

Molecular biology of *Tomato spotted wilt virus*: An update

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Abstract

Advances in understanding of *Tomato spotted wilt virus* (TSWV) molecular biology are reviewed. TSWV, a type species of the genus *Tospovirus*, is an enveloped virus that causes high economical losses in many crops worldwide. It is transmitted by several species of thrips and multiplies in insect cells. The most important vector is *Frankliniella occidentalis* which transmits TSWV in a persistent propagative manner. Several factors are known from both virus and vector side which plays important role in virus acquisition by thrips and its subsequent transfer. TSWV is a segmented negatively strand RNA virus. RNA of TSWV is partitioned among three negative or ambisense single stranded RNA (ssRNA) labeled as L, M and S in order of decreasing size, (approximately 8897, 4821 and 2916 nucleotides long, respectively). These RNA segments encode various proteins like N and Ns by S RNA; NSm and G1/G2 by M RNA and RdRp by L RNA. Intergenic region present in M and S RNA of TSWV helps in proper transcription of different genes encoded by M and S RNA. The different proteins encoded by TSWV genome help the virus in protection, cellular movements, vector transmission, replication and recently in RNA silencing suppressor activity. The present review focuses on basic structure, genome organization, molecular basis of transmission and recent advances in TSWV detection.

Key words: TSWV, Thrips, L RNA, M RNA, S RNA, Intergenic region

Abbreviations: AMV=*Alfalfa mosaic virus*; ArMV=*Arabidopsis mosaic virus*; CERV=*Carnation etched ring virus*; cDNA= complementary DNA; DAS ELISA = double antibody sandwich enzyme linked immunosorbent assay; ds = double stranded; ELISA = enzyme linked immunosorbent assay; FoTf = *Frankliniella occidentalis* putative transcription factor; GA = Georgia; GFP = green fluorescent protein; GP = glycoprotein precursor; IGR = intergenic regions; IRES = internal ribosomal entry site; FL = Florida; FRET = fluorescence resonance energy transfer; FLIM = fluorescence lifetime imaging microscopy; miRNA = micro-RNA; MP = movement protein; NC = nucleocapsid; ORF = open reading frame; PNGase F = peptide:N-glycosidase F; PTGS = post transcriptional gene silencing; PNRSV = *Prunus necrotic ring spot virus*; PCR = polymerase chain reaction; RNPs = ribonucleoproteins; RdRp = RNA dependant RNA polymerase; RT-PCR = real time polymerase chain reaction; siRNA = small interfering RNA; ss = single stranded; SLRSV = *Strawberry latent ringspot virus*; SDS-PAGE = sodium dodecyl sulphate- polyacrylamide gel electrophoresis; TSWV = *Tomato spotted wilt virus*; TMMV = *Tuberose mild mottle virus*; UTR = untranslated region; vc = virus complementary; WFT = western flower thrips

Introduction

Tomato spotted wilt virus (TSWV) is found in almost all continents and is the type species of the plant viruses belonging to the genus *Tospovirus* within the arthropod-borne Bunyaviridae family (Van Regenmortel *et al.*, 2000). TSWV is one of the most economically important members of the genus *Tospovirus* (Peters and Goldbach, 1995; Moyer, 2000). From family *Bunyaviridae*, genus *Tospovirus* is the only genus consisting of viruses infecting plants (Pappu, 2008). TSWV is transmitted by multiple species of thrips (Ullman, 1997) and the most important vector is *Frankliniella occidentalis* Pergande, the western flower thrips (WFT) which transmits TSWV in a persistent propagative fashion (Gera *et al.*, 2000). It was not until mid-1960 that the enveloped virions morphology was revealed and the molecular characterization and genome organization did not occur until after 1990 (Moyer, 1999).

It seems that disease caused by the TSWV was first observed in 1906 (Sakimura, 1962). Brittlebank (1919) published the first description of this new disease, detected on tomatoes in 1915 in the state of Victoria (Australia) and he called it "spotted wilt of tomato". In the same year, Osborn also observed this disease on tomatoes in the south of Australia and in 1920 its presence was reported in all the Australian states (Best, 1968). The first

characterization of this virus as the causal agent of the disease was reported by Samuel *et al.* (1930), who gave it its current name "*Tomato spotted wilt virus*". Since then it was reported from several tropical and temperate regions and it is considered worldwide in distribution. Worldwide loss caused due to this virus, mainly of commercial vegetable crops, is around one billion dollars annually (Scott, 2000). TSWV ranks among the top 10 of the most detrimental plant viruses worldwide (Prins and Goldbach, 1998). In general, the two main concomitant factors that make TSWV one of the most destructive and widely distributed plant viruses are the highly polyphagous nature of its vectors and the relative lack of host specificity of the virus. Consequently, TSWV has the widest host ranges of any known plant viruses and its control remains problematic. Major crops susceptible to TSWV infection are tomato, pepper, lettuce, potato, papaya, peanut, tobacco and chrysanthemum (German *et al.*, 1992). Symptoms vary with the host plant, time of year and environmental conditions and include stunting, necrosis, chlorosis, ring spots and ring/line patterns affecting leaves, stems and fruit (German *et al.*, 1992; Mumford *et al.*, 1996). The current list of TSWV hosts consists of 1090 plants species belonging to 15 families of monocotyledonous plants, 69 families of dicotyledonous plants and one family of pteridophytes (Parrella *et al.*, 2003).

