transmission (alternating replication) of plant viruses like TSWV which replicate in the vector species.

Genetic analysis of TSWV has been impeded due to the lack of a reverse genetic system. Given this limitation, we employed an alternative approach to map the viral genomic region responsible for thrips transmission. To identify mutations responsible for thrips transmission and assess the effect of serial mechanical or thrips transmission on TSWV evolution, genetic changes in the M RNA from TSWV isolates were determined prior to and after serial mechanical or thrips transmission. This is the first investigation demonstrating

TSWV genetic changes accrued during prolonged replication in a plant host and alternating replication in plant and insect hosts.

Materials and methods

Virus isolates and thrips colony

Three TSWV isolates, TSWV-RG2, -GT, and -CFL [22], were used for mechanical or thrips passaging. It was recently found that a subisolate of TSWV-GT (TSWV-GTN) could not be transmitted by TT or WFT (G. G. Kennedy, unpublished). The TSWV-GTN was used for sequence comparison to that of transmissible TSWV-GT. WFT was used in all thrips transmission assays. Thrips were maintained on pole bean (*Phaseolus vulgaris*) pods in a white plastic container held at 24°C, 65% relative humidity and a photoperiod of 14:10 hr (light/dark). The tops of the containers were covered with Bugbed 110 (Green Thumb Group, Downers Grove, IL) fine mesh thrips screening.

Passaging of virus isolates

Each of the three TSWV isolates was initially divided into three subgroups. One subgroup was stored frozen at -80 °C for analysis as the starting control. The second subgroup of each isolate was continually maintained in plants via 24 mechanical passages (plant lines; TSWV-RG2P24, -GTP24, and -CFLP24) through *Nicotiana benthamiana* and the third subgroup was passaged 24 times by thrips (thrips lines; TSWV-RG2T24, -GTT24, and -CFLT24) using WFT through *Emilia sonchifolia* plants. Subisolates whose thrips transmissibilities had reverted to original levels (reverted lines; TSWV-RG2R, -GTR, and -CFLR) were used for sequence comparison to locate genetic change associated with the loss of thrips transmissibility [22]. Inoculation buffer (10mM Tris-HCl, pH 7.8, 10mM Na₂SO₃, 0.1% cysteine-HCl) was used in all mechanical inoculations. All plants were kept individually in cylindrical, insect-proof cages. Thrips-based inoculations were performed as described previously [22]. A 2-day acquisition access period and a 3-day inoculation access period were used for each thrips transmission. Ten potentially viruliferous adult thrips were transferred to one healthy *E. sonchifolia* plant and ten plants were inoculated per each transmission.

RT-PCR and sequence analysis

Total RNA was extracted from systemically infected leaves of *N. benthamiana* (plant lines) or *E. sonchifolia* (thrips lines and reverted lines) using RNA ISOLATORTM (Genosys,

Woodlands, TX) according to the manufacturer's protocol. The first strand cDNA was synthesized using *Avian myeloblastosis virus* reverse transcriptase (Promega, Madison, WI) as described previously [29]. The primers used for the cDNA synthesis and PCR were chosen from a published sequence of TSWV isolate BR-01 and are shown in Table 1 [18]. The reverse primers were used for the first strand of cDNA synthesis. PCR was performed as described previously [29]. The M RNA sequence of each TSWV isolate was determined by cycle sequencing of overlapping PCR products. Each PCR product was sequenced on both strands using forward and reverse primers. Amplified fragments were sequenced on an ABI 3700 automated sequencer with the BigDye Ver. 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequence of each PCR product was aligned using Vector NTI Ver. 7.0 software (InformaxTM, Frederick, MD). Pairwise and multiple sequence alignment of the TSWV isolates was performed using ClustalW [12]. The sequences obtained in this study were compared to those of previously reported TSWV isolates. GenBank accession number of those are S48091 (TSWV-BR01), AF208497 (TSWV-D), and AB010996 (TSWV-Japan). TSWV-D is a known, thrips-nontransmissible isolate, while TSWV-BR01 and -Japan are transmitted by thrips. The mutation frequency of the ORF was calculated as the number of mutations found per the number of nucleotides of the ORF. The mean mutation frequency of the isolates was measured as the sum of mutation frequencies of the isolates per the number of the isolates. The mean mutation frequency per passage was determined as the mean mutation frequency per the number of transmission cycles. Numbering system to establish coordinates of mutations began with the start codon of the ORF due to variability in the number of nucleotides in the IGR. The first nucleotide of noncoding regions of M RNA was used to establish coordinates of mutations within the noncoding regions.

Results

Sequence identity among TSWV isolates

Sequences of NSm and G_N/G_C ORFs on the M RNA of the TSWV isolates were compared to the sequences of two reference isolates, TSWV-BR01 and TSWV-D; the type isolate for TSWV and a nontransmissible isolate used in previous investigations, respectively [13, 18]. The differences in nucleotide sequence were dispersed within each ORF (See Appendix II). Percent identities of the both ORFs between the 2 reference isolates were 96.7 - 97.4% in nucleotide sequence. The 3 isolates shared 99.6 - 100% of nucleotide and amino acid sequence of the both ORFs, whereas the percent identities of the

both ORFs between the 3 isolates and the 2 reference isolates were 92 - 93%. However, percent identities of the IGR were 100% among the 3 isolates, 70% between the 3 isolates and the 2 reference isolates (See Appendix II).

Mutations accumulated after mechanical or thrips-based passaging

The accumulation of mutations in the M RNAs of TSWV-RG2, GT, and CFL was assessed after 24 sequential mechanical or thrips transmissions by comparing the nucleotide sequences of control populations of each isolate with that of the respective population after repeated serial transmission (Table 2 - 3). The number of mutations that accumulated was determined as mutation frequency for the plant or thrips lines of each isolate (Table 4). No mutations were found in the 5'NTR in the TSWV-RG2P24 or TSWV-RG2T24 isolates, while two substitutions were found in TSWV-GTT24 and one substitution was observed in TSWV-CFLP24. Similarly, no mutations were detected in the 3' NTR for the three isolates. A thirteen-nucleotide deletion and 1 substitution were found in the IGR of the TSWV-GTP24 and the TSWV-GTT24, respectively. In TSWV-CFLT24, three single nucleotide deletions and two substitutions were found throughout the IGR sequence.

Mutation frequencies in the NSm ORF of TSWV-RG2 were the same after both mechanical and thrips passages. However, three times as many mutations were dispersed in the NSm ORF after thrips passages than mechanical passages in TSWV-GT. No mutations were observed in TSWV-CFLT24 (Table 4). After mechanical transfers, single NS mutation was found in the NSm ORF of each plant line, while the number of S mutations was different for each isolate (Table 3). No conserved mutations were found in the NSm ORF during mechanical or thrips passages. The distinctiveness in pattern of mutation for each TSWV isolate was also found in the other ORF on the M RNA. Four times more mutations were observed in the G_N/G_C ORF of TSWV-RG2P24 than TSWV-RG2T24, whereas two times more mutations were found in TSWV-GTT24 than TSWV-GTP24. Mutation frequencies in the G_N/G_C ORF of TSWV-CFL were similar for both mechanical and thrips transmission, respectively.

A total of thirty-three and thirty-eight mutations were found in the NSm and G_N/G_C ORFs across the mechanical and thrips lines of the three isolates. Of the 33 mutations in the plant lines, 16 were NS, whereas 18 were NS in the 38 substitutions in the thrips lines. A strong bias towards transitions over transversions was apparent for the mechanical or thrips lines, as 30 out of 33 substitutions and 37 out of 38 substitutions were transitions in the mechanical or thrips lines, respectively. More mutations accumulated in the G_N/G_C ORF

during thrips transmission than mechanical transmission except in TSWV-RG2. Interestingly, two NS mutations and one synonymous (S) mutation were conserved in the G_N/G_C ORF of all three isolates after mechanical transmission. Two NS mutations were a substitution of C to U at nucleotide 86 (C86U) which changes T to I at amino acid 29 (T29I) and a substitution of U1373C (M458T), while one synonymous mutation was a substitution G492A.

The mean mutation frequency in NSm ORFs (909 nts) was 2.2×10^{-3} for the three isolates after both repeated mechanical and thrips transmission (Table 4). As for the G_N/G_C ORF (3408 nts), the mean mutation frequency of 2.6×10^{-3} or 3.1×10^{-3} was calculated after 24 mechanical or thrips passages, respectively. The mean mutation frequency per passage for the NSm ORF was 9.2×10^{-5} nucleotide substitutions per base per mechanical or thrips passage. A similar frequency was observed in the G_N/G_C ORF with 1.1×10^{-4} and 1.3×10^{-4} nucleotide substitutions per base per mechanical or thrips passage, respectively. The days needed for the completion of each mechanical or thrips transmission were not recorded exactly. Assuming that the days for each mechanical or thrips passage were 10 or 30 days, respectively, the mean mutation frequency for the three TSWV isolates can be expressed as the mutation frequency per nucleotide per year. The mean mutation frequency of the NSm ORF per nucleotide per year is 3.3×10^{-3} or 1.1×10^{-3} for mechanical passage or thrips passage, respectively. As for the G_N/G_C ORF, the mean mutation frequency per nucleotide per year is 4.0×10^{-3} or 1.6×10^{-3} for mechanical passage or thrips passage, respectively.

The mean mutation frequency accumulated in the M RNA of the 3 isolates after 24 mechanical or thrips passages was 4.2×10^{-3} or 3.2×10^{-3} , respectively. The mean mutation frequency per nucleotide in the M RNA per passage was 1.7×10^{-4} or 1.3×10^{-4} substitutions per each mechanical or thrips passage for our populations, respectively. We calculated 2.7×10^{-4} or 6.8×10^{-5} substitutions per nucleotide per year to be the evolutionary rate for mechanical or thrips lines of TSWV isolates in our investigation.

Mutations found in the thrips-transmissible subisolates (reverted lines)

Mutation profile in the M RNAs of the transmissibility-recovered lines was determined by comparing the nucleotide sequences of the recovered ones (TSWV-RG2R, -GTR, and -CFLR) with those of the plant lines (TSWV-RG2P24, -GTP24, and -CFLP24). No mutations were found in the 5' and 3' NTR or IGR or NSm ORFs, except for a U58G mutation in the 5' NTR of TSWV-CFLR. In the G_N/G_C ORF, one S mutation (C1860U) and one NS mutation

(G2833U [V945F]) were observed in TSWV-CFLR. One S mutation (U1683C) occurred in the G_N/G_C ORF of the TSWV-RG2R, while there were no nucleotide changes in TSWV-GTR.

Mutations found in a nontransmissible isolate, TSWV-GTN

The nucleotide and amino acid sequence of the TSWV-GTN M RNA, a nontransmissible isolate, was compared to that of TSWV-GTT24 in an attempt to identify mutations responsible for the loss of thrips transmissibility (Table 5). Of two mutations in the NSm ORF, a G88A (D30N) NS substitution may not be associated with the loss of thrips transmissibility, as the same change was also found in the control and thrips line. One S mutation (G825A) occurred only in TSWV-GTN. Of 7 mutations in the G_N/G_C ORF, one S substitution (A1704G) and two NS substitutions of U1976C (V659A) and A2150G (Y717C) were observed only in TSWV-GTN that did not occur in the other TSWV isolates.

