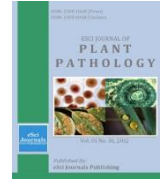




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COMPLETE GENOME SEQUENCE OF *TOMATO SPOTTED WILT VIRUS* FROM PAPRIKA IN KOREA

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ABSTRACT

We isolated *tomato spotted wilt virus* (TSWV-KP) from a diseased *Capsicum annuum* var. *grossum* with malformed leaves and necrotic spotted fruits. TSWV-KP produced necrosis or necrotic ring spots on inoculated leaves along with mosaic, vein necrosis, or death on the upper leaves on *Datura stramonium*, *Nicotiana clevarandii*, *N. rustica*, and *N. tabacum* cvs. Ultrastructurally, typical tospovirus particles were observed in the cytoplasm. The virion contained three molecules of genomic RNAs of approximately 9.0, 4.9, and 3.0 kb. The nucleocapsid (N) protein of the purified virion migrated as a single band with ~29 kDa molecular weight in SDS-PAGE. Complete nucleotide sequences of the large (L) genome segments of TSWV-KP were determined. Defective forms of L-RNA containing core polymerase regions were observed. L-RNA (8,917 nucleotides) contained a single open reading frame (ORF) in the viral complementary (vc) strand and encoded a 330-kDa protein. The L-protein had high identity in the “core-polymerase domain” with the corresponding regions of other tospoviruses. The complete nucleotide sequence of TSWV-KP medium-sized (M) RNA comprised 4,768 nucleotides and indicated a typical tospovirus with two genes in ambisense arrangement. The vRNA ORF coded for a potential cell-to-cell movement (NSm) 34.8-kDa protein; and the vcRNA ORF, for the viral glycoprotein (G1/G2) 128.0-kDa precursor. Multiple sequence alignment of the M-RNA showed highest homologies to TSWV-BR01. Amino acid sequences of TSWV-KP NSm and G1/G2 exhibited 48.7–85.3% and 34.9–96.2% identity, respectively. TSWV-KP small (S) RNA comprised 2,991 nucleotides with ambisense coding strategy. The sequence contained two ORFs—one in the viral sense, encoding a protein with predicted 52.4-kDa Mr; and another in the viral complementary sense, encoding the viral nucleocapsid protein of 28.8-kDa Mr. Amino acid sequences of TSWV-KP of S-RNA NSs and N exhibited 35.9–87.9% and 19.9–98.4% identity, respectively.

Keywords: *Bunyaviridae*, *Tospovirus*: Tomato spotted wilt virus, paprika, genome structure.

INTRODUCTION

Thus far, 13 species of the genus *Tospovirus* have been reported worldwide. *Tomato spotted wilt virus* (TSWV) comprises enveloped spherical particles (diameter, about 80–110 nm); these particles are attached to the endoplasmic reticulum. Nucleotide sequence and translational analyses showed that TSWV has a large negatively stranded genomic RNA (IRNA) (de Haan *et al.*, 1991) and 2 ambisense RNAs—sRNA and mRNA (de Haan *et al.*, 1990; Kormelink *et al.*, 1992). TSWV transmission by thrips commonly occurs in temperate and subtropical regions throughout the world (Ie, 1970). TSWV is distributed worldwide and has a very broad

host range of approximately 900 plant species in 80 families (Goldbach and Peters 1994).

In pepper crops with TSWV infection, yellowing and chlorotic rings appear on leaves. The incidence of TSWV infection in pepper- and tomato-producing areas can be as high as 60% (Greenough *et al.*, 1990). Twenty-five species of weed, including *Malva parviflora*, *Verbescina encelioides*, *Bidens pilosa*, and *Ipomoea congesta*, are considered to be the main reservoirs of TSWV (Cho *et al.*, 1986).