Basic Structure: TSWV particles can be readily detected by electron microscope observations of leaf dip preparations with or without gold-immunolabelling (Milne, 1993). With the help of electron microscope, it has been shown that TSWV virions are spherical shaped (between 80 to 110 nm diameter). Each viral particle consists of granular core bounded by an envelope with surface projections (Best and Palk, 1964; Ie, 1964; Martin, 1964; Kitajima, 1965). Like the other bunyaviruses, membrane envelope of TSWV contains virally encoded glycoproteins, a feature quite uncommon for plant-infecting viruses but rather typical among animal viruses. Virion composition is 5% nucleic acid (RNA), 70% protein, 5% carbohydrate, and 20% lipid. Very little information was available concerning the nucleic acid of TSWV till 1965. Later, it was concluded that the nucleic acid was RNA on the basis of positive orcinol and negative diphenylamine reactions (Van Kammen *et al.*, 1966). Best (1968) arrived at the same conclusion from data obtained by paper chromatography and estimation of the base composition of the nucleic acid (adenine 35 %, cytosine 9 %, guanine 38 % and uracil 18 %).

Genome organization

Tomato spotted wilt virus (TSWV) is an enveloped segmented negatively strand RNA virus. Negative strand RNA viruses are also known as antisense strand RNA viruses. The term 'negative strand viruses' is derived from the coding strategy adopted by segments of viral genomes. RNA molecules of negative strand RNA viruses are transcribed first to give positive coding strand. So, in negative strand RNA viruses during amplification cycle, transcription is the first step (Van knippenberg, 2005). This explains the presence of viral polymerase inside virus particle (Van Poelwijk *et al.*, 1993). In contrast, RNA molecule is of

coding or sense polarity (mRNA) in positive strand RNA viruses. This RNA molecule can be directly utilized for the synthesis of proteins if provided with a 5' cap structure or internal ribosomal entry site (IRES) (Van knippenberg, 2005). Within the group of segmented negative sense RNA viruses, a distinct sub-group is formed by the so-called ambisense viruses. These possess at least one genome segment that is of dual polarity, *i.e.* containing one reading frame in the viral-sense RNA and one in the viral-complementary (vc) RNA. The *Tospovirus* genome is a tripartite, partitioned among three negative or ambisense single-stranded RNA (ssRNA) segments named L, M, and S in order of decreasing size (Fig. 1) (de Haan *et al.*, 1989). In addition, both the M RNA and S RNA possess the characteristic A-U rich intergenic regions (IGR) capable of forming stable hairpin structure (de Haan *et al.*, 1990). A conserved sequence found at the top of the hairpins of TSWV S and M RNAs indicates a possible role of amino acid signal for transcription termination of the open reading frames (ORFs) within the ambisense RNA segments (de Haan *et al.*, 1990; Kormelink *et al.*, 1992a). The IGR of M RNA is smaller in length compared to the IGR of S RNA (Bhatt *et al.*, 1999). Recently, it has been studied that hairpin structure sequence of S ambisense RNA in 3' untranslated region (UTR) of mRNA plays important role in translation also (Geerts-Dimitriadou *et al.*, 2012). The RNAs have partially complementary terminal sequences. This complementarity for each individual segment is extended to ~65 nt, that allows the RNAs to adopt a pseudocircular or panhandle conformation (de Haan *et al.*, 1989, 1990; Kormelink *et al.*, 1992a; Elliott *et al.*, 2000). The 5' and 3' terminal 8 nucleotides (nt) of each genomic RNA segment are complementary and conserved: 5'-AGAGCAAU and 3'-UCUCGUUA. The assembly