Discussion

The high mutation rate during genome replication is one of the intrinsic properties of RNA viruses. Misincorporation error rates have been assessed in the range of 10^{-3} to 10^{-5} substitutions per nucleotide per round of replication [10]. RNA-dependent RNA polymerases without or with low proof-reading activities is the main factor responsible for high mutation rates of RNA viruses [10]. The mean mutation frequency of our populations was within the range of other RNA viruses. After 12 mechanical passages, a mutation frequency of 3.1×10^{-4} substitutions per base per year was calculated for TMV. This experiment examined 9.2% of the TMV genome [14]. A rate of 5×10^{-4} substitutions per year was estimated for the haemagglutinin glycoprotein gene of *Measles virus* by determining sequences of 75 isolates [30]. A strong bias towards transitions over transversions was found both in mechanical (90.9%) and thrips lines (97.4%). Because transitions exchange the same kind of nucleotides (either purines or pyrimidines), this bias may be due to conservation of a certain structure in the viral RNA [14].

The degree of mutation accrued during 24 mechanical passages was comparable with that of thrips lines. The evolutionary rate of the M RNA of our TSWV populations passaged by mechanical means was higher than that of thrips lines by 10 times. This indicates that extended mechanical passaging of the TSWV may serve as a more vigorous selective force to increase heterogeneity than thrips passage (alternating replication). The mean mutation frequency per nucleotide for the G_N/G_C ORF was larger than that for the NSm ORF after either mechanical or thrips passages. The NSm has been associated with

movement function in a plant host [19, 37, 38]. The cell-to-cell movement of the virus is considered one of the essential functions during the viral life cycle in plant hosts irrespective of transmission method. Therefore, this function of the NSm protein may make the NSm ORF more conserved than the G_N/G_C under the selective force such as prolonged passaging through plant hosts, whether by mechanical means or by a thrips vector.

In all three isolates, 2 NS mutations (C86T and T1373C) and one S substitution (G492A) in the G_N/G_C ORF were conserved after 24 mechanical passages. The fixation of these mutations over the three isolates can be explained by adaptive evolution under continuous mechanical passaging. However, corresponding conserved mutations were not found in any isolates after serial thrips passages. It is not surprising that there are no conserved mutations in the M RNA of the TSWV after prolonged passaging by thrips, as the experimental design of repeated thrips transmission in this study mimics the thrips transmission of the TSWV in nature, which is considered the only way of TSWV spread in fields.

Sequences of the recovered subisolates were compared with the plant lines of the three isolates, in an attempt to identify the viral genomic region responsible for the loss of thrips transmissibility. One NS mutation (G2833T) in TSWV-CFLR may not be implicated in the loss of thrips transmissibility, as this change was not observed in TSWV-CFL and -CFLT24. However, two NS substitutions were found in G_N/G_C ORF, when the sequence of the thrips-nontransmissible isolate, TSWV-GTN was compared to that of TSWV-GTT24 (Table 5). Two NS substitutions, T1976C (V659A) and A2150G (Y717C), may be linked to the loss of thrips transmissibility of (at least) the TSWV-GT, because these changes were found only in TSWV-GTN.

There was no specific pattern on genetic changes following repeated mechanical or thrips transmission of TSWV in this study, though 3 isolates shared 99.4 - 99.7% sequence identity in the M RNA (See Appendix). Mutation frequencies in the NSm ORFs of TSWV-RG2P24 and -RG2T24 were the same, while no mutation was observed in TSWV-CFLT24. In the G_N/G_C ORF, four times as many mutations accumulated during serial mechanical passages of TSWV-RG2 than in thrips passages, whereas two times more mutations were observed in TSWV-GTT24 than TSWV-GTP24. Further studies are needed to demonstrate this variance among TSWV isolates in terms of TSWV evolution.

The mutation frequency and the evolutionary rate of TSWV populations in this study can be used for modeling TSWV evolution under extended passaging by mechanical means or thrips. Our results demonstrated that the evolutionary rate of the M RNA passaged by

mechanical means was larger than that of thrips lines by 10 times. Development of efficient reverse genetic system for TSWV would help to confirm candidate mutations for the loss of thrips transmissibility of the TSWV-GT.

Table 1. List of primers used for RT-PCR amplification and sequencing of the M RNA from TSWV isolates

Primer name	Forward or reverse	Sequence	Annealing temperature, °C
1	F	AGAGCAATCAGTGCATCAGAAATATACCTATTA TACA	55
1	R	CACTACCAAAGAAACCCC	
2	F	GAAGATGAACAACACCCC	40
3	F	TCAGTTGAAGAGGAAGA	41
3	R	TATGTTAATGAAAGATACAA	
4	F	TGAGAGAAATCCATAGG	55
4	R	GGTTTAGAGCAAATATCAG	
5	F	CCGCATAGAAGACAGCC	55
5	R	TACAGGAAACTGCGACAC	
6	F	AGAAGTTGGTGTACACA	46
6	R	GATGCTTTTATGTTCCAGG	
7	F	GTGCCAAAGATACTCTCTATG	46
7	R	CTGAGGAAATGTTGGATGG	
8	F	GCAATCTCTGACTCTTT	52
8	R	ATGATGATTCTGCTGAG	
9	F	GTATCTGACGGGTTCCAGG	55
9	R	AGAGCAATCAGTGCAAACAAAAACCTTAATCC	

Table 2. The type of mutations accumulated in the M RNA after 24 repeated mechanical or thrips transmissions of TSWV-RG2, GT, and CFL

TSWV Isolate	Viral genomic Region ^a	Control to Mechanical ^b		Control to Thrips ^b	
		NS ^c	S ^c	NS ^c	S ^c
RG2	5' NTR	No mutation		No mutation	
	NSm	G to A at 832 (D ²⁷⁸ to N) ^d	C to U at 72 C to U at 738	U to C at 62 (L ²¹ to S) A to G at 139 (T ⁴⁷ to A)	C to U at 454
	IGR ^e	No mutation		No mutation	
	G _N /G _C	C to U at 86 (T ²⁹ to I) ^f U to G at 138 (D ⁴⁶ to E) U to C at 1373 (M ⁴⁵⁸ to T) G to A at 3331 (E ¹¹¹¹ to K)	C to U at 252 G to A at 306	A to G at 184 (I ⁶² to V)	G to A at 450 G to A at 2163
			U to C at 432 G to A at 492		
			C to U at 1804 G to A at 2163		
			A to G at 3006		
	3' NTR	No mutation		No mutation	

Table 2. (continued)

TSWV Isolate	Viral genomic Region ^a	Control to Mechanical ^b		Control to Thrips ^b	
		NS ^c	S ^c	NS ^c	S ^c
GT	5' NTR	No mutation		A to G at 52 and 98	
	NSm	A to G at 80 (H ²⁷ to R)	None	A to G at 88 (N ³⁰ to D)	A to G at 615 and 699
	IGR	Deletion from 104 to 116		U to C at 43	
	G _N /G _C		G to A at 492		
			C to A at 1431	G to A at 67 (V ²³ to I)	A to G at 189
		C to U at 86 (T ²⁹ to I)		A to G at 172 (T ⁵⁸ to A)	G to A at 294, C to U at 432
		A to U at 747 (L ²⁴⁹ to F)	C to U at 1804	A to G at 1031 (K ³⁴⁴ to R)	C to U at 519, G to A at 618
		U to C at 1373 (M ⁴⁵⁸ to T)		C to A at 1257 (N ⁴¹⁹ to K)	A to G at 1392,
		G to A at 2384 (S ⁷⁹⁵ to N)	C to U at 2391	G to A at 1549 (G ⁵¹⁷ to S)	U to C at 1743, U to C at 1767
		C to U at 2897 (S ⁹⁶⁶ to F)	G to A at 3099	G to A at 1649 (R ⁵⁵⁰ to Q)	A to G at 2016, C to U at 2391,
				C to U at 2267 (T ⁷⁵⁶ to I)	G to A at 2568, U to C at 2793
				G to A at 2384 (S ⁷⁹⁵ to N)	G to A at 3099
	3' NTR	No mutation		No mutation	

Table 2. (continued)

TSWV Isolate	Viral genomic Region ^a	Control to Mechanical ^b		Control to Thrips ^b	
		NS ^c	S ^c	NS ^c	S ^c
CFL	5' NTR	G to A at 52		No mutation	
	NSm	A to G at 860 (E ²⁸⁷ to G)	G to A at 186	No mutation	No mutation
	IGR	Deletion from 104 to 116		A deletion at 7, 91 and 116 C to A at 13, U to C at 43	
	G _N /G _C	C to U at 86 (T ²⁹ to I)		C to U at 241 (P ⁸¹ to S)	
		A to G at 817 (T ²⁷³ to A)		U to C at 785 (I ²⁶² to T)	
		U to C at 1373 (M ⁴⁵⁸ to T)		A to G at 1031 (K ³⁴⁴ to R)	
		G to A at 2969 (G ⁹⁹⁰ to D)		G to A at 1549 (G ⁵¹⁷ to S)	
				G to A at 3043 (V ¹⁰¹⁵ to I)	
				C to U at 3052 (P ¹⁰¹⁸ to S)	
		U to C at 432		U to C at 1804	
		G to A at 492		U to C at 2361	
	3' NTR	No mutation		No mutation	

^aNSm, G_N/G_C, and IGR mean a nonstructural protein coded, the precursor for glycoproteins, and intergenic region separating NSm and G_N/G_C ORFs in the M RNA, respectively. 5' and 3' NTR represent non-translated region in the 5' and 3' terminus of the M RNA, respectively.

^bControl, mechanical, and thrips mean control population, mechanically or thrips-maintained populations of TSWV-RG2, GT, and CFL, respectively.

^cNS and S represent nonsynonymous and synonymous mutation, respectively.

^dG to A at 832 (D to N) means that the nucleotide at 832 was substituted from G to A, which changes amino acid, Asp (D) to Asn (N). The nucleotide of the NSm was numbered from the 5' viral RNA excluding 5' NTR.

^eThe nucleotide of the IGR was numbered from the starting nucleotide of the IGR itself.

^fThe nucleotide of the G_N/G_C was numbered from the 3' viral RNA excluding 3' NTR because the G_N/G_C its ORF is transcribed from viral sense RNA.

Table 3. The number and types of mutations accumulated in the M RNA accrued during 24 repeated mechanical or thrips transmissions of TSWV-RG2, -GT, and -CFL

TSWV isolate	Viral genomic region ^a (nts)	Mechanical line		Thrips line (alternated passages)	
		NS ^b	S ^b	NS ^b	S ^b
RG2	5' NTR (100)	0		0	
	NSm (909)	1	2	2	1
	IGR (270)	0		0	
	G _N /G _C (3408)	4	7	1	2
	3' NTR (84)	0		0	
GT	5' NTR (100)	0		2	
	NSm (909)	1	0	1	2
	IGR (270)	13		1	
	G _N /G _C (3408)	5	5	8	13
	3' NTR (84)	0		0	
CFL	5' NTR (100)	1		0	
	NSm (909)	1	1	0	0
	IGR (270)	13		5	
	G _N /G _C (3408)	4	2	6	2
	3' NTR (84)	0		0	

^aNSm, G_N/G_C, and IGR mean a nonstructural protein, the precursor of glycoproteins, and intergenic region separating NSm and G_N/G_C ORFs in the M RNA, respectively. 5' and 3' NTR represent nontranslated region in the 5' and 3' terminus of the M RNA, respectively. The number of nucleotide of IGR is for control population of each isolate.