The analyses of purified viral particles show that the envelope is lipidic in nature. It surrounds 3 types of single-stranded RNA molecules: sRNA (2.9 kb), mRNA (5.4 kb), and IRNA (8.9 kb) (van Den Hurk *et al.*, 1977; Mohamed 1981; de Haan *et al.*, 1990, 1991). Four structural proteins with different molecular masses

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(28.8, 58, 78, and 331.5 kDa) are found in the viral particles. The 28.8-kDa protein, named as the nucleocapsid (N) protein, is tightly associated with the RNA genome, both together forming a stable pseudocircular structure, i.e., the nucleocapsid. These nucleocapsids with few copies (between 10 and 20) of the 311.4-kDa protein—named as large (L) protein—form the interior of the viral particle. The 78- and 58-kDa proteins named as G1 and G2, respectively, are glycosides. They are located on the viral particle surface, forming the spikes observed under the electron microscope (Mohamed *et al.*, 1973; Tas *et al.*, 1977; Mohamed 1981; van Poelwijk *et al.*, 1993).

The complete nucleotide sequence of the genome of the TSWV Brazilian isolate, BR01, has recently been determined. The L-RNA segment has negative polarity (–). Thus, the viral complementary strand (+) has to be copied before the L-RNA segment can be read. A promoter sequence not coded by the virus is added in this transcription process. This promoter is necessary to start the translation process. The L-RNA viral complementary strand contains a single open-reading frame (ORF) (de Haan *et al.*, 1990). This ORF codes for the RNA-polymerase RNA-dependent L protein (Adkins *et al.*, 1995). Medium-sized (M) and small (S) RNA segments both have an ambisense codifying strategy. Each of them have two ORFs separated by a central intercistronic region rich in A-U links and bent in the form of a curl. M-RNA encodes in the viral sense a non-structural protein called NSm (33.6 kDa); and in the viral complementary sense, a precursor of the two glycoproteins. S-RNA encodes in the viral sense a nonstructural protein named NSs (53.4 kDa); and in the viral complementary sense, the N protein. Recently, the genus *Tospovirus* has been classified into five serogroups based on the serological cross reactivity and sequence homology of the N protein. Serogroup I consists of TSWV (de Haan *et al.*, 1990; Maiss *et al.*, 1991), which reacts weakly with the antibodies to Serogroup II members [TSWV-B; *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV)] (de Avila *et al.*, 1993; Pang *et al.*, 1993) and does not react with antibodies to *Impatiens necrotic spot virus* (INSV), which is a serogroup III member (Law *et al.*, 1990).

The serogroup IV members, *Groundnut bud necrosis virus* (GBNV) and *Watermelon silver mottle virus* (WSMoV), are serologically distinct from the members of serogroups I, II, and III (Adam *et al.*, 1993). The N

protein of TSWV (serogroup I) showed 77–78% sequence identity with GRSV and TCSV (serogroup II) (de Avila *et al.*, 1993), 55% sequence identity with INSV (serogroup III) (de Haan *et al.*, 1992) and 33–35% sequence identity with *Peanut bud necrosis virus* (PBNV) and WSMoV (serogroup IV) (Yeh and Chang 1995). In this study, we identified TSWV from diseased paprika (*Capsicum annuum* var. *grossum*) for the first time, and its complete genome sequence was determined and analyzed.

MATERIALS AND METHODS

Host range of TSWV-KP: Virus-infected leaves were homogenized in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.01 M sodium sulfite and rubbed onto healthy indicator plant leaves with a wooden trowel, after dusting with 600 mesh carborundum. After inoculation, the test plants were immediately rinsed with tap water, and maintained for visual inspection of virus symptoms in the green house at $25 \pm 2^\circ\text{C}$ for at least 3 weeks.

Electron microscopy: Leaf-dip preparation was prepared by crushing ~0.1 g of virus-infected leaf tissue in a drop of 2% sodium phosphotungstic acid (pH 6.8) on a parafilm. Formavar-coated grids were made to float on the drops for 30 sec, and then drained with a piece of filter paper. For ultrathin sections, samples were embedded in LR-White medium by using a modification of the method described by Cho (2003). Systemically infected leaves were cut at 1×3 mm and fixed in 3% glutaraldehyde in 0.01 M cacodylate buffer for 2 h. The fixed samples were washed 3 times in sodium phosphate buffered salts (pH 7.2) and post-fixed with 2% osmium tetroxide for 2 h at 4°C and dehydrated with an ascending series of 50–100% ethanol in 6 steps for 30 min each. The hydrated samples were embedded in LR-White medium and polymerized at 60°C for 24 h. Ultrathin sections of 80-nm thickness were stained with 2% uranyl acetate and 0.5% lead citrate and rinsed with distilled water. The leaf-dip preparation and ultrathin sections were viewed and photographed using a Zeiss LEO 906 transmission electron microscope at 80 kV.