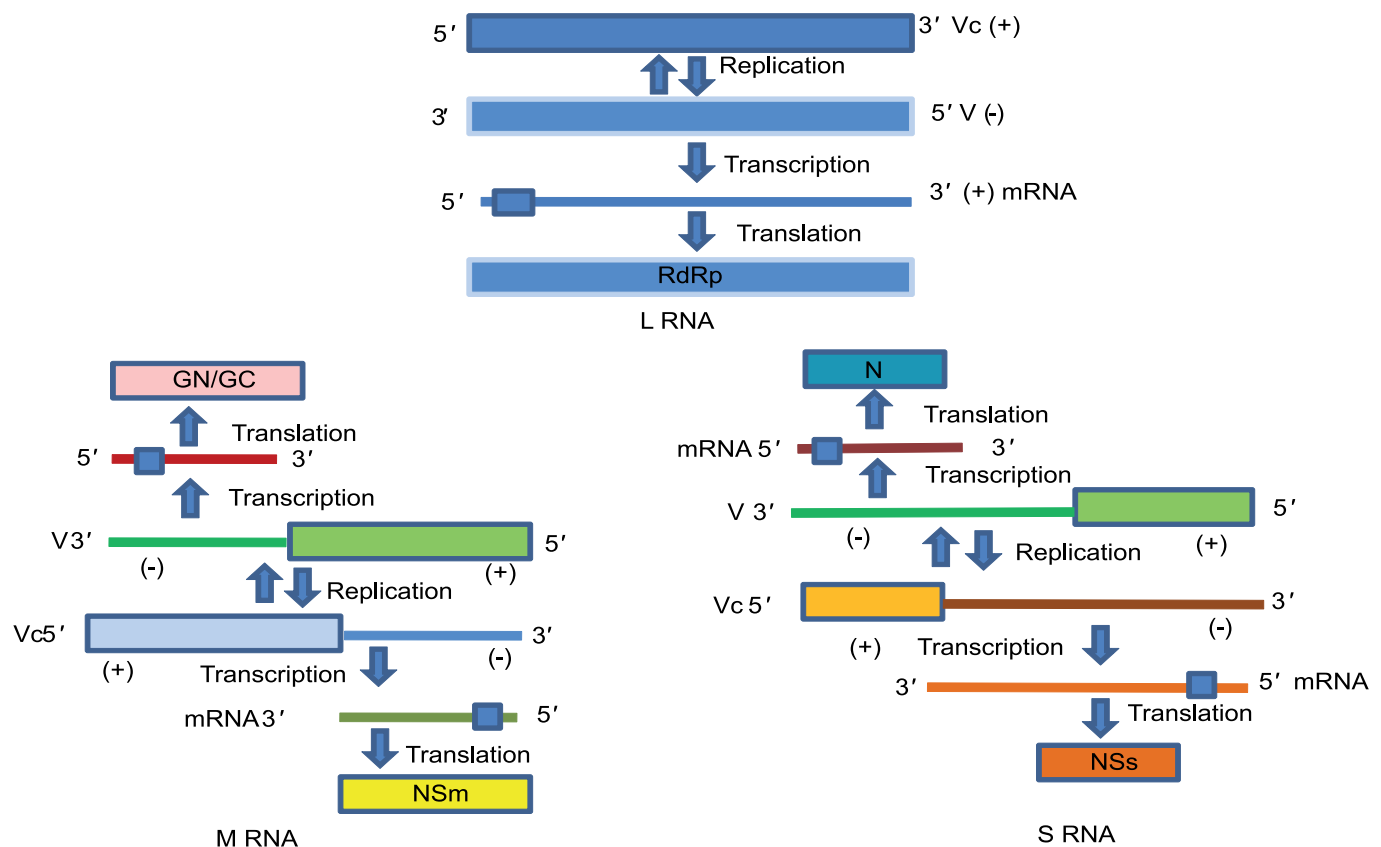


Fig. 1. Replication strategy for tripartite genome of TSWV (data taken from de Aliva, 1992; de Haan *et al.* 1990; 1991; Kormelink *et al.*, 1992c; Roselló *et al.*, 1996)

of virus particles first requires the formation of ribonucleoproteins (RNPs). These RNPs are complexes of viral genomic RNA, N protein and a few copies of the L protein (Elliott, 1990). The RNPs are enclosed by a lipid membrane envelope, which contains the viral-encoded glycoproteins. The viral genome contains five open reading frames coding for six mature proteins (Table 1).

L RNA: The largest RNA (L RNA) is negative sense and monocistronic. L RNA serves as a multifunctional, replication-associated protein and is believed to function cooperatively with host-encoded factors. L RNA is approximately 9 kb and has a single open reading frame (ORF) in the viral complementary sense. The RNA is 8897 nucleotides long coding for a 330 kDa protein, which is the putative RNA-dependent RNA polymerase (RdRp) or L protein (de Haan *et al.*, 1991; Lee *et al.*, 2011). This 330-kDa protein has been implied in several enzymatic activities such as transcriptase, replicase and endonuclease (Adkins *et al.*, 1995; Van Poelwijk, 1996; Chapman *et al.*, 2003). RNA viruses show extremely high mutation rates, because of lack of proofreading ability of their polymerases (Moya *et al.*, 2000). Although, the mutation or error rate of viral RdRp has not been estimated for plant viruses, it has been measured for animal RNA viruses and it is approximately 10^{-4} , or one error per genome per replication cycle (Roossinck, 1997). As Tospovirus has both negative and ambisense coding strategies, the RNA dependent RNA polymerase has to be co-transported with the viral RNA to allow transcription and replication within the newly infected cells (Soellick *et al.*, 2000).

M RNA: The M RNA is approximately 4.8 kb (Lee *et al.*, 2011) and encodes a 34 kDa protein in viral sense designated nonstructural NSm (commonly called as movement protein) proposed to be involved in cell to cell movement of nonenveloped ribonucleocapsid structures (Storms *et al.*, 1995; Soellick *et al.*, 2000; Silva *et al.*, 2001) and stimulation of tubule formation in protoplasts. Viral movement proteins facilitate transport of infectious material through plasmodesmata, the intercellular connection for the plant cell (Griffiths *et al.*, 1992). In plant tissue, the NSm protein of TSWV functions as viral movement protein (MP), aggregating into plasmodesma-penetrating tubules to establish cell-to-cell movement. Upon heterologous expression NSm was able to form similar tubules on the surface of insect (*Spodoptera frugiperda*) cells, during expression and cellular manifestation of this protein in infected thrips tissue. It is shown that NSm, though detectably expressed in both the L2 larval and adult thrips stages, does not aggregate into tubules, indicating that this requirement is associated to its function as MP in plants, and raising the question if NSm has a function at all during the insect life cycle of TSWV (Storms *et al.*, 2002). TSWV NSm domains required for tubule formation, movement

and symptoms were identified previously by deletion-mapping and alanine-substitution mutagenesis using the TMV-based system. Mutagenesis studies of TSWV NSm amino acids that are conserved in other tospovirus were conducted by Li *et al.* (2010) and suggested that functional domains of NSm protein may be conserved across the genus. Recent findings suggest that these movement proteins, which recognize and transport the viral genomes as naked nucleic acid or in complex with other viral proteins, resemble plant proteins that are involved in selective trafficking of protein and protein- nucleic acid complexes through plasmodesmata as part of fundamental transport and signaling process (de Haan *et al.*, 1990). The molecular basis of NSm function was studied by expressing the protein in *Escherichia coli* and investigated protein-protein and protein-RNA interactions of NSm protein *in vitro*. NSm specifically interacts with TSWV N protein and binds single-stranded RNA in a sequence-nonspecific manner. Using NSm as bait in a yeast two-hybrid screen, two homologous NSm-binding proteins of the DnaJ family from *Nicotiana tabacum* and *Arabidopsis thaliana* were identified (Soellick *et al.*, 2000).