^bNS and S represent nonsynonymous and synonymous mutation, respectively.

Table 4. The mutation frequency for the two ORFs on the M RNA of TSWV-RG2, GT, and CFL which occurred during 24 serial, mechanical or thrips transmission

ORF	TSWV isolate	Mutation frequency (X 10 ⁻³)	
		Mechanical passage	Thrips passage (alternated passages)
NSm (909 nt)	RG2	3.3	3.3
	GT	1.1	3.3
	CFL	2.2	0
	Mean mutation frequency ^a	2.2	2.2
	Mean mutation frequency per passage ^b	0.092	0.092
G _N /G _C (3408 nt)	RG2	3.2	0.88
	GT	2.9	6.2
	CFL	1.8	2.3
	Mean mutation frequency ^a	2.6	3.1
	Mean mutation frequency per passage ^b	0.11	0.13

^aMean mutation frequency for three TSWV isolates was calculated.

^bMean mutation frequency per passage means mutation frequency per each passage for three isolates.

Table 5. The mutations found in the M RNA of TSWV-GTN, a nontransmissible isolate, which was originated from the TSWV-GT

		Position of the change in the nucleotide (amino acid) ^a		
		NSm	G _N /G _C	
		825	1976 (659)	2150 (717)
TSWV isolate	GT	G	U (Val)	A (Tyr)
	GTP24	G	U (Val)	A (Tyr)
	GTT24	G	U (Val)	A (Tyr)
	GTN	A	C (Ala)	G (Cys)
	RG2	G	U (Val)	A (Tyr)
	RG2P24	G	U (Val)	A (Tyr)
	RG2T24	G	U (Val)	A (Tyr)
	CFL	G	U (Val)	A (Tyr)
	CFLP24	G	U (Val)	A (Tyr)
	CFLT24	G	U (Val)	A (Tyr)
	BR-01	G	U (Val)	A (Tyr)
	D	G	U (Val)	A (Tyr)
	Japan	G	U (Val)	A (Tyr)

^aNumbering system for mutation began with the start codon of the each ORF.

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**Viral Genetic Determinants for Thrips Transmission of
*Tomato spotted wilt virus*²**

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Abstract

Tomato spotted wilt virus (TSWV) is transmitted exclusively by thrips in nature. Using a reassortment-based viral genetic system, transmissibility by thrips was mapped to the M RNA of TSWV. To locate determinants of thrips transmission in the M RNA, thirty single lesion isolates (SLIs) were generated from a TSWV isolate with low thrips transmissibility. Of these 30 SLIs, three were readily transmitted by thrips and 27 were not. Sequence analysis of the M RNA, thrips transmission assays, Western blot 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. This is the first report of a specific viral determinant associated with thrips transmission. In addition, it was demonstrated that loss of transmissibility is associated with the accumulation of defective haplotypes in the isolate rather than reduced transmission efficiency of a dominant haplotype. These results also indicate that the glycoproteins may be dispensable for TSWV infection of plant hosts, but not for transmissibility by thrips.

INTRODUCTION

Viruses in the *Bunyaviridae* are among the most medically and agronomically important viruses. Many included 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 this family 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 are quite distinct (2). This virus-host relationship has significant consequences for the epidemiology of the virus and the potential strategies for control. The absence of an efficient system for reverse genetics for any of the members of this family has severely limited our understanding of this relationship. It is as yet unclear if there are indeed separate strategies of pathogenesis requiring separate viral genes, or viral genes that are multifunctional or if there are in fact similar pathways for pathogenesis in these divergent hosts. To begin to unravel this relationship, we are taking advantage of the genetic system developed for *Tomato spotted wilt virus* (TSWV) (3-6).

TSWV is a plant-infecting *Tospovirus* in the family *Bunyaviridae*. Tospoviruses replicate in both their plant hosts and their insect (thrips) vectors. Since its first description in 1915 (7), TSWV and other tospoviruses have been causing tremendous economic loss in a large variety of crops. Over 900 species spanning both monocotyledonous and dicotyledonous plants are susceptible to TSWV (8). The TSWV virion is a quasi-spherical particle, which 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 (9). The L RNA is of negative polarity and encodes an RNA-dependent RNA polymerase (RdRp) (10, 11). The putative cell-to-cell movement protein and the precursor of surface glycoproteins on the envelope are encoded by the M RNA in the viral and viral complementary sense, respectively (12, 13). The glycoprotein precursor is further glycosylated and cleaved into two glycoproteins, G_N and G_C by an unknown mechanism (14). The S RNA encodes a nonstructural protein (NSs) which was recently reported to suppress gene silencing, and the nucleocapsid protein (15-17).

Thrips are severe insect pest of plants as well as efficient vectors of plant viruses. A broad overlapping host range and inefficient vector control have made TSWV one of the most threatening plant viruses worldwide (8). TSWV is spread in a propagative manner by at least seven thrips species, all of which belong to the genera *Frankliniella* and *Thrips* in the family *Thripidae*. (18-20). Among those, *Frankliniella occidentalis* (western flower thrips

[WFT]) and *Frankliniella fusca* (tobacco thrips [TT]) are the most efficient vectors in the southeastern USA (21). 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 (22, 23). The midgut epithelial cells of thrips are the initial site of TSWV entry and infection (19, 24). After replication in the midgut, TSWV moves to muscle fibers surrounding the midgut and then migrates to the salivary glands (19, 20). Even though little is known about the pathway by which TSWV migrates from the midgut to the salivary glands, the favored hypothesis is that the salivary glands become infected by a process of TSWV migration involving ligaments (24-26).

Until now, most studies of the TSWV-thrips interactions focused on elucidating cellular and biochemical determinants for acquisition and movement of TSWV in thrips (27-29). Several reports suggest that a receptor-mediated endocytosis governs TSWV entry to thrips midgut cells and that the TSWV G_C and a 50 kDa thrips protein may coordinate the TSWV entry process (27, 29). However, the G_N was also identified as a viral attachment protein that binds to a 94 kDa thrips protein (28). Further circumstantial 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 following ingestion and was not transmitted by thrips (30). It was also shown that thrips transmissibility of TSWV declined following repeated mechanical passages (31). These reports suggest that the M RNA or its translational products may be determinants of thrips transmissibility of TSWV. However, the other TSWV RNAs or proteins are possible control elements for thrips transmission. For example, the TSWV RdRp has been implicated in thrips transmission. A TSWV isolate containing defective interfering (DI) RNA, which had a truncated L RNA, was not transmitted by thrips (30).

In this investigation, we used a system for the viral genetic analysis including reassortment that we previously used to map several viral phenotypes to specific genome segments of TSWV (3-6), combined with the analysis of transmission deficient mutants to identify thrips transmission determinants. Here we present genetic evidence confirming that the M RNA encodes determinants of thrips transmission and identify changes in the M RNA that are associated with the loss of transmissibility by thrips. These investigations characterize specific mutations in the glycoprotein precursor coding region associated with the loss of thrips transmissibility of TSWV. We demonstrated that the decline in thrips transmissibility of TSWV following repeated mechanical passages is due to the accumulation of M RNA segments in the viral population with sequence specific mutations.

Specific point mutations identified here may be indicative of sites responsible for mediating thrips transmission of TSWV. 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.

Material and Methods

TSWV Isolates and Thrips Colony. Two TSWV isolates were used as parental isolates to generate reassortants. TSWV isolate D (TSWV-D) originating from dahlia (*Dahlia hybrida*) in the Netherlands is not transmitted by thrips. TSWV isolate RG2 (TSWV-RG2) was obtained from tobacco (*Nicotiana tabacum* cv. 'Burley 21') in North Carolina and is transmissible by WFT and TT. Parental isolates and reassortants between them were maintained in *Nicotiana benthamiana* or *Emilia sonchifolia* by mechanical means. Inoculation buffer (10mM Tris-HCl, pH 7.8, 10mM Na₂SO₃, 0.1% cysteine-HCl) was used in all mechanical inoculations in this study. All plants used were kept individually in cylindrical, insect-proof cages. WFT was used in all thrips transmission assays. Thrips were originally collected from carnation (*Dianthus caryophyllus*) in a greenhouse at North Carolina State University and maintained on pole bean (*Phaseolus vulgaris*) pods in plastic containers held at 24 °C, 65% relative humidity and a photoperiod of 14/10 hr (light/dark). The tops of the containers were covered with Bugbed 110 (Green Thumb Group, Downers Grove, IL) fine mesh thrips screening.

Generation of TSWV Reassortants. Reassortants between thrips-transmissible TSWV-RG2 and nontransmissible TSWV-D were made from a dual infection in *N. benthamiana* and subsequent serial passages through *N. 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, ground in inoculation buffer and 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 has 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 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 RT-PCR and segment-specific RFLP.

Genetic Markers and Genotyping of Reassortants. Total RNA was extracted from systemically infected leaves of *N. benthamiana* infected with each reassortant using RNA ISOLATOR™ (Genosys) according to the manufacturer's protocol. The first strand cDNA was synthesized using *Avian myeloblastosis virus* reverse transcriptase (Promega) as described previously (6). 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 previously (6). The amplified regions by RT-PCR were parts of RdRp, G_N/G_C, and NSm open reading frames of the L, M, and S RNA segment, respectively. The PCR products were cleaned on PCR purification columns (Qiagen). Purified PCR products were digested with *TaqI* for the L RNA fragment, *MseI* for the M RNA fragment, and *TaqI* 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 WFT first instar larvae 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 hr at 25±3 °C, after which they were transferred to fresh pods of *P. vulgaris* at 28±3 °C for 8-9 days and reared to adult. Potentially viruliferous adult thrips were then confined in groups of 10 per plant to 10 or 30 caged 2-week old non-infected *E. sonchifolia* plants. Then plants were held in a greenhouse under 28 °C/20 °C (light/dark) and 14 hr/10 hr (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 specific thrips nontransmissible mutants, a TSWV isolate (TSWV-RG2P24), was selected following 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 following 3 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 and are shown in Table 1 (13). Amplification of IGR was conducted as described previously (32). Sequencing of the amplified fragments was performed on an ABI3700 automated sequencer with the BigDye Ver. 3.1 Cycle Sequencing kit (Applied Biosystems). Fragments were aligned by using Vector NTI Ver. 7.0 software (InformaxTM, Frederick, MD). Numbering system for mutation in G_N/G_C began with the start codon of the ORF.

Transmission Electron Microscopy. Small pieces of the symptomatic leaf tissues infected with SLIs obtained from the TSWV-RG2P24 were fixed and processed for transmission electron microscopy (TEM) as described by Urban *et al.* with modification (33). Leaf tissue pieces of *E. sonchifolia* plant infected with the SLI from the TSWV-RG2P24 were fixed with 1% glutaraldehyde (v/v) and 2.5% paraformaldehyde (w/v) in 0.1 M potassium phosphate (pH 7.4) overnight at 4 °C. After postfixation in 1% OsO₄, 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 in a JEOL transmission electron microscopy JEM-100S.