Purification of virions: The nucleocapsid of TSWV was purified by a modification of the method previously described (Tsuda *et al.*, 1992). All work was performed on ice. Batches of 100 g of TSWV-infected *N. rustica* leaves were homogenized for 5 min in a blender at a high speed with 0.1 M Tris-HCl (pH 7.0; 1:5, w/v) containing 10 mM sodium sulfite, 10 mM glycine, 0.1%

(w/v) L-cysteine and Triton X-100. These were centrifuged at 10,000 $\times g$ for 10 min. Then, 5% polyethylene glycol (mol. wt., 8,000) and 0.2 M NaCl was added to the supernatant. The precipitate after centrifugation at 7,500 $\times g$ for 10 min was resuspended with 10 mM Tris-HCl (pH 7.0) containing 1 mM glycine, and then centrifuged at 65,000 $\times g$ for 120 min through a 20% sucrose cushion. The pellets were resuspended in 10 mM Tris-HCl (pH 7.0) containing 1 mM glycine and further purified by centrifugation in 20–50% sucrose density gradient at 77,000 $\times g$ in a Beckman SW28 rotor for 3 h. The opalescent zones containing virus nucleocapsids were collected, further centrifuged at 130,000 $\times g$ for 90 min, and finally resuspended in 10 mM Tris-HCl (pH 7.0) as purified nucleocapsids.

SDS-polyacrylamide gel electrophoresis: The molecular weight of the coat protein for the purified viruses of TSWV was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified virus (1 mg/ml) was mixed with equal volumes of sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β -mercaptoethanol, and 0.002% bromophenol blue) and then heated for 3 min at 100°C. The sample was electrophoresed at 100 V for 1 h by using a 4% stacking gel on a 12% discontinuous polyacrylamide slab gel containing 0.1% SDS following the method of Laemmli and Farre (1973). After the electrophoresis, the gel was stained with Coomassie brilliant blue R 250.

Isolation of viral dsRNAs: The isolation procedure of dsRNAs from the virus-infected *N. rustica* was similar to that described by Morris and Dodds (1979). The dsRNAs were eluted from the CF-11 cellulose column and precipitated by ethanol and sodium acetate. The pellet was washed with 80% ethanol, dried under vacuum, and then dissolved in RNase-free water. Total dsRNAs were loaded on 0.8% agarose gel and separated by electrophoresis. The dsRNAs bands were visualized under ultraviolet light.

Extraction of viral RNA from partially purified virions: All materials and solutions used for RNA isolation were sterilized by autoclaving, backing, filtering, or treating with diethyl pyrocarbamate (DEPC) solution prior to use. All samples were kept on ice and all centrifugation steps were performed at 4°C. Viral RNA was extracted from the purified virions by using a modification of the method described by Ryu *et al.*, (1995). Equal volumes of the virus (0.5 mg/ml) and

extraction solution (100 mM Tris-HCl, pH 7.6, 20 mM EDTA, 100 mM NaCl, 2 mM DTT, 1% SDS) were mixed, and proteinase K was added to the final concentration of 100 $\mu\text{g/ml}$. It was mixed by gentle agitation and incubated at 37°C for 45 min. Equal volumes of a phenol/chloroform/isoamylalcohol mixture (PCI, 25:24:1, v/v/v) were added, and the sample was vortexed and centrifuged at 14,000 rpm for 20 min. The aqueous phase was carefully collected and kept on ice. The PCI extraction was then repeated. The final aqueous phase was recovered and re-extracted once with chloroform:isoamylalcohol (CI, 24:1, v/v). The viral RNA was precipitated with half volume of 7.5 M ammonium acetate and 2.5 volumes of cold absolute ethanol. This mixture was stored at -70°C for 2 h. After centrifugation at 14,000 rpm for 20 min, the precipitate of viral RNA was rinsed with 80% ice-cold ethanol and centrifuged at 14,000 rpm for 10 min. The pellets were dried in a Speedvac for 5 min and then suspended in 50 μl of DEPC-treated distilled water. This was divided into small aliquots, and stored at -70°C.