The viral complementary sense (vc RNA) ORF codes for glycoprotein precursor GP, is post-translationally cleaved into the spikes or glycoproteins G1 and G2 (Kormelink *et al.*, 1992a). For TSWV, the 58 kDa G2 is also referred to as G(N) and 78 kDa G1 as G(C) (N and C refer to the amino and carboxy terminal position within the glycoprotein precursor, respectively) (Snippe *et al.*, 2007). These glycoproteins are required for virus infection of the arthropod vector. Other members of the Bunyaviridae enter host cells by pH-dependent endocytosis. During this process, the glycoproteins are exposed to conditions of acidic pH within endocytic vesicles causing the G(C) protein to change its conformation. This conformational change renders G(C) more sensitive to protease cleavage. TSWV virions were subjected to varying pH conditions and determined that TSWV G(C), but not G(N), was cleaved under acidic pH conditions and this phenomenon was not observed at neutral or alkaline pH. This provides evidence at low pH, G(C) conformation changes, which results in altered protease sensitivity. Furthermore, sequence analysis of G(C) predicts the presence of internal hydrophobic domains, regions that are characteristic of fusion proteins (Whitfield *et al.*, 2005). The presence of the membrane glycoproteins is essential for the virus's ability to replicate alternately in its plant host and its thrips vector (Wijkamp, 1996). Evidence for the involvement of the glycoproteins in thrips transmission is provided by the interaction of the glycoproteins with the proteins of the thrips vector (Bandla *et al.*, 1998), the loss of thrips transmissibility of envelope-deficient mutants (Resende *et al.*, 1991) and the presence of a sequence motif that is characteristic for cellular attachment

Table 1. Genome organization of *Tomato spotted wilt virus*

Type (RNA)	Orientation	Size (kb)	Gene / Products	Function	References
Small (S)	Ambisense	3.0	NSs / Non-structural protein	First RNA silencing suppressor	Takeda <i>et al.</i> , 2002; Bucher <i>et al.</i> , 2003; Schnettler <i>et al.</i> , 2010
			N / Nucleocapsid protein	Protective Envelope	de Haan <i>et al.</i> , 1990
Medium (M)	Ambisense	4.8	NSm / Movement protein	Cell-to-cell Movement	Storms <i>et al.</i> , 1995; Silva <i>et al.</i> , 2001
			G1, G2 / Glycoproteins	Vector transmission	Griffiths <i>et al.</i> , 1992; Petterson, 1991; Stephens and Compans, 1988
Large (L)	Negative	~ 9.0	RNA dependent RNA Polymerase	Replication	Adkins <i>et al.</i> , 1995; Chapman <i>et al.</i> , 2003; Van Poelwijk, 1996

domains (Kormelink *et al.*, 1992a). TSWV glycoproteins were also reported to induce the formation of endoplasmic reticulum and Golgi-derived pleomorphic membrane structures in plant cells (Ribeiro *et al.*, 2008). Interactions between TSWV glycoproteins and nucleocapsid (N) proteins were studied using fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) techniques. Interaction was demonstrated between G(C) and N and not in G (N) and N using both the techniques (Snippe *et al.*, 2007). Recently by using FLIM technique, it has been studied that nucleocapsid protein interacts with both viral glycoproteins (Riberio *et al.*, 2009). Glycoproteins G(N) and G(C) were examined for their lectin binding affinity (mannose binding lectins, N-Acetylglucosamine lectins and fucose binding lectins) and their sensitivities to glycosidases to know the nature of present oligosaccharides residues on them. Result showed that G(C) has strong binding to three lectin molecules whereas G(N) has lesser affinity to mannose lectin but not to other two. After treatment with two glycosidases (endoglycosidase H and peptide:N-glycosidase F (PNGase F), there is significant decrease in the binding of G(C) but no such effect was observed on G(N). Due to difference in binding properties of two glycoproteins it has been suggested that glycoprotein G(C) is heavily glycosylated as compare to G(N). There is no evidence observed for the presence of O-linked oligosaccharides on G (N) or G(C) (Naidu *et al.*, 2004).

During viral infection of a plant cell, the two glycoproteins eventually accumulate in the Golgi complex. So, site of TSWV particle morphogenesis was determined to be the Golgi system of host plant (Kikkert *et al.*, 1999). Golgi stacks containing the two glycoproteins then wrap around viral ribonucleoprotein (RNP) complexes consisting of viral RNA, associated with nucleocapsid (N) protein and the putative viral RNA-dependent RNA polymerase (RdRp) to form doubly enveloped virus particles. These are thought to fuse with each other and with ER-derived membranes, resulting in the formation of large intracellular vesicles containing singly enveloped virus particles (Kikkert *et al.*, 1999).

The formation of the enveloped virus particles is strongly regulated by the viral glycoproteins. They generally accumulate independently at a particular cellular membrane by targeted transport through the secretory pathway, to facilitate the interaction with the viral nucleocapsids and the initiation of budding (Stephens and Compans, 1988; Petterson, 1991; Griffiths *et al.*, 1992).

S RNA: The S segment is approximately 3 kb and contains two ORFs in ambisense orientation separated by a large intergenic region. The ORF nearer the 5' end of the RNA, codes for a nonstructural protein in the viral sense designated as NSs (54 kDa). The role of the NSs protein has long been enigmatic, but recently NSs was shown to be a suppressor of gene silencing required to protect the virus against the plant's anti-viral response of post-transcriptional gene silencing (PTGS) and also affects symptom expression in TSWV-infected plants (Takeda *et al.*, 2002; Bucher *et al.*, 2003). TSWV NSs is the first RNA silencing suppressor identified in negative-strand RNA viruses. This protein suppressed sense transgene- induced PTGS but did not suppress inverted repeat transgene- induced PTGS (Takeda *et al.*, 2002). Recently, biochemical analysis of NSs proteins from different *tospoviruses* using purified NSs or NSs containing cell extracts showed that NSs proteins have affinity to small dsRNA molecules *i.e.*, small interfering RNAs (siRNAs) and micro-RNA (miRNA).