Western Blot. Western blot analysis was performed to detect the presence of G_C in the TSWV mutants. Sample preparation, electrophoresis, and electro-transfer were conducted as described by Law *et al.* (34). 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 1:1000 and detection of the band was carried out using enhanced chemiluminescence system (Amersham).

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 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 ($L_D M_D S_D$) and the TSWV-RG2 genotype ($L_R M_R S_R$) 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 $L_R M_D S_R$, $L_R M_R S_D$, and $L_R M_D S_D$ were generated when equal amounts of inoculum of two parental isolates were coinoculated, whereas $L_D M_D S_R$, $L_D M_R S_D$, and $L_D M_R S_R$ were generated when TSWV-D inoculum was increased two 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 WFT of the six reassortants revealed that reassortants, $L_R M_R S_D$, $L_D M_R S_D$, and $L_D M_R S_R$ were transmitted by thrips, while $L_R M_D S_D$, $L_D M_D S_R$, and $L_R M_D S_R$, were not (Table 2). Reassortant isolates with the M RNA from TSWV-D, a thrips-nontransmissible isolate, could not be transmitted, whereas reassortants with the M RNA from TSWV-RG2, a thrips-transmissible isolate, could be transmissible, irrespective of the origin of the L and S RNA. The TSWV-RG2 and three transmissible reassortants, $L_R M_R S_D$, $L_D M_R S_D$, and $L_D M_R S_R$ induced concentric, necrotic ringspots followed by systemic chlorosis in *E. sonchifolia* after thrips-mediated inoculation. The viral genotypes of the thrips-transmitted reassortants following 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.

Isolation and Sequence Analysis of Transmission-deficient Mutants. To locate the site or region necessary for thrips transmission, single lesion isolates (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 thirty SLIs produced the same type of symptoms of 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 WFT, whereas two (37 and 41) were transmitted. To identify the viral

genomic region responsible for thrips transmission in the M RNA, the whole M RNA sequences of these eight SLIs were determined and compared to that of TSWV-RG2P24 (Table 3). Mutations were found in the G_N/G_C ORF, while no mutation was observed in the NSm ORF, the intergenic region (IGR) between the NSm and the G_N/G_C ORF, or 5' and 3' nontranslated regions (NTR) of the M RNA of the eight SLIs (Table 3). 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). SLI 4 could be transmitted, whereas the other twenty-one SLIs could 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. 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 SLIs (Table 3 and 4). No mutations were observed in the M RNA of SLI 4, while 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 9 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 are 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 mechanically.

Virion Formation. Symptomatic leaf tissues infected with each of the SLIs from the TSWV-RG2P24 were processed for transmission electron microscopy to determine if 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. 2A). Transmissible SLIs (37 and 41) also formed virions in the infected leaf tissues (Fig. 2B). 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. 2C). No virions were observed in leaf tissues infected with SLIs (2, 39, 61, 79, and 80), which had frameshift or nonsense mutation or both (Fig. 2D). It is well established that cytopathological structures including viroplasm and crystalline filaments are a manifestation of TSWV infection in host plants (33, 35-37). 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) which 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 which only had the C1375A mutation in G_N/G_C ORF (81), while G_C was not found in *E. sonchifolia* leaf tissues infected with SLIs, which had nonsense or frameshift mutation 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 2 NS mutations, A1202G and G2294U, indicating these mutations may also be linked to the loss of G_C. The western blot analysis of three nontransmissible SLIs with nonsense or frameshift mutation 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 have been limited by the absence of an efficient system for 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 (38). 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 (27-30). This association was based on the accumulation of truncated L RNAs and the absence of virion formation in nontransmissible isolates (30). In this study, we developed a system for genetic analysis based on the gradual loss of thrips transmissibility following sequential mechanical passages from plant to plant. We hypothesized that the loss in transmissibility was the result of the accumulation of defective RNAs in the viral isolate and that these could be separated into transmissible and nontransmissible isolates following 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 utilizing 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 non-random distribution of segment reassortment was similar to that reported for *Snowshoe hare virus* and *La Crosse virus* as well as for TSWV in our previous studies (3, 6, 39). Differential replication efficiency of the two corresponding segments of parental isolates and host selective influences on reassortant genotypes have been suggested as possible causes (39, 40). Competitiveness of genome segment exchange of TSWV has been linked to the IGR of the S RNA (6).

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 (27-29). Point mutations or small deletions in the G_N/G_C ORF have been suggested as the cause of non-transmissibility and loss of envelope formation, although no specific mutations have been identified (30, 41). It has also been suggested that a TSWV isolate supporting DI RNA, which suppresses normal L RNA functions and attenuates symptom expression, was not transmitted by WFT (30). However, other DI RNA-containing isolates that minimally interfered with symptom expression were transmitted as efficiently as a wild-type isolate (30). We did not encounter any symptom attenuation between the two parental isolates and six reassortants on *N. benthamiana*, *N. tabacum* cv. 'Burley 21', and *E. sonchifolia*. While 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 thrips transmission of TSWV (Table 2).

While the M RNA has been associated with thrips transmission, there is a limited body of knowledge regarding the molecular determinants controlling thrips transmission and/or changes responsible for the loss of thrips transmissibility of TSWV. 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 3). 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 suggests strongly that the 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 which 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 (13, 42-44). The C1375A mutation may not be involved with the glycoprotein processing, even though there is a previous report that amino acid 484 of TSWV G_N/G_C may be a cleavage site of the glycoprotein precursor (45). It may be significant that amino acid 459 resides within a putative transmembrane domain of the glycoproteins, which spans from amino acid 428 to 484 (45). The nonsense and frameshift mutations that were also observed in nontransmissible SLIs would prevent the G_N/G_C from being translated by terminating translation prematurely or frameshifting of the G_N/G_C ORF, respectively. Transmission electron microscopic 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 bands 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 plant *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 (41, 46). It was also reported that when TSWV is transmitted by repeated mechanical passages, mutants can be generated that lose their thrips transmissibility but infect plant hosts (47). However, there has been no sequence determination for those mutants. Our results support that TSWV mutants without an envelope can be generated by repeated mechanical transmission and have characterized specific point mutations or nonsense mutations in the G_N/G_C ORF of the M RNA associated with the lack of an envelope. Our report is the first to demonstrate the presence of specific mutations in transmission-deficient isolates generated by serial mechanical passages of TSWV. These results also indicate that the glycoproteins may be dispensable for TSWV replication in plants but necessary for thrips transmission.

The low transmissibility of TSWV isolates after repeated, mechanical passages can be explained with sequence analysis and thrips transmission assay of the SLIs, which originated from TSWV-RG2P24. As mechanical passage proceeded, nontransmissible

mutants accumulated in the TSWV population. The low proportion of the transmissible SLIs, 3 SLIs out of 30 isolated, was consistent with the reduced transmission efficiency of TSWV isolates after serial mechanical transmission (31). The increased frequency of nontransmissible SLIs from the TSWV-RG2P24 may explain the decreased transmissibility of TSWV after repeated mechanical transmission as the nontransmissible SLIs were accumulated during serial mechanical transmission. Our results support quantitatively that the TSWV-RG2P24, which was used as a parental isolate for SLIs, may be composed of a high proportion of nontransmissible mutants, c.a. 90%.

In conclusion, we used a reassortment-based viral genetic analysis system to demonstrate the critical importance of the M RNA of TSWV in determining the thrips transmissibility. Furthermore, we have identified a putative, single NS mutation of C1375A associated with thrips transmissibility and have identified specific frameshift mutations or nonsense mutations in the G_N/G_C ORF that interfered with glycoprotein synthesis and thrips transmissibility. The formation of enveloped TSWV virions by nontransmissible SLIs with only the C1375A mutation in the G_N/G_C ORF also suggests that, in addition to G_N/G_C , there may be other determinants governing thrips transmissibility of TSWV. Sequence analysis, TEM observation of ultrathin sections, and western blot analysis of the SLIs strongly suggest that the glycoproteins, at least G_C , may be dispensable for TSWV multiplication in its plant host but not for transmissibility by its thrips vector. Further investigation of the nature of the TSWV glycoproteins, and the morphogenesis of the TSWV virion are needed for the improved understanding of the mechanisms controlling TSWV movement in thrips vector. Progress toward this end would be greatly enhanced by the development of reliable reverse genetic system.

Table 1. Primers used for RT-PCR amplification and sequencing of the M RNA of the TSWV isolates

Primer name	Forward or reverse	Sequence	Annealing temperature, °C
1	F	AGAGCAATCAGTGCATCAGAAATATACCTATTATACA	55
1	R	CACTACCAAAAGAAACCCC	
2	F	GAAGATGAACAACACCCC	40
3	F	TCAGTTGAAGAGGAAGA	41
3	R	TATGTTAATGAAAGATACAA	
4	F	TGAGAGAAATCCATAGG	55
4	R	GGTTTAGAGCAAATATCAG	
5	F	CCGCATAGAAGACAGCC	55
5	R	TACAGGAAACTGCGACAC	
6	F	AGAAGTTGGTGTTACA	46
6	R	GATGCTTTTATGTTCCAGG	
7	F	GTGCCAAAGATACTCTCTATG	46
7	R	CTGAGGAAATGTTGGATGG	
8	F	GCAATCTCTGACTCTTT	52
8	R	ATGATGATTCTGCTGAG	
9	F	GTATCTGACGGGTTCCAGG	55
9	R	AGAGCAATCAGTGCAAACAAAAACCTTAATCC	

Table 2. 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 _D M _D S _D	0/10 ; 0/10 ; 0/10	N/A
L _R M _R S _R	6/9 ; 3/10 ; 2/10	L _R M _R S _R
L _R M _R S _D	4/10 ; 2/10 ; 2/10	L _R M _R S _D
L _R M _D S _D	0/10 ; 0/10 ; 0/10	N/A
L _R M _D S _R	0/10 ; 0/10 ; 0/10	N/A
L _D M _D S _R	0/10 ; 0/10 ; 0/10	N/A
L _D M _R S _R	4/10 ; 3/10 ; 3/10	L _D M _R S _R
L _D M _R S _D	4/10 ; 2/10 ; 2/10	L _D M _R S _D

*Transmission was confirmed by symptom development and RT-PCR and RFLP. Individual experiments are separated by semicolon.

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

Table 3. Mutation in the M RNA and thrips transmissibility of 8 single lesion isolates (SLIs) from the TSWV-RG2P24 which was obtained following 24 mechanical transmission cycles

SLI	Thrips transmissibility*	Mutation						
		NSm, IGR, 5' and 3' NTR	1726-3408 (1726 – 2616 for G _C [†])		811 – 1725 [‡]		1– 810 (175-810 for G _N [‡])	
			S [§]	NS [§]	S	NS	S	NS
2	0/30; 0/30; 0/30	- [¶]	-	-	-	C1375A (P459T)	-	a deletion at 310
11	0/30; 0/30; 0/30	-	-	-	-	C1375A (P459T)	-	a deletion at 270
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	-	-	-	-	C1375A (P459T)	-	A776G (H259R)
80	0/30; 0/30; 0/30	-	-	addition of UUU at 1977 G1975U (V659L)	U1683C	C1054U (L352F)	-	U144A (stop)
81	0/30; 0/30; 0/30	-	-	-	-	C1375A (P459T)	-	-

*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 Ref. 13 and Ref. 45.