Rapid amplification of cDNA end (RACE): A 5'-end cDNA synthesis was performed according to the methods described in the GIBCO BRL Protocols and Applications Guide. For first strand synthesis, approximately 1.5–3.0 μg (total volume, 20 μl) of each viral RNA of TSWV-KP was denatured for 2 min at 70°C in a reaction volume containing 1 μl of gene-specific primer (GSP), SMART II oligo (5'-CGTATCTCACCTGTCTGGCGCTC-3'), and 5 μl of dH₂O. After cooling on ice for 10 min, the following first strand cDNA synthesis mixture [1 \times first strand reaction buffer, 0.5 mM DTT, 1 mM dNTP, 0.8 units RNase inhibitor, 0.2 units AMV (TaKaRa)] was added and incubated at 42°C for 1 h. For amplification of cDNA, 48 μl of the PCR reaction mixture [1 \times Taq polymerase buffer (TaKaRa), 2 mM dNTP, 5 mM MgCl₂, 0.5 μM Universal primer mix (UPM; Clontech), 0.1 μM , GSPs (TSWV-KP TFR 10, TMR 6, and TSR 4), and EX-Taq DNA polymerase (TaKaRa)] was added to the 2 μl of the first strand cDNA solutions. PCR cycling consisted of 94°C for 40 sec, 52°C for 40 sec, and 68°C for 10 min (35 cycles). The last cycle was followed by a prolonged extension for 20 min at 68°C to complete synthesis of the cDNA products. The amplified DNAs were purified for nucleotide sequence determination.

Polyadenylation of viral RNA and amplification of full-length Cdna: Genomic RNA of viruses in the

tospovirus contains a nopoly (A) tract in the 3'-end. To produce cDNA by reverse transcription from the 3'-terminal of TSWV, genomic RNAs were polyadenylated at their 3' ends *in vitro* by the method of Sippel (1973). Approximately 3.0–5.0 µg (in 50 µl) of viral RNA of TSWV-KP were incubated at 75°C for 90 sec for denaturation and quickly chilled on ice. After cooling on ice for 10 min, the polyadenylation mixture [0.8 units RNase inhibitor (TaKaRa), 0.5 M Tris-HCl, pH 8.0, containing 25 mM MnCl₂, 10 mM DTT, 10 mM ATP (Roche), and 2 µl of *E. coli* poly (A) polymerase (7 units, GIBCO BRL)] was added directly to the denatured viral RNAs and incubated at 37°C for 20 min. After incubation, the polyadenylation reaction was terminated by adding Na₂-EDTA solution (pH 8.0) to the final concentration of 10 mM.

The reaction mixture was extracted once with PCI and once with CI. Half volume of 7.5 M ammonium acetate was added to the supernatant and precipitated with 3 volumes of absolute ethanol. The resulting pellets of viral RNAs were dissolved in 10 µl volumes of DEPC-treated RNase-free water. Primers for full-length sequencing of TSWV-KP were designed corresponding to TSWV-BR01 (L RNA [NC_002052], M-RNA [NC_002050], and S-RNA [NC_002051]), respectively. RT-PCR was carried out with primer pairs from viral RNA extracted from purified virus particles by SDS-phenol extraction methods. The relative position of the pairs in the TSWV genomes is schematically shown in Fig. 2, and each PCR product is used as a template for sequencing.

RT-PCR was performed in the same tube containing 50 µl reaction mixture containing 500 ng of purified viral RNA, 10 µl 5 × reaction buffer, 10 ng each of reverse primer and forward primer, 1 mM deoxynucleotide triphosphate (dNTP), 10 units of RNase inhibitor, 10 units of AMV reverse transcriptase (TaKaRa), and 5 units of Ex Taq polymerase (TaKaRa). The RT-PCR cycle was as follows: 50°C, 45 min; 94°C, 2 min (1 cycle); 94°C, 30 sec; 50–55°C, 30 sec; 72°C, 2 min (40 cycles); and 72°C, 10 min. The synthesized RT-PCR products were directly analyzed on 1% agarose gel with a 1-kb DNA ladder (Promega). Each template was purified by MicroSpin™ S-400 H Columns (Amersham Biosciences).