The NSs protein of TSWV was shown to be capable of inhibiting Dicer- mediated cleavage of long ds RNA *in vitro*. In addition, it suppressed the accumulation of green fluorescent protein (GFP)-specific siRNAs during coinfiltration with an inverted-repeat-GFP RNA construct in *N. benthamiana* (Schnettler *et al.*, 2010). The ORF near the 3' end is in viral complementary (vc) sense and encodes for the nucleocapsid protein (N) (29 kDa) which encapsidates the viral RNAs within the viral envelope (de Haan *et al.*, 1990). The nucleocapsid protein (N) contributes to the viral replication cycle in a structural and, perhaps, regulatory manner by participating in the complex interactions among the RNP components leading to the initiation of viral RNA transcription and replication. Consistent with its role in fulfilling this putative function, the N protein has been shown to form dimers in the absence of RNA (Uhrig *et al.*, 1999; Kainz *et al.*, 2004) and to cooperatively bind ssRNA but not dsRNA (Richmond *et al.*, 1998). So, each RNA segment of tospovirus is associated with nucleocapsid (N) proteins (29kDa) and a few copies of the large (L) protein to form pseudo-circular nucleocapsid (NC) structures or ribonucleic protein particles (RNPs) (Kormelink *et al.*, 1992a; Peters, 2003). These particles result from the homopolymerization of the N protein, and are highly stable in plant cells and can be easily purified from TSWV infected plant cells by ultracentrifugation (de Aliva *et al.*, 1990). On the basis of these two properties of N protein (homopolymerization and high stability), a gene fusion approach was explored to increase the stability of foreign proteins produced in plants, thereby offering a possible purification alternative for a target protein as a gene fusion. These results show that the homopolymerization properties of the N protein can be used as a fast and simple way to purify large amounts of proteins from plants (Lacorte *et al.*, 2007). By using FRET and FLIM techniques homotypic interactions of nucleocapsid proteins were studied (Snippe *et al.*, 2005). Mutated forms of N protein serve as potent dominant-negative inhibitors of virus replication (Rudolph *et al.*, 2003). The complete nucleotide sequence of the TSWV is now available, allowing the precise comparison with the other animal infecting members of the *Bunyaviridae* family and with other families of plant infecting viruses (Table 2). The negative polarity of L RNA is 8897 nucleotide long whereas ambisense M and S RNAs are approximately 4821 and 2916 nucleotides long, respectively (de Hann *et al.*, 1990; 1991; Kormelink *et al.*, 1992a).

Intergenic region (IGR): The oppositely located ORFs on the ambisense S and M RNA segments are separated by intergenic regions (IGRs) of several hundred nucleotides and are regarded as the most hyper variable regions of the genome. Analysis of the intergenic region (IGR) of S and M RNAs of tospoviruses (Family *Bunyaviridae*) indicated their heterogeneity both in length and sequence. Both IGRs contain a long stretch of mainly A residues followed by a long stretch of mainly U residues, and are predicted to form large stable hairpin structures (~120 bp for the S RNA, ~75 bp for the M RNA;) (de Haan *et al.*, 1990; Kormelink *et al.*, 1992a). In addition, a sequence (CCAAUUUGG for S and GCAAACUUUGG for M) that is conserved between different tospoviruses is located near the top of these intergenic hairpins (Maiss *et al.*, 1991; de Haan *et al.*, 1992; Kormelink *et al.*, 1992a). More specifically the 5' and 3'ends of the IGRs are conserved, separated by variable sequences, deletions and insertions that appear as gaps in alignments (Bhatt *et al.*, 1999; Heinze *et al.*, 2001). From the estimated sizes of the mRNAs, transcription

Table 2. Complete sequence information of tripartite genome of TSWV

Genome segment	Strain No	Nucleotides (bp)	References	Database Accession No
L	-	8897 bp	De Haan <i>et al.</i> , 1991	NC_002052
	-	8917 bp	Unpublished*	AB190813
	YN	8910bp	Hu <i>et al.</i> , 2011	JF 960237
	NJ-JN	8913bp	Lee <i>et al.</i> , 2011	HM581934
	CG-1	8917bp	Unpublished*	JN664254
M	Rib1TL1	4786 bp	Lopez <i>et al.</i> , 2011	HM015524
	Gr5TL1	4791 bp	Lopez <i>et al.</i> , 2011	HM015523
	ViTL3	4787 bp	Lopez <i>et al.</i> , 2011	HM015522
	NC-7	4774 bp	Tsompana <i>et al.</i> , 2005	AY744490
	NC-6	4773 bp	Tsompana <i>et al.</i> , 2005	AY744489
	NC-5	4787bp	Tsompana <i>et al.</i> , 2005	AY744488
	NC-4	4773 bp	Tsompana <i>et al.</i> , 2005	AY744487
	CA-7	4766 bp	Tsompana <i>et al.</i> , 2005	AY744485
	-	4821 bp	Kormelink <i>et al.</i> , 1992a	S48091
	-	4821 bp	Kormelink <i>et al.</i> , 1992a	NC_002050
	M	4763 bp	Naidu <i>et al.</i> , 2008	AY870390
	T	4774 bp	Naidu <i>et al.</i> , 2008	AY870389
	-	4768 bp	Unpublished*	AB190818

* Taken from www.ncbi.nlm.nih.gov (nucleotide database)

is thought to terminate somewhere in the IR (de Haan *et al.*, 1990; Kormelink *et al.*, 1992a), and it has been suggested that the hairpin structure or the conserved sequence motif may be involved in transcription termination (van Knippenberg *et al.*, 2005).