[‡]The nucleotide of the G_N/G_C was numbered from the 3' viral RNA excluding 3' NTR.

[§]S and NS represent synonymous and nonsynonymous mutation, respectively.

[¶]- means no mutation was found in the region.

^{||}C to A at 1375 (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.

Table 4. Mutation in the G_N/G_C ORF and thrips transmissibility of 22 single lesion isolates (SLIs) from the TSWV-RG2P24 which was obtained following 24 mechanical transmission cycles

SLI	Thrips transmissibility*	Mutation					
		1726-3408 (1726 – 2616 for G _C)		811 – 1725		1– 810 (175-810 for G _N)	
		S [§]	NS [§]	S	NS	S	NS
3	0/10; 0/10	A2541G	C3229U (L1077F)	-	C1375A (P459T) U1385C (M462T)	-	C253U (stop [¶])
4	3/10; 3/10	-	-	-	-	-	-
10	0/10; 0/10	-	G1784A (G595E) addition of UUUU at 1975	U1683C	deletion at 816	-	C235U (stop [¶]) G325U (D109Y) deletion at 676
13	0/10; 0/10	-	U3154A (F1052 I)	-	C1375A (P459T)	-	-
18	0/10; 0/10	-	addition of UUUU at 1975	U1683C	G838A (E280K)	-	a deletion at 310 C524U (T175I)
20	0/10; 0/10	-	deletion at 1814, 1971	U1683C	G1047A (stop [¶])	-	C259U (stop [¶]) A389G (Y130C)
30	0/10; 0/10	U2547C	G1975U (V659L) addition of U at 1977 deletion at 2580	U1683C	-	-	C304U (stop [¶])
33	0/10; 0/10	-	a deletion at 1974	-	G1143U (L381F) C1375A (P459T)	A540G	a deletion at 108 C601U (P201S)

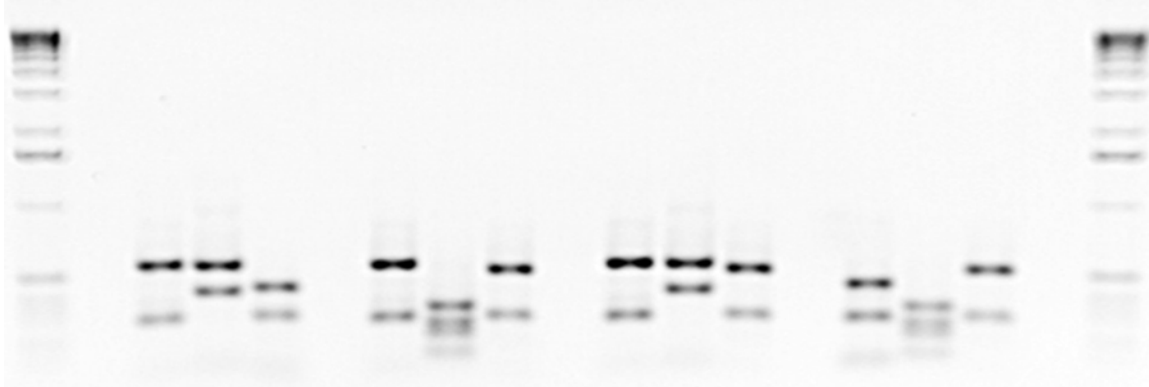
Table 4 (continued)

SLI	Thrips transmissibility*	Mutation					
		1726-3408 (1726 – 2616 for G _C)		811 – 1725		1– 810 (175-810 for G _N)	
		S [§]	NS [§]	S	NS	S	NS
39	0/10; 0/10	-	-	-	C1375A (P459T)	A540G	U119G (I40S) G262U (stop [¶]) a deletion at 284 C332U (P111L) A389G (Y130C) deletion of 45 nts (762-806)
54	0/10; 0/10	-	U1826C (I609T) A1971U (K657N) addition of TTT at 1975	-	A1003G (I335V) C1156A (H386N) C1375A (P459T)	-	C235U (stop [¶])
58	0/10; 0/10	-	G2318A (R773K)	U1404C G1623A	C1375A (P459T) A1384G (M462V)	C669U	a deletion at 108
60	0/10; 0/10	-	G2294U (G765V)	U1683C	A1202G (K401R)	-	-
61	0/10; 0/10	-	-	U1683C	A824G (E275G) deletion at 874, 1974	-	C259U (stop [¶]) A389G (Y130C)
63	0/10; 0/10	-	-	-	C1375A (P459T)	-	-
65	0/10; 0/10	-	A2216G (N739S)	-	C1375A (P459T)	-	-

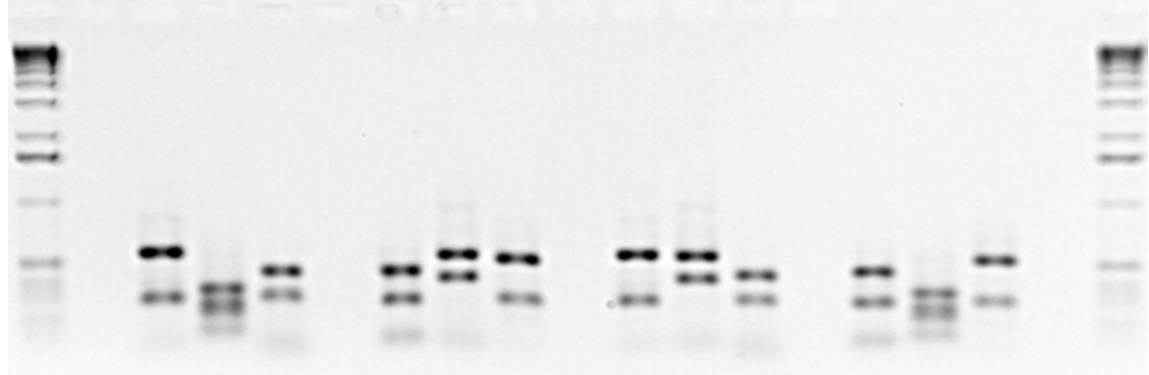
Table 4 (continued)

SLI	Thrips transmissibility*	Mutation					
		1726-3408 (1726 – 2616 for G _C)		811 – 1725		1– 810 (175-810 for G _N)	
		S [§]	NS [§]	S	NS	S	NS
68	0/10; 0/10	-	-	-	C1375A (P459T)	-	-
71	0/10; 0/10	-	G1975U (V659L) addition of U at 1977	U1683C	C869A (A290D)	-	C304U (stop [¶]) G637A (V213I)
73	0/10; 0/10	-	-	-	A839G (E280G) C1375A (P459T)	-	-
75	0/10; 0/10	-	U3017C (V1006A)	-	C1375A (P459T) G1711A (V571I)	-	-
76	0/10; 0/10	-	G1975U (V659L) addition of UU at 1977 addition of A at 2581	-	A981G (I327M) U 1672G (F558V)	G183U	addition of AA at 311 A389G (Y130C)
79	0/10; 0/10	U3246C	A1763G (N588S) addition of UUU at 1975 U2129C (F710S) addition of AA at 2581 C3152U (P1051L)	A1122G U1578C	A923G (K308R) C1375A (P459T)	-	addition of A at 12
83	0/10; 0/10	G1731A G3006A	C1820U (T607I)	-	C1375A (P459T)	-	G467U (C156F)

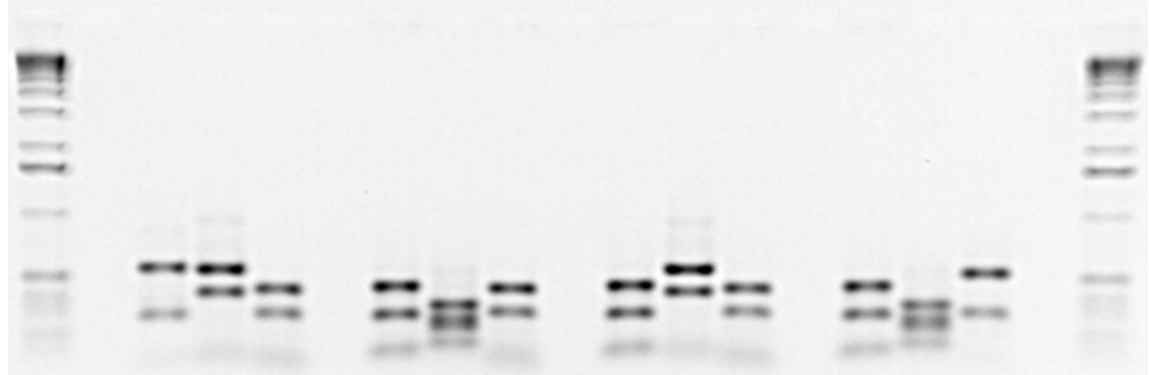
* See Table. 2 for legend.