Full-length cDNAs were sequenced by using ABI PRISM BigDye™ Terminator v. 3.0 Cycle Sequencing Kits (Roche) directly with the eight templates and designed primers. Sequencing reactions were run on a fluorescent

DNA sequencer ABI3100 (Perkin-Elmer). Sequences were analyzed by using the Sequencer program (Gene Code Corporation, USA). Analyses of nucleotides and the deduced amino acid sequences of TSWV-KP were performed by using the DNASTar (Medison, WI, USA) and DNAMAN Sequence Analysis Program (Version 5.1, Lynnon Biosoft Co., Canada). The nucleotide and amino acid sequences of TSWV-KP and TSWV-BR01 and those of other viruses of Tospovirus group were surveyed by using Genbank (<http://www.ncbi.nih.gov>).

RESULTS

Symptomatology and host range: TSWV-infected paprika crops showed mosaic and, at times, necrotic spots on the upper leaves. Infected fruits had generally large chlorotic or necrotic spots and were not commercially viable (Fig. 1-A). Inoculated leaves of *N. rustica* showed necrotic lesions with chlorotic rings, followed by systemic mosaic and necrotic spots. Chlorotic local lesions on the inoculated leaf of *N. benthamiana* appeared 4–5 d after inoculation, followed by yellowing. *Petunia hybrida* and *Gomphrena globosa* showed necrotic local and white necrotic local lesions on inoculated leaves 3–4 d after inoculation, respectively. *N. tabacum* cv. White burley and cv. Ky-57 showed local lesions on the inoculated leaves with systemic symptoms. Finally, all the plants died with severe necrosis (Table 1).

Cytology of TSWV-infected pepper leaf tissue: In ultrathin sections of the tissue of *C. annuum* infected with TSWV-KP, the virus particles of TSWV-KP accumulated in the cistae of endoplasmic reticulum (ER). Single-enveloped virions (SEV) were present in the cytoplasm. SEV concentration in leaf tissue was abundant; further, double-enveloped virions as well as fiber and fibrous structures could also be found in most parts of the cytoplasm of the infected cells (Fig. 1-B).

Molecular weight of N protein: The nucleocapsid of TSWV-KP was purified from *N. rustica* plants that showed typical tospovirus symptoms. The preparation, analyzed by SDS-PAGE, gave a single protein band (Fig. 1-C, left). The N protein of the TSWV-N, TSWV-M, and TSWV-P isolates had a molecular weight of 30 kDa, while the molecular weight of the TSWV-W and TSWV-K isolates was 32 kDa (Tsuda *et al.*, 1996). The molecular size pattern of TSWV-KP was similar to those of the Japanese isolates of N, M, and P of TSWV.

Analysis of dsRNAs: The total dsRNA segments extracted from the leaves of *N. rustica* infected with