There is the first report on detailed sequence analysis of IGRs of S and M RNAs of known tospoviruses (Pappu *et al.*, 2000). In general, IGRs of M RNA were shorter in length compared to the IGRs of their respective S RNA species. Per cent identity among the S RNA IGR sequences of distinct tospovirus species varied from 42 to 57%, whereas it was 79 to 99% among isolates of the same species. Similarly, when IGRs of M RNAs were compared, there was higher sequence identity among isolates of the same tospovirus species (84 to 98%) than among distinct tospovirus species (46 to 59%). Per cent nucleotide identities and maximum likelihood trees of IGR sequences of S and M RNAs indicated that their sequence divergence is similar to that of nucleocapsid gene at inter and intra-species levels (Pappu *et al.*, 2000). The intergenic region (IGR) of the medium (M) RNA of TSWV isolates naturally infecting peanut (groundnut), pepper, potato, stokesia, tobacco and watermelon in Georgia (GA) and a peanut isolate from Florida (FL) was analysed. The IGR sequences were compared with one another and with respective M RNA IGRs of TSWV isolates from Brazil and Japan and other *tospoviruses*. The length of M IGR of GA and FL isolates varied from 271 to 277 nucleotides. IGR sequences were more conserved (95-100%) among the populations of TSWV from GA and FL, than when compared with those of TSWV isolates from other countries (83-94%). Cluster analysis of the IGR sequences showed that all GA and FL isolates are closely clustered and are distinct from the TSWV isolates from other countries as well as from other *tospoviruses* (Bhatt *et al.*, 1999).

Transcription: TSWV initiates transcription of their genome by a mechanism called cap snatching. All TSWV genes are expressed by the synthesis of mRNAs that can be discriminated from the (anti) genomic RNA strands by the presence of non-

viral leader sequences (Kormelink *et al.*, 1992b and 1992c). For these leader sequences, TSWV has evolved a cap stealing mechanism known as cap snatching. During this process the viral RNA dependent RNA polymerase (RdRp), encompassing an endonuclease activity, cleaves a host mRNA at a position 10-20 nucleotides from the capped 5' end of host mRNA. The short capped fragments act as primers for viral mRNA transcription (Kormelink *et al.*, 1992c; Van Poelwijk *et al.*, 1996; Duijsings *et al.*, 2001). This mechanism is used by all segmented negative strand RNA viruses to initiate transcription of their genome and was first described for *Influenza virus* (Plotch *et al.*, 1981; Nguyen and Haenni, 2003). This mechanism was investigated for TSWV by extensive *in planta* studies (Duijsings *et al.*, 1999; 2001), resulting in the model for transcription initiation. It has been demonstrated that *Alfalfa mosaic virus* (AMV) RNAs can be utilized by TSWV as cap donors during a mixed infection of *N. benthamiana* (Duijsings *et al.*, 1999). Furthermore, it was shown that suitable cap donors require a single base complementarity to the ultimate or penultimate residue of the TSWV template (Duijsings *et al.*, 2001). It has been demonstrated that *in vitro* ongoing transcription of TSWV requires the presence of reticulocyte lysate. This dependence was further investigated by testing the occurrence of transcription in the presence of two translation inhibitors: edeine, an inhibitor that still allows scanning of nascent mRNAs by the 40S ribosomal subunit, and cycloheximide, an inhibitor that completely blocks translation including ribosome scanning. Neither of these inhibitors blocked TSWV transcription initiation or elongation *in vitro*, as demonstrated by de novo-synthesized viral mRNAs with globin mRNA-derived leader sequences, suggesting that TSWV transcription *in vitro* requires the presence of (a component within) reticulocyte lysate, rather than a viral protein resulting from translation (van Knippenberg *et al.*, 2004).

Thrips -vector of TSWV: Cell walls present in plants are major barrier to viral infection. Some plant viruses depend upon vector (vehicles) for their movement from infected host plants to healthy ones. Insects (thrips) play a very important role as vector in virus transmission. Thrips are important members of the ecosystems as herbivores and predators. Thrips are minute slender bodied insects that have ability to transmit plant viruses. They are categorized as important agricultural pest. Thrips belong to the insect order *Thysanoptera* and the main TSWV vector species all belong to family Thripidae. Thrips feed on plant tissue with piercing and sucking mouth parts. Thrips acquire TSWV in two larval stages and it is only when larvae feed on infected plant host. Once thrips acquire the virus they remain viruliferous (infected with virus) throughout their life span. Virus acquired by the larvae renders the thrips infectious, and transmission of the virus is mainly ascribed to adults (Sakimura, 1962). It has been studied that TSWV infection alters the feeding behavior of its insect vector. Data reveals that viruliferous males are good feeder as compare to non- viruliferous males and for females no change was observed for their behavior (Stafford *et al.*, 2010). Wijkamp *et al.* (1993) showed that larvae of *Frankliniella occidentalis* also transmit the virus efficiently. The virus upon acquisition was shown to move through the midgut and subsequently reaches the salivary glands. It is hypothesized that the close proximity of midgut and salivary glands in the thrip's larval stage facilitates the virus movement whereas the virus fails to do so as the thrips reaches adult stage. This may explain the inability of adult thrips to transmit the virus if the virus is acquired for the first time in its adult life (Filho *et*