M	D	D	D	D	R	R	D	D	R	R	R	M
	L	M	S	L	M	S	L	M	S	L	M	S



M	D	R	D	R	D	R	D	D	D	R	R	R	M
	L	M	S	L	M	S	L	M	S	L	M	S	



M	D	D	D	R	R	D	R	D	D	R	R	R	M
	L	M	S	L	M	S	L	M	S	L	M	S	

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

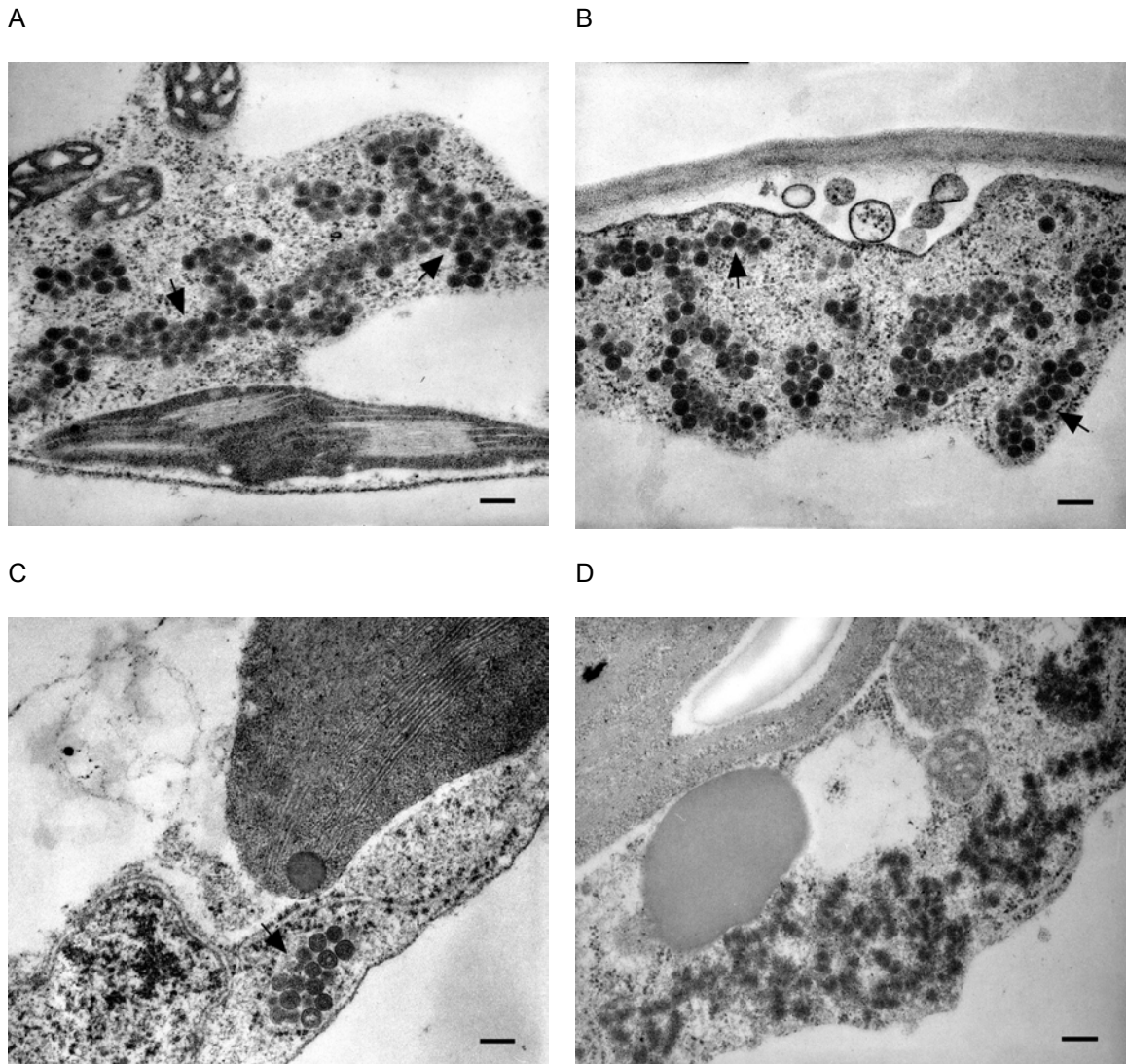


Fig. 2. Transmission electron micrographs of TSWV-infected tissue and mutant SLIs. Arrow indicates an example of groups of enveloped TSWV virions. Bar = 200nm. A. Enveloped TSWV virions in TSWV-RG2P24-infected tissue. B. Enveloped TSWV virions in tissue infected with SLI 37, a thrips transmissible isolate. C. Enveloped TSWV virions in tissue infected with SLI 81, a thrips non-transmissible isolate with a mutation of C to A at 1375 which changes Pro⁴⁵⁹ to Thr. 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; lane 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; lane 1, 5, and 6) which have nonsense or frameshift mutation 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 (Lys401Arg) and G2294U (Gly765Val) 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 (lane 8 and 9).

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APPENDICES

APPENDIX I

Table 1. The thrips transmissibility of TSWV-RG2P24, -CFLP24, and -GTP24, which were generated by 24 serial mechanical transmissions of TSWV-RG2, -CFL, and -GT

TSWV Isolate	Transmission (infected/inoculated, P_T) ^a
RG2P24	(4/10, 0.050) ; (5/10, 0.067) ; (4/10, 0.050)
GTP24	(4/10, 0.050) ; (5/10, 0.067) ; (3/10, 0.035)
CFLP24	(3/10, 0.035) ; (3/10, 0.035) ; (4/10, 0.050)
RG2 ^b	(5/10, 0.067) ; (4/10, 0.050) ; (4/10, 0.050)
D ^b	(0/10, 0) ; (0/10, 0) ; (0/10, 0)

^aTransmission was confirmed by symptom development and RT-PCR. Rate of transmission by thrips was represented as the probability (P_T) of TSWV transmission by a single adult thrips, which was estimated by $P_T = 1 - (1 - S/N)^{1/K}$, where N = number of plant inoculated, S = number of plants that became infected, and K = number of adult thrips per plant. Each of the experiments is separated by semicolon.

^bTSWV-RG2 and -D were used as a positive and negative control, respectively.

APPENDIX II

Identity matrix table of TSWV isolates in Chapter 1 including TSWV-BR01, -D, and -Japan.

Table 1. Nucleotide identity of 5'NTR among TSWV isolates.

ID means identical and GenBank accession number of previously reported isolates are S48091 (TSWV-BR01), AF208497 (TSWV-D), and AB010996 (TSWV-Japan).

	RG2	RG2P24	RG2T24	RG2R	CFL	CFLP24	CFLT24	CFLR	GT	GTP24	GTT24	GTR	GTN	BR-01	D	Japan
RG2	ID	1.000	1.000	1.000	0.990	1.000	0.990	0.990	1.000	1.000	0.980	1.000	0.980	0.970	0.980	0.990
RG2P24	1.000	ID	1.000	1.000	0.990	1.000	0.990	0.990	1.000	1.000	0.980	1.000	0.980	0.970	0.980	0.990
RG2T24	1.000	1.000	ID	1.000	0.990	1.000	0.990	0.990	1.000	1.000	0.980	1.000	0.980	0.970	0.980	0.990
RG2R	1.000	1.000	1.000	ID	0.990	1.000	0.990	0.990	1.000	1.000	0.980	1.000	0.980	0.970	0.980	0.990
CFL	0.990	0.990	0.990	0.990	ID	0.990	1.000	0.980	0.990	0.990	0.990	0.990	0.990	0.960	0.970	0.980
CFLP24	1.000	1.000	1.000	1.000	0.990	ID	0.990	0.990	1.000	1.000	0.980	1.000	0.980	0.970	0.980	0.990
CFLT24	0.990	0.990	0.990	0.990	1.000	0.990	ID	0.980	0.990	0.990	0.990	0.990	0.990	0.960	0.970	0.980
CFLR	0.990	0.990	0.990	0.990	0.980	0.990	0.980	ID	0.990	0.990	0.970	0.990	0.970	0.970	0.980	0.980
GT	1.000	1.000	1.000	1.000	0.990	1.000	0.990	0.990	ID	1.000	0.980	1.000	0.980	0.970	0.980	0.990
GTP24	1.000	1.000	1.000	1.000	0.990	1.000	0.990	0.990	1.000	ID	0.980	1.000	0.980	0.970	0.980	0.990
GTT24	0.980	0.980	0.980	0.980	0.990	0.980	0.990	0.970	0.980	0.980	ID	0.980	1.000	0.970	0.960	0.970
GTR	1.000	1.000	1.000	1.000	0.990	1.000	0.990	0.990	1.000	1.000	0.980	ID	0.980	0.970	0.980	0.990
GTN	0.980	0.980	0.980	0.980	0.990	0.980	0.990	0.970	0.980	0.980	1.000	0.980	ID	0.970	0.960	0.970
BR-01	0.970	0.970	0.970	0.970	0.960	0.970	0.960	0.970	0.970	0.970	0.970	0.970	0.970	ID	0.970	0.960
D	0.980	0.980	0.980	0.980	0.970	0.980	0.970	0.980	0.980	0.980	0.960	0.980	0.960	0.970	ID	0.970
Japan	0.990	0.990	0.990	0.990	0.980	0.990	0.980	0.980	0.990	0.990	0.970	0.990	0.970	0.960	0.970	ID

Table 2. Nucleotide identity of 3'NTR among TSWV isolates

	RG2	RG2P24	RG2T24	RG2R	CFL	CFLP24	CFLT24	CFLR	GT	GTP24	GTT24	GTR	GTN	BR-01	D	Japan
RG2	ID	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
RG2P24	1.000	ID	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
RG2T24	1.000	1.000	ID	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
RG2R	1.000	1.000	1.000	ID	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
CFL	1.000	1.000	1.000	1.000	ID	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
CFLP24	1.000	1.000	1.000	1.000	1.000	ID	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
CFLT24	1.000	1.000	1.000	1.000	1.000	1.000	ID	1.000	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
CFLR	1.000	1.000	1.000	1.000	1.000	1.000	1.000	ID	1.000	1.000	1.000	1.000	1.000	0.928	0.928	0.964
GT	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	ID	1.000	1.000	1.000	1.000	0.928	0.928	0.964
GTP24	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	ID	1.000	1.000	1.000	0.928	0.928	0.964
GTT24	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	ID	1.000	1.000	0.928	0.928	0.964
GTR	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	ID	1.000	0.928	0.928	0.964
GTN	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	ID	0.928	0.928	0.964
BR-01	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	ID	1.000	0.894
D	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	0.928	1.000	ID	0.894
Japan	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.964	0.894	0.894	ID

Table 3. Nucleotide identity of IGR among TSWV isolates

	RG2	RG2P24	RG2T24	RG2R	CFL	CFLP24	CFLT24	CFLR	GT	GTP24	GTT24	GTR	GTN	BR01	D	Japan
RG2	ID	1.000	1.000	1.000	1.000	0.951	0.981	0.951	1.000	0.951	0.996	0.951	0.996	0.702	0.701	0.889
RG2P24	1.000	ID	1.000	1.000	1.000	0.951	0.981	0.951	1.000	0.951	0.996	0.951	0.996	0.702	0.701	0.889
RG2T24	1.000	1.000	ID	1.000	1.000	0.951	0.981	0.951	1.000	0.951	0.996	0.951	0.996	0.702	0.701	0.889
RG2R	1.000	1.000	1.000	ID	1.000	0.951	0.981	0.951	1.000	0.951	0.996	0.951	0.996	0.702	0.701	0.889
CFL	1.000	1.000	1.000	1.000	ID	0.951	0.981	0.951	1.000	0.951	0.996	0.951	0.996	0.702	0.701	0.889
CFLP24	0.951	0.951	0.951	0.951	0.951	ID	0.940	1.000	0.951	1.000	0.948	1.000	0.948	0.662	0.661	0.841
CFLT24	0.981	0.981	0.981	0.981	0.981	0.940	ID	0.940	0.981	0.940	0.985	0.940	0.985	0.690	0.685	0.871
CFLR	0.951	0.951	0.951	0.951	0.951	1.000	0.940	ID	0.951	1.000	0.948	1.000	0.948	0.662	0.661	0.841
GT	1.000	1.000	1.000	1.000	1.000	0.951	0.981	0.951	ID	0.951	0.996	0.951	0.996	0.702	0.701	0.889
GTP24	0.951	0.951	0.951	0.951	0.951	1.000	0.940	1.000	0.951	ID	0.948	1.000	0.948	0.662	0.661	0.841
GTT24	0.996	0.996	0.996	0.996	0.996	0.948	0.985	0.948	0.996	0.948	ID	0.948	1.000	0.699	0.698	0.886
GTR	0.951	0.951	0.951	0.951	0.951	1.000	0.940	1.000	0.951	1.000	0.948	ID	0.948	0.662	0.661	0.841
GTN	0.996	0.996	0.996	0.996	0.996	0.948	0.985	0.948	0.996	0.948	1.000	0.948	ID	0.699	0.698	0.886
BR01	0.702	0.702	0.702	0.702	0.702	0.662	0.690	0.662	0.702	0.662	0.699	0.662	0.699	ID	0.902	0.681
D	0.701	0.701	0.701	0.701	0.701	0.661	0.685	0.661	0.701	0.661	0.698	0.661	0.698	0.902	ID	0.670
Japan	0.889	0.889	0.889	0.889	0.889	0.841	0.871	0.841	0.889	0.841	0.886	0.841	0.886	0.681	0.670	ID