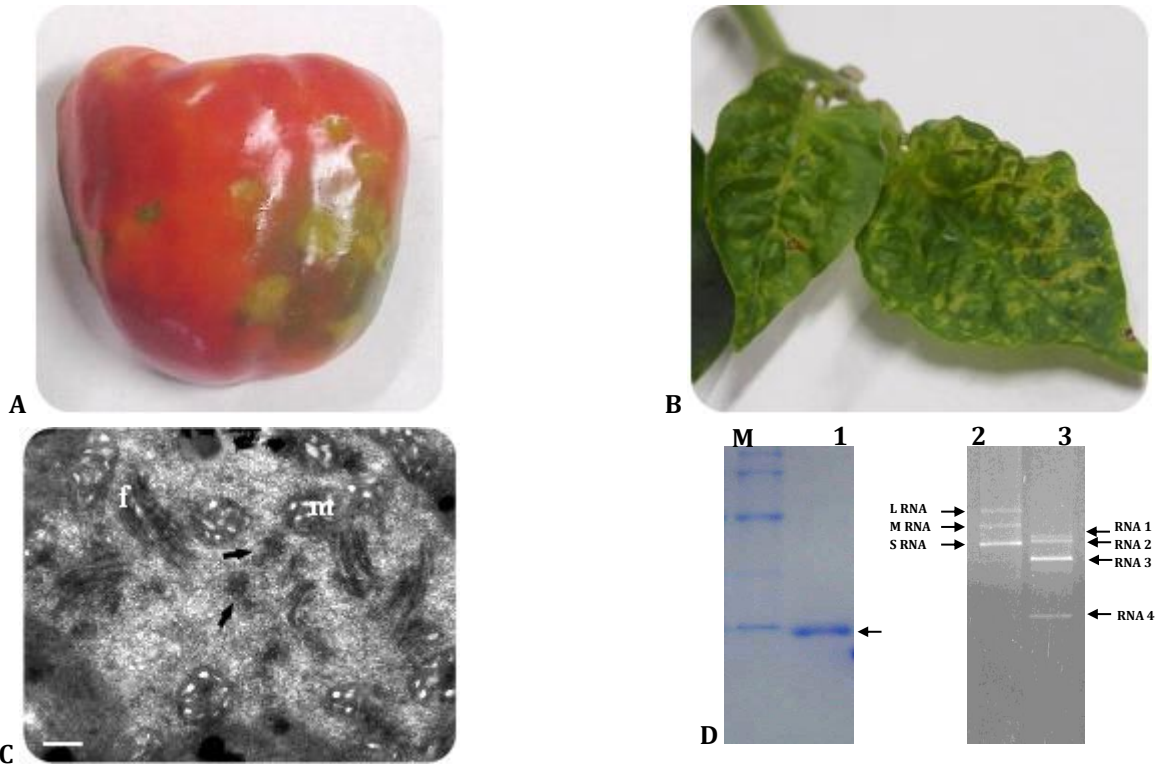
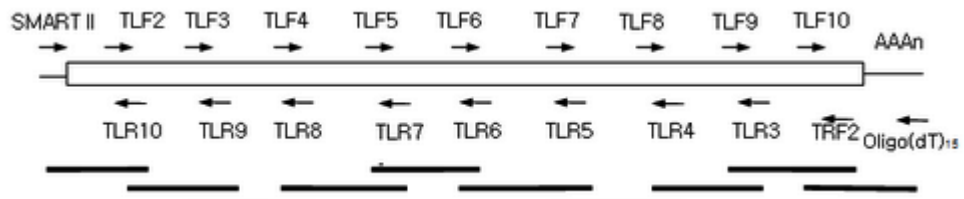
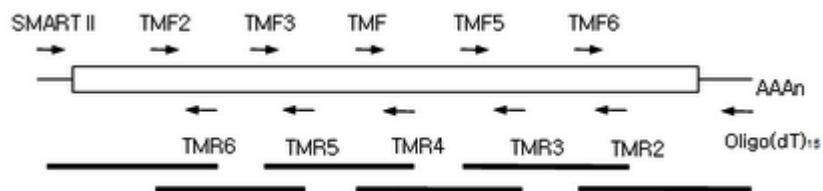


Figure 1. Symptoms, cytology, N protein and dsRNAs analysis of TSWV-KP. A, Large chlorotic and necrotic spots on the fruit, severe mosaic and malformation on the leaves of paprika. B, Ultrastructure of the tissue of *C. annuum* infected with TSWV-KP. Formation of single enveloped virion (arrows) by fusion of double enveloped virion. f, filamentous inclusion; m, mitochondria. Bars, 300nm. C, Electrophoresis of coat protein and genomic RNA of TSWV-KP. M, standard protein; 1, The purified nucleocapsid protein; 2, TSWV dsRNAs; 3, CMV-Mf dsRNAs.