al., 2004). The specificity of TSWV and thrips vectors may be due to the presence of a receptor in the vector species which may be absent in non-vector species. *F. occidentalis* and *Thrips tabaci* have been found to be vectors of at least four important plant virus groups including the bunyaviruses (Ullman *et al.*, 1997). Earlier, eight species of thrips were reported to transmit TSWV (Wijkamp *et al.*, 1995). *F. occidentalis* Pergande (the western flower thrips), *T. tabaci* Lindeman (the onion thrips), *T. palmi* Karny (melon thrips), *T. setosus* Moulton, *F. schultzei* Trybom (the common blossom or cotton bud thrips), *F. intonsa* Trybom and *F. fusca* Hinds (the tobacco thrips) were reported to be vector of TSWV (Wijkamp *et al.*, 1995; Ullman *et al.*, 1997). Webb *et al.* (1997) also reported *F. bispinosa* Morgan (Florida flower thrips) as a vector of TSWV. *F. tenuicornis* Uzel (European grass thrips) and *Scirtothrips dorsalis* Hood (the chilli thrips) had been previously reported to be vector of TSWV, but experimental verification had not been done for all species as has been done for *F. occidentalis* and *T. tabaci* (Ullman *et al.*, 1997). *F. occidentalis* and *T. tabaci* are common and important vectors of multiple plant viruses in many regions of the world (Ullman *et al.*, 1997).

Molecular mechanism of transmission: Insect vectors play a key role in dissemination of viruses that cause important diseases in humans, animals and plants (Ullman *et al.*, 2005). With the discovery that TSWV multiplies in insect vectors, the complex nature of the interplay between thrips, tospoviruses and their plant hosts was first recognized by Ullman *et al.* (1993) and Wijkamp *et al.* (1993). TSWV is able to infect both its botanical hosts and its insect vector (thrips). It has been demonstrated by using the approach of Reddy and Black (1966) that tospovirus multiply in their insect vector (*F. occidentalis*). The evidence that genetic determinants for TSWV transmissibility reside on middle RNA which encode viral glycoproteins came by creating reassortants by coinoculating plants with thrips-transmissible isolate (TSWV-RG2) and a thrips-nontransmissible TSWV isolate (TSWV-D) (Sin *et al.*, 2005). Insect inoculation of tospoviruses into their plant hosts cannot occur without viral passage across at least three insect organs (the midgut, visceral muscle cells and salivary glands) that include six membrane barriers (Whitfield *et al.*, 2005). Replication of the TSWV in midgut, its movement from midgut to visceral muscles and then the salivary glands are crucial factor determining the vector competency (Nagata *et al.*, 2002). Several lines of experiments demonstrated that TSWV GPs bind to the insect midgut during TSWV acquisition by thrips and plays a critical role in TSWV transmission while TSWV G(N) binds the insect vector midgut and inhibits TSWV acquisition (Ullman *et al.*, 2005). Whitfield *et al.* (2008) also reported that soluble form of the envelope glycoprotein GN (GN-S) specifically bound thrips midguts and reduced the amount of detectable virus inside midgut tissues.

An increase in the concentration of two TSWV encoded proteins (N & NSs), by S RNA firmly demonstrate replication of TSWV in its vector. The accumulation of N protein is indicative of the production of virus particles, but the accumulation of the NSs protein, which has not been found in virus particles (Kormelink *et al.*, 1991) can only occur after transcription of its mRNA from the complementary viral RNA strand which is formed during the replication of viral RNA. Hence, the presence and increase of this protein give conclusive proof that TSWV replicates in its vector (*F. occidentalis*) (Wijkamp *et al.*, 1993).

Screening of a cDNA library of *F. occidentalis* using fragments of TSWV RdRp, a *F. occidentalis* putative transcription factor (FoTF) was identified that binds to TSWV RdRp, which was shown to bind to TSWV RNA and enhance TSWV replication (*in vitro*). Mammalian cells expressing this putative transcription factor supported TSWV replication. So, this factor which supports TSWV replication *in vivo* and *in vitro* could be used to compare molecular defense mechanisms in plant, insect and mammalian cell lines against the same pathogen for better understanding of evolutionary studies (Medeiros *et al.*, 2005).

Molecular detection and characterization: TSWV has certain unique biological properties that are useful for diagnosis. A turning point in TSWV detection and diagnosis came with the production of high quality polyclonal antisera and development of an enzyme-linked immunosorbent assay (ELISA) (Gonsalves and Trujillo, 1986). High quality specific polyclonal antisera has also been developed for virus detection using *in vitro* expressed coat protein of different viruses like *Carnation etched ring virus* (CERV) and *Prunus necrotic ring spot virus* (PNRSV) (Raikhy *et al.*, 2007; Kulshrestha *et al.*, 2009). Adam *et al.* (1995) have described an assay which could detect tospoviruses generally, based on antibodies to the G proteins of the virus. Dot-blot immunoassay and tissue-print immunoassay has also been used for specific detection of TSWV (Hsu & Lawson, 1991; Louro, 1995). TSWV detection by molecular methods has been developed using cDNA probes (Ronco *et al.*, 1989; Rice *et al.*, 1990) and riboprobes (Huguenot *et al.*, 1990), both of which have proved useful for the sanitary certification of plant material (Saldarelli *et al.*, 1996). Several PCR-based methods have also been developed for the specific detection of TSWV. The first PCR-based assay was developed by Mumford *et al.* (1994). Immunocapture PCR and RT-PCR were developed by Nolasco *et al.* (1993) and Weekes *et al.* (1996), respectively. A very sensitive protocol for the detection and quantification of TSWV is the real-time RT-PCR assay which is based on TaqMan™ chemistry, on both “leaf soak” and total RNA extracts from infected plants (Roberts *et al.*, 2000). A comparison between ELISA and RT-PCR assays was done to detect TSWV in field-grown chrysanthemum (Matsuura *et al.*, 2002; 2004) and recently for peanut also (Dang *et al.*, 2009). Similarly potential of RT-PCR was evaluated by comparing its sensitivity with DAS-ELISA for the detection of TSWV among 22 Australian plant species. DAS ELISA was found to be less sensitive as compare to RT-PCR method (Dietzgen *et al.*, 2005). One-step multiplex reverse transcription-polymerase chain reaction (multi-PCR) (modified) was also utilized for simultaneous identification of five tospovirus species (Kuwbara *et al.*, 2010). For the detection of TSWV in individual thrips, a sensitive and robust real time fluorescent (RT-PCR Taqman) technique was developed (Boonham *et al.*, 2002). Similarly, by using RT-PCR technique TSWV from a single infected thrip has been reported by Mason *et al.* (2003). A quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) procedure using a general primer set and three TaqMan(®)MGB probes was developed for general and genotype-specific detection and quantization of the genomic M segment of TSWV (Debreczeni *et al.*, 2011).