Table 4. Nucleotide identity of NSm ORF among TSWV isolates

	RG2	RG2P24	RG2T24	RG2R	GT	GTP24	GTT24	GTN	GTR	CFL	CFLP24	CFLT24	CFLR	BR01	Japan	D
RG2	ID	0.996	0.996	0.996	0.996	0.995	0.993	0.993	0.995	0.996	0.994	0.996	0.994	0.928	0.981	0.940
RG2P24	0.996	ID	0.993	1.000	1.000	0.998	0.996	0.996	0.998	1.000	0.997	1.000	0.997	0.931	0.984	0.943
RG2T24	0.996	0.993	ID	0.993	0.993	0.992	0.990	0.990	0.992	0.993	0.991	0.993	0.991	0.927	0.977	0.939
RG2R	0.996	1.000	0.993	ID	1.000	0.998	0.996	0.996	0.998	1.000	0.997	1.000	0.997	0.931	0.984	0.943
GT	0.996	1.000	0.993	1.000	ID	0.998	0.996	0.996	0.998	1.000	0.997	1.000	0.997	0.931	0.984	0.943
GTP24	0.995	0.998	0.992	0.998	0.998	ID	0.995	0.995	1.000	0.998	0.996	0.998	0.996	0.930	0.983	0.942
GTT24	0.993	0.996	0.990	0.996	0.996	0.995	ID	0.997	0.995	0.996	0.994	0.996	0.994	0.928	0.981	0.940
GTN	0.993	0.996	0.990	0.996	0.996	0.995	0.997	ID	0.995	0.996	0.994	0.996	0.994	0.928	0.981	0.940
GTR	0.995	0.998	0.992	0.998	0.998	1.000	0.995	0.995	ID	0.998	0.996	0.998	0.996	0.930	0.983	0.942
CFL	0.996	1.000	0.993	1.000	1.000	0.998	0.996	0.996	0.998	ID	0.997	1.000	0.997	0.931	0.984	0.943
CFLP24	0.994	0.997	0.991	0.997	0.997	0.996	0.994	0.994	0.996	0.997	ID	0.997	1.000	0.929	0.984	0.941
CFLT24	0.996	1.000	0.993	1.000	1.000	0.998	0.996	0.996	0.998	1.000	0.997	ID	0.997	0.931	0.984	0.943
CFLR	0.994	0.997	0.991	0.997	0.997	0.996	0.994	0.994	0.996	0.997	1.000	0.997	ID	0.929	0.984	0.941
BR01	0.928	0.931	0.927	0.931	0.931	0.930	0.928	0.928	0.930	0.931	0.929	0.931	0.929	ID	0.936	0.974
Japan	0.981	0.984	0.977	0.984	0.984	0.983	0.981	0.981	0.983	0.984	0.984	0.984	0.984	0.936	ID	0.948
D	0.940	0.943	0.939	0.943	0.943	0.942	0.940	0.940	0.942	0.943	0.941	0.943	0.941	0.974	0.948	ID

Table 5. Amino acid identity of NSm protein among TSWV isolates

	RG2	RG2P24	RG2T24	RG2R	GT	GTP24	GTT24	GTN	GTR	CFL	CFLP24	CFLT24	CFLR	BR01	Japan	D
RG2	ID	0.996	0.993	0.996	0.996	0.993	0.993	0.996	0.993	0.996	0.993	0.996	0.993	0.956	0.986	0.966
RG2P24	0.996	ID	0.990	1.000	1.000	0.996	0.996	1.000	0.996	1.000	0.996	1.000	0.996	0.960	0.990	0.970
RG2T24	0.993	0.990	ID	0.990	0.990	0.986	0.986	0.990	0.986	0.990	0.986	0.990	0.986	0.950	0.980	0.960
RG2R	0.996	1.000	0.990	ID	1.000	0.996	0.996	1.000	0.996	1.000	0.996	1.000	0.996	0.960	0.990	0.970
GT	0.996	1.000	0.990	1.000	ID	0.996	0.996	1.000	0.996	1.000	0.996	1.000	0.996	0.960	0.990	0.970
GTP24	0.993	0.996	0.986	0.996	0.996	ID	0.993	0.996	1.000	0.996	0.993	0.996	0.993	0.956	0.986	0.966
GTT24	0.993	0.996	0.986	0.996	0.996	0.993	ID	0.996	0.993	0.996	0.993	0.996	0.993	0.960	0.986	0.966
GTN	0.996	1.000	0.990	1.000	1.000	0.996	0.996	ID	0.996	1.000	0.996	1.000	0.996	0.960	0.990	0.970
GTR	0.993	0.996	0.986	0.996	0.996	1.000	0.993	0.996	ID	0.996	0.993	0.996	0.993	0.956	0.986	0.966
CFL	0.996	1.000	0.990	1.000	1.000	0.996	0.996	1.000	0.996	ID	0.996	1.000	0.996	0.960	0.990	0.970
CFLP24	0.993	0.996	0.986	0.996	0.996	0.993	0.993	0.996	0.993	0.996	ID	0.996	1.000	0.956	0.986	0.966
CFLT24	0.996	1.000	0.990	1.000	1.000	0.996	0.996	1.000	0.996	1.000	0.996	ID	0.996	0.960	0.990	0.970
CFLR	0.993	0.996	0.986	0.996	0.996	0.993	0.993	0.996	0.993	0.996	1.000	0.996	ID	0.956	0.986	0.966
BR01	0.956	0.960	0.950	0.960	0.960	0.956	0.960	0.960	0.956	0.960	0.956	0.960	0.956	ID	0.970	0.976
Japan	0.986	0.990	0.980	0.990	0.990	0.986	0.986	0.990	0.986	0.990	0.986	0.990	0.986	0.970	ID	0.980
D	0.966	0.970	0.960	0.970	0.970	0.966	0.966	0.970	0.966	0.970	0.966	0.970	0.966	0.976	0.980	ID

Table 6. Nucleotide identity of G_N/G_C ORF among TSWV isolates

	RG2	RG2P24	RG2T24	RG2R	GT	GTP24	GTT24	GTN	GTR	CFL	CFLP24	CFLT24	CR_G	BR-01	Japan	D
RG2	ID	0.996	0.999	0.996	0.997	0.996	0.993	0.992	0.996	0.997	0.996	0.996	0.996	0.929	0.979	0.928
RG2P24	0.996	ID	0.996	0.999	0.997	0.998	0.993	0.992	0.998	0.998	0.999	0.995	0.998	0.931	0.980	0.928
RG2T24	0.999	0.996	ID	0.996	0.997	0.995	0.993	0.992	0.995	0.997	0.996	0.995	0.995	0.930	0.979	0.928
RG2R	0.996	0.999	0.996	ID	0.997	0.998	0.992	0.991	0.998	0.997	0.999	0.995	0.998	0.931	0.980	0.928
GT	0.997	0.997	0.997	0.997	ID	0.997	0.993	0.992	0.997	0.998	0.997	0.996	0.997	0.930	0.980	0.928
GTP24	0.996	0.998	0.995	0.998	0.997	ID	0.992	0.991	1.000	0.997	0.998	0.995	0.998	0.931	0.980	0.928
GTT24	0.993	0.993	0.993	0.992	0.993	0.992	ID	0.998	0.992	0.994	0.993	0.993	0.992	0.927	0.976	0.926
GTN	0.992	0.992	0.992	0.991	0.992	0.991	0.998	ID	0.991	0.993	0.992	0.992	0.991	0.926	0.975	0.925
GTR	0.996	0.998	0.995	0.998	0.997	1.000	0.992	0.991	ID	0.997	0.998	0.995	0.998	0.931	0.980	0.928
CFL	0.997	0.998	0.997	0.997	0.998	0.997	0.994	0.993	0.997	ID	0.998	0.997	0.997	0.931	0.980	0.928
CFLP24	0.996	0.999	0.996	0.999	0.997	0.998	0.993	0.992	0.998	0.998	ID	0.995	0.999	0.931	0.980	0.928
CFLT24	0.996	0.995	0.995	0.995	0.996	0.995	0.993	0.992	0.995	0.997	0.995	ID	0.995	0.928	0.978	0.927
CR_G	0.996	0.998	0.995	0.998	0.997	0.998	0.992	0.991	0.998	0.997	0.999	0.995	ID	0.931	0.980	0.928
BR-01	0.929	0.931	0.930	0.931	0.930	0.931	0.927	0.926	0.931	0.931	0.931	0.928	0.931	ID	0.934	0.967
Japan	0.979	0.980	0.979	0.980	0.980	0.980	0.976	0.975	0.980	0.980	0.980	0.978	0.980	0.934	ID	0.933
D	0.928	0.928	0.928	0.928	0.928	0.928	0.926	0.925	0.928	0.928	0.928	0.927	0.928	0.967	0.933	ID

Table 7. Amino acid identity of G_N/G_C protein among TSWV isolates

	RG2	RG2P24	RG2T24	RG2R	GT	GTP24	GTT24	GTN	GTR	CFL	CFLP24	CFLT24	CR_G	BR-01	Japan	D
RG2	ID	0.996	0.999	0.996	0.998	0.995	0.992	0.991	0.995	0.998	0.996	0.992	0.995	0.960	0.980	0.946
RG2P24	0.996	ID	0.995	1.000	0.996	0.997	0.991	0.989	0.997	0.996	0.998	0.991	0.997	0.960	0.980	0.945
RG2T24	0.999	0.995	ID	0.995	0.997	0.994	0.992	0.990	0.994	0.997	0.995	0.992	0.994	0.959	0.979	0.945
RG2R	0.996	1.000	0.995	ID	0.996	0.997	0.991	0.989	0.997	0.996	0.998	0.991	0.997	0.960	0.980	0.945
GT	0.998	0.996	0.997	0.996	ID	0.995	0.992	0.991	0.995	0.998	0.996	0.992	0.995	0.960	0.980	0.946
GTP24	0.995	0.997	0.994	0.997	0.995	ID	0.990	0.988	1.000	0.995	0.997	0.990	0.996	0.959	0.979	0.944
GTT24	0.992	0.991	0.992	0.991	0.992	0.990	ID	0.998	0.990	0.992	0.991	0.991	0.990	0.955	0.975	0.942
GTN	0.991	0.989	0.990	0.989	0.991	0.988	0.998	ID	0.988	0.991	0.989	0.989	0.988	0.953	0.973	0.940
GTR	0.995	0.997	0.994	0.997	0.995	1.000	0.990	0.988	ID	0.995	0.997	0.990	0.996	0.959	0.979	0.944
CFL	0.998	0.996	0.997	0.996	0.998	0.995	0.992	0.991	0.995	ID	0.996	0.994	0.995	0.960	0.980	0.946
CFLP24	0.996	0.998	0.995	0.998	0.996	0.997	0.991	0.989	0.997	0.996	ID	0.991	0.999	0.960	0.980	0.945
CFLT24	0.992	0.991	0.992	0.991	0.992	0.990	0.991	0.989	0.990	0.994	0.991	ID	0.990	0.955	0.975	0.942
CR_G	0.995	0.997	0.994	0.997	0.995	0.996	0.990	0.988	0.996	0.995	0.999	0.990	ID	0.960	0.980	0.945
BR-01	0.960	0.960	0.959	0.960	0.960	0.959	0.955	0.953	0.959	0.960	0.960	0.955	0.960	ID	0.962	0.965
Japan	0.980	0.980	0.979	0.980	0.980	0.979	0.975	0.973	0.979	0.980	0.980	0.975	0.980	0.962	ID	0.949
D	0.946	0.945	0.945	0.945	0.946	0.944	0.942	0.940	0.944	0.946	0.945	0.942	0.945	0.965	0.949	ID