L RNA



M RNA



S RNA

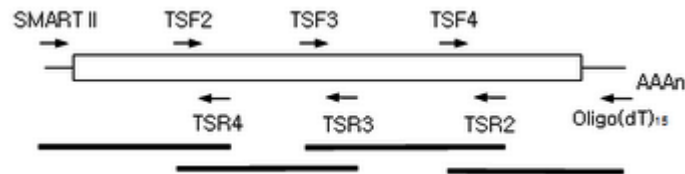


Figure 2. Schematic representation of nucleotide sequencing strategy for TSWV-KP L RNA, M RNA and S RNA. Horizontal lines represent templates for sequencing. Arrows represent the sequencing position and orientation of each primers.

TSWV-KP were analyzed by electrophoresis in 0.8% agarose gel. A typical dsRNA virus is shown in Figure 1-C, right side (lane 2). The dsRNAs extracted from CMV-infected tobacco were used as markers (Fig. 1-C, lane 3). The sizes of the three dsRNAs of TSWV-KP were estimated to be 9.0 kb, 4.9 kb, and 3.0 kb. Based on the molecular size of the dsRNAs (Tsuda *et al.*, 1992), they were considered the replication forms of genomic L-, M-, and S-RNA, respectively.

Sequence of L-RNA: The complete nucleotide sequence of the TSWV-KP L-RNA had 8,917 nucleotides, with a base composition of 37.7% A, 28.4% U (T), 14.7% C, and 19.6% G (data not shown). The length was in very good agreement with the previously estimated size, deduced from electrophoretic mobility (Van den Hurk *et al.*, 1977; de Haan *et al.*, 1991a). It was similar to that of L-RNA of TSWV-BR01 (8,897 nt; NC_002052), INSV (8,776 nt; NC_003625), GBNV (8,911 nt; NC_003614), and WSMoV (8,917 nt; NC_3832).

Sequence analysis showed that the L-RNA of TSWV contained a single large ORF initiating at the ATG codon at nt position 34 and terminating at the TAA codon at nt position 8,676 from the 5' end of the viral strand. It was encoded in a protein comprising 2,880 amino acids with a predicted molecular weight of 330.9 kDa. No other reading frame in viral or viral complementary sense contained ORFs of significant size. Thus, the L-RNA of TSWV-KP appears as a negative-sense RNA (Fig. 3). The predicted size of the L-RNA protein was similar to that of TSWV-BR01. The identities in the nucleotide and amino acid sequence of the L protein exhibited 54.2–92.2% and 45.6–93.6% identity with other tospoviruses, respectively (Fig. 4).

The predicted translation product of the TSWV-KP L-RNA appeared to be the viral polymerase. This is based on the similarities between the sequence and size of other tospovirus L proteins and the presence of conserved signature sequences in the “core polymerase region” (Fig. 5, A–E). This is a characteristic of the viral RNA polymerases of the negative-stranded *Bunyaviridae* (Poch *et al.*, 1989).

Sequence of M-RNA: The M-RNA of TSWV-KP contains 4,768 nucleotides showing an ambisense arrangement typical of tospoviruses (Fig. 3). The M-RNA has a base composition of 31.7% A, 32.6% T (U), 17.7% C, and 18.0% G (data not shown). The ORF within the viral RNA strand starts with an ATG codon at nucleotide (nt) 101 and stops at a TAG triplet on nt 1,009 from the 5' end of

the viral sense RNA. It encoded a protein of 301 amino acids due to its homology with the same ORF of TSWV-BR01, INSV, GBNV, and WSMoV M-RNA, which represented the potential role in cell-to-cell movement protein (NSm).

The ORF within the viral complementary strand of the RNA starts with an ATG at nt position 4,683 and stops with a TGA stop codon at nt position 1,276 from the 5' end of the viral sense RNA. It encodes for the precursor to the glycoproteins G1 and G2, and encompasses 1,134 amino acids.

The complete sequence of TSWV-KP M-RNA allowed for comparison among the six tospovirus M-RNA sequences. The homologies for the TSWV-KP NSm protein were higher than those of TSWV-BR01 and INSV but lower than those of GBNV and WSMoV (Table 2).

Moreover, higher homologies were noted throughout the NSm protein for all tospoviruses (40–70%), with the exception of the first 60 N-terminal amino acids (Fig. 6). The “D-motif” found in the “30K-superfamily” of virus movement proteins (Melcher 2000; Mushegian *et al.*, 1993) was also present in all the NSm amino acid sequences of Brazilian tospoviruses as well as in TSWV, INSV, GBNV, and WSMoV (Fig. 6).

In addition to the “D-motif,” the “P/D-L-X motif” and a glycine residue (“G-residue”) that were almost completely conserved in the C-termini of several movement proteins from the “30K superfamily” (Melcher 2000) were also present in NSm sequences (Fig. 6). While the “P/D-L-X motif” was present only in GRSV, TCSV, CSNV, TSWV, and INSV, the “G-residue” was present in all NSm sequences except in INSV.

In addition to the typical “30K superfamily” motifs, a homologous region to the phospholipase A2 (PLA2) catalytic site was observed only in GRSV, TCSV, CSNV, and TSWV, i.e., viruses that had a relatively broad host range (Fig. 6). In some PLA2 catalytic sites, there was a histidine residue at the center and three cysteine residues involved in disulphide bonds at the edges (Dennis 2005; Silva *et al.*, 2001). A different amino acid pattern was present in TSWV-KP and BR-01 NSm sequences (Fig. 6).

Sequence of S-RNA: The complete nucleotide sequence of the TSWV-KP S-RNA comprised 2,991 nucleotides. This was similar to the size estimated by relative electrophoretic migration in denaturing agarose gels (Mohamed 1981). It had a base composition of 32.0% A, 33.8% U, 18.4% C, and 15.8% G.

Table 1. Symptoms on indicator plant species induced by TSWV-KP isolate.

Indicator plants	Host reaction ^a	
	Inoculated leaves	Upper leaves
<i>Chenopodium amaranticolor</i>	NL	-
<i>C. quinoa</i>	NL	-
<i>Gomphrena globosa</i>	CWL	-
<i>Physalis floridana</i>	NL	-
<i>Datura stramonium</i>	NR	NS
<i>Nicotiana benthamiana</i>	CS	M
<i>N. glutinosa</i>	NL	-
<i>N. clevarandii</i>	NS	NS
<i>N. rustica</i>	NR	NS
<i>N. tabacum</i>	cv. Xanthi nc.	NS
	cv. White burley	NS
	cv. Ky-57	NS
	cv. Samsun	NS
<i>Petunia x hybrida</i>	NL	-
<i>Lycopersicon esculentum</i>	+	M

^a -, no infection; +, latent symptom; CS, chlorotic spot; CWL, chlorotic white lesion; LD, lethal death; M, mosaic; NL, necrotic local infection; NR, necrotic ring; NS, necrotic spot; VN, vein necrosis.

Table 2. Identities of nucleotide sequence (nt), amino acid (aa) of TSWV-KP M RNA and those of other tospoviruses.

Virus ^a	5'UTR	NSm		IGR	G1/G2		3'UTR
		nt	aa		nt	aa	
TSWV-BR01	97.1	92.3	85.3	90.3	93.4	96.2	92.0
GRSV	-	-	-	-	74.8	81.1	80.5
TCSV	-	-	-	-	74.6	79.9	80.5
INSV	54.5	67.8	66.1	50.4	65.2	63.9	56.8
GBNV	64.8	40.6	51.3	45.9	49.0	35.1	54.2
WSMoV	64.2	37.3	48.7	45.0	48.8	34.9	54.2

^aThe following sequences were obtained from NCBI GenBank database. TSWV-BR01 [NC_002050], GRSV [AY574055], TCSV [AY574054], INSV [NC_003616], GBNV [NC_003620], WSMoV [NC_003841].

The 5' and 3' ends of the viral RNA contained two untranslated regions of 88 and 151 nt, respectively, with 58-nt complementary sequences at the terminal which could form a strong panhandle structure characteristic of the members of *Bunyaviridae* (data not shown). The sequence analysis of TWSV-KP S-RNA revealed two ORFs with an ambisense arrangement (Fig. 3).

There was a large ORF of 1,404 nt located on the 5' end of the viral sense strand. This was initiated with an AUG codon at nucleotide 89 and terminated at an UAA stop codon at nucleotide 1,492 from the 5' end of the viral strand. This ORF