Similarly PCR based detection methods were also developed for the detection of other economically important viruses like PNRSV in pelargonium (Kulshrestha *et al.*, 2005c), *Tuberose*

mild mottle virus in *Tuberose* (Kulshrestha *et al.*, 2005b). Nematode transmitted viruses like *Arabidopsis mosaic virus* (ArMV) and *Strawberry latent ringspot virus* (SLRSV) has also been detected in vector nematodes by RT-PCR (Kulshrestha *et al.*, 2005a; Adekunle *et al.*, 2005). In Korea, TSWV is characterized from paprika (*Capsicum annuum* var. *grossum*). TSWV-KP nucleocapsid (N) protein of the purified virion migrated as a single band with molecular weight of about 29 kDa in SDS-PAGE. The N gene of TSWV-KP showed 96.5-97.2% and 97.7-98.5% identities to the three different TSWV isolates of Genbank Database at the nucleotide and amino acid, respectively (Kim *et al.*, 2004). The complete cDNA genomic libraries were prepared from complete genomic RNA of Bulgarian L3 isolate of TSWV. S RNA specific clones were selected which covered approximately 2.8kb which is 95% of this RNA and S RNA to be ambisense in nature (Maiss *et al.*, 1991). In Israel, virus identity was characterized by host range, serology and electron microscopy. Serological reaction with the isolates, found in Israel, using antisera from different sources as well as the sequence analysis of the nucleocapsid gene, demonstrated that the Israeli isolates of TSWV are a member of tospovirus serogroup I, type I (BR-01 strain) (Antignus *et al.*, 1997). There was a first report of molecular characterization of TSWV on pepper (*Capsicum* sp.) in South Africa (SA) by taking six isolates from different geographical locations of the country. The identity of this virus was confirmed by using ELISA, electron microscopy and protein analysis. Phylogenetic analysis based on multiple alignments of N gene sequences revealed the branching of TSWV in two distinct clusters designated as the American and European groups. TSWV from SA showed high sequence similarity with TSWV isolates from Europe. Among themselves, the N gene of six isolates revealed high similarity (> 90%) (Sivprasad and Gubba, 2008). Genetic variability was analyzed for TSWV from different crop species in Australia. It was found that genetic variability was small among Australian isolates (Dietzgen *et al.*, 2005). In Jordan, TSWV infection was recorded for the first time and sequence analysis of isolates from Jordan shared nucleotide similarities with isolates from different countries (Anfoka *et al.*, 2006). In China, complete nucleotide sequence of TSWV isolate was determined and phylogenetic relationship of the Chinese isolate was also analyzed from Brazilian and Korean isolates on the basis of nucleotide sequences of the glycoprotein and nucleocapsid genes (Hu *et al.*, 2011).

The review specifically focuses on the molecular biology of *Tomato spotted wilt virus* (TSWV). Earlier reviews which have been published focuses on Tospovirus genus (Adkins, 2000; Elliot, 1990; Goldbach and Peters, 1996; Pappu, 2008; Tsompana and Moyer, 2008). Important focus of this review is on the function of different TSWV genome encoded proteins like N and NSs by small RNA. N proteins functions as protective envelope and NSs protein (non structural protein) has been recently identified as first RNA silencing suppressor which is responsible for inhibition of dicer mediated cleavage (Bucher *et al.*, 2003; Schnettler *et al.*, 2010 and Takeda *et al.*, 2002). Medium RNA encodes NSm which helps in cell to cell movement; G1/G2 glycoproteins help in vector transmission. Similarly L RNA segment of TSWV encodes for RNA dependant RNA polymerase which help in replication. Out of these proteins, amino acid sequences of N protein were used for the identification of new tospovirus species, though exceptions

remain (Hassani-Mehraban *et al.*, 2007). This review also discusses possible mechanism of TSWV transmission by thrips (*F. occidentalis*) along with different known factors from both virus and vector side which plays critical role in virus acquisition by thrips and its subsequent transfer.

Till date there is no report of TSWV infection from India, but it has been reported from several parts of the world. During our survey in different districts of Himachal Pradesh, TSWV like symptoms were observed on capsicum and tomato, so there is an urgent need for an extensive survey to rule out the possibility of TSWV infection in India. There is also an urgent need to have strict quarantine norms to restrict the entry of TSWV from other parts of the world.

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