Table 8. Nucleotide identity of whole M RNA among TSWV isolates

	RG2	RG2P24	RG2T24	RG2R	GT	GTP24	GTT24	GTN	GTR	CFL	CFLP24	CFLT24	CFLR	BR01	Japan	D
RG2	ID	0.997	0.998	0.996	0.994	0.993	0.993	0.992	0.993	0.997	0.993	0.994	0.993	0.915	0.974	0.915
RG2P24	0.997	ID	0.996	0.999	0.995	0.996	0.993	0.993	0.996	0.998	0.996	0.994	0.995	0.917	0.976	0.916
RG2T24	0.998	0.996	ID	0.996	0.993	0.992	0.992	0.991	0.992	0.996	0.993	0.993	0.992	0.915	0.974	0.915
RG2R	0.996	0.999	0.996	ID	0.994	0.996	0.993	0.992	0.996	0.998	0.996	0.994	0.995	0.917	0.975	0.916
GT	0.994	0.995	0.993	0.994	ID	0.991	0.998	0.997	0.991	0.995	0.992	0.993	0.991	0.914	0.973	0.915
GTP24	0.993	0.996	0.992	0.996	0.991	ID	0.990	0.989	1.000	0.995	0.998	0.991	0.997	0.914	0.972	0.913
GTT24	0.993	0.993	0.992	0.993	0.998	0.990	ID	0.998	0.990	0.994	0.990	0.992	0.990	0.913	0.972	0.913
GTN	0.992	0.993	0.991	0.992	0.997	0.989	0.998	ID	0.989	0.994	0.989	0.991	0.989	0.913	0.971	0.913
GTR	0.993	0.996	0.992	0.996	0.991	1.000	0.990	0.989	ID	0.995	0.998	0.991	0.997	0.914	0.972	0.913
CFL	0.997	0.998	0.996	0.998	0.995	0.995	0.994	0.994	0.995	ID	0.995	0.996	0.994	0.917	0.975	0.916
CFLP24	0.993	0.996	0.993	0.996	0.992	0.998	0.990	0.989	0.998	0.995	ID	0.991	0.999	0.914	0.973	0.913
CFLT24	0.994	0.994	0.993	0.994	0.993	0.991	0.992	0.991	0.991	0.996	0.991	ID	0.991	0.913	0.972	0.913
CFLR	0.993	0.995	0.992	0.995	0.991	0.997	0.990	0.989	0.997	0.994	0.999	0.991	ID	0.913	0.972	0.913
BR01	0.915	0.917	0.915	0.917	0.914	0.914	0.913	0.913	0.914	0.917	0.914	0.913	0.913	ID	0.918	0.965
Japan	0.974	0.976	0.974	0.975	0.973	0.972	0.972	0.971	0.972	0.975	0.973	0.972	0.972	0.918	ID	0.918
D	0.915	0.916	0.915	0.916	0.915	0.913	0.913	0.913	0.913	0.916	0.913	0.913	0.913	0.965	0.918	ID

APPENDIX III

Table 1. Comparison of nucleotide and amino acid (parenthesis) in NSm ORF of TSWV isolates in Chapter 1

The nucleotide of the NSm and the G_N/G_C was numbered as Appendix 1.

	Isolate	RG2	RG2P24	RG2T24	GT	GTP24	GTT24	GTN	CFL	CFLP24	CFLT24	BR01	D
Position of the change in the nucleotide (amino acid)	32 (11)	C (S)	U (F)	U (F)	U (F)	U (F)	U (F)	U (F)	U (F)	U (F)	U (F)	U (F)	U (F)
	62 (21)	U (L)	U (L)	C (S)	U (L)	U (L)	U (L)	U (L)	U (L)	U (L)	U (L)	U (L)	U (L)
	72	C	U	C	U	U	U	U	U	U	U	U	U
	80 (27)	A (H)	A (H)	A (H)	A (H)	G (R)	A (H)	A (H)	A (H)	A (H)	A (H)	A (H)	A (H)
	88 (30)	A (N)	A (N)	A (N)	A (N)	A (N)	G (D)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)
	139 (47)	A (T)	A (T)	G (A)	A (T)	A (T)	A (T)	A (T)	A (T)	A (T)	A (T)	A (T)	A (T)
	186	G	G	G	G	G	G	G	G	A	G	G	G
	454	C	C	U	C	C	C	C	C	C	C	C	C
	493	C	U	C	C	C	C	C	C	C	C	C	C
	615	A	A	A	A	A	G	G	A	A	A	A	A
	699	A	A	A	A	A	G	G	A	A	A	A	A
	705	C	C	U	C	C	C	C	C	C	C	U	U
	738	U	U	C	U	U	U	U	U	U	U	U	U
	825	G	G	G	G	G	G	A	G	G	G	G	G
	832 (278)	G (D)	A (N)	G (D)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)

Table 2. Comparison of nucleotide and amino acid (parenthesis) in G_N/G_C ORF of TSWV isolates in this study

	Isolate	RG2	RG2P24	RG2T24	GT	GTP24	GTT24	GTN	CFL	CFLP24	CFLT24	BR01	D
Position of the change in the nucleotide (amino acid)	67 (23)	G (V)	G (V)	G (V)	A (I)	G (V)	G (V)	A (I)	G (V)	G (V)	G (V)	G (V)	G (V)
	86 (29)	C (T)	U (I)	C (T)	C (T)	U (I)	C (T)	C (T)	C (T)	U (I)	C (T)	U (I)	C (T)
	138 (46)	U (D)	G (E)	U (D)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)
	173 (58)	A (T)	A (T)	A (T)	A (T)	A (T)	G (A)	G (A)	A (T)	A (T)	A (T)	A (T)	A (T)
	184 (62)	A (I)	A (I)	G (V)	A (I)	A (I)	A (I)	A (I)	A (I)	A (I)	A (I)	A (I)	A (I)
	241 (81)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	U (S)	C (P)	C (P)
	252	C	U	C	U	U	U	U	U	U	U	U	U
	294	G	G	G	G	G	A	A	G	G	G	G	G
	300	U	U	U	U	U	U	A	U	U	U	A	A
	306	G	A	G	A	A	A	A	A	A	A	A	A
	432	U	C	U	C	C	U	U	U	C	U	C	C
	450	G	G	A	G	G	G	G	G	G	G	A	A
	492	G	A	G	G	A	G	G	G	A	G	G	G
	519	C	C	C	C	C	U	U	C	C	C	C	U
	618	G	G	G	G	G	A	A	G	G	G	A	A
	747 (249)	G (L)	G (L)	G (L)	G (L)	U (F)	G (L)	G (L)	G (L)	G (L)	G (L)	G (L)	G (L)
	785 (262)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	C (T)	U (I)	U (I)
	817 (273)	A (T)	A (T)	A (T)	A (T)	A (T)	A (T)	A (T)	G (A)	A (T)	A (T)	A (T)	A (T)
	1031 (344)	A (K)	A (K)	A (K)	A (K)	A (K)	G (R)	G (R)	A (K)	A (K)	G (R)	A (K)	A (K)
	1257 (419)	C (N)	C (N)	C (N)	C (N)	C (N)	A (K)	A (K)	C (N)	C (N)	C (N)	C (N)	C (N)
	1373 (458)	U (M)	C (T)	U (M)	U (M)	C (T)	U (M)	U (M)	U (M)	C (T)	U (M)	U (M)	U (I)
	1374 (458)	G (M)	G (M)	G (M)	G (M)	G (M)	G (M)	G (M)	G (M)	G (M)	G (M)	G (M)	U (I)
	1392	U	U	U	U	U	U	U	U	U	U	A	A
	1431	C	C	C	C	A	C	C	C	C	C	C	C
	1549 (517)	G (G)	G (G)	G (G)	G (G)	G (G)	A (S)	A (S)	G (G)	G (G)	A (S)	G (G)	G (G)
	1649 (550)	G (R)	G (R)	G (R)	G (R)	G (R)	A (Q)	A (Q)	G (R)	G (R)	G (R)	G (R)	A (Q)

Table 2. (Continued)

	Isolate	RG2	RG2P24	RG2T24	GT	GTP24	GTT24	GTN	CFL	CFLP24	CFLT24	BR01	D
	1704	A	A	A	A	A	A	G	A	A	A	A	A
	1743	U	U	U	U	U	C	C	U	U	U	U	U
	1767	U	U	U	U	U	C	C	U	U	U	U	U
	1794	A	A	A	A	A	A	U	A	A	A	C	A
	1804	C	U	C	C	U	C	C	U	U	C	U	U
	1976 (659)	U (V)	U (V)	U (V)	U (V)	U (V)	U (V)	C (A)	U (V)	U (V)	U (V)	U (V)	U (V)
	2016	A	A	A	A	A	G	G	A	A	A	A	A
	2150 (717)	A (Y)	A (Y)	A (Y)	A (Y)	A (Y)	A (Y)	G (C)	A (Y)	A (Y)	A (Y)	A (Y)	A (Y)
	2163 (721)	G	A	A	A	A	A	A	A	A	A	A	A
	2267 (756)	C (T)	C (T)	C (T)	C (T)	C (T)	U (I)	U (I)	C (T)	C (T)	C (T)	C (T)	C (T)
Position of	2277	A	A	A	A	A	A	A	A	A	U	A	A
the change	2361	U	U	U	U	U	U	U	U	U	C	U	U
in the	2384 (795)	A (N)	A (N)	A (N)	G (S)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)	A (N)
nucleotide	2391	U	U	U	C	U	U	U	U	U	U	U	U
(amino acid)	2568 (856)	G (A)	G (A)	G (A)	G (A)	G (A)	A (A)	G (A)	G (A)	G (A)	G (A)	G (A)	A (T)
	2570 (857)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	U (I)	C (T)
	2793	U	U	U	U	U	C	C	U	U	U	U	U
	2833 (945)	G (V)	G (V)	G (V)	G (V)	G (V)	G (V)	G (V)	G (V)	U (F)	G (V)	C (L)	A (I)
	2897 (966)	C (S)	C (S)	C (S)	C (S)	U (F)	C (S)	C (S)	C (S)	C (S)	C (S)	C (S)	C (S)
	2969 (990)	A (D)	A (D)	A (D)	A (D)	A (D)	A (D)	A (D)	G (G)	A (D)	G (G)	A (D)	A (D)
	3006	A	G	A	G	G	G	G	G	G	G	G	A
	3043 (1015)	G (V)	G (V)	G (V)	G (V)	G (V)	G (V)	G (V)	G (V)	G (V)	A (I)	G (V)	A (I)
	3052 (1018)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	C (P)	U (S)	C (P)	C (P)
	3099	A	A	A	G	A	A	A	A	A	A	A	A
	3331 (1111)	G (E)	A (K)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)	G (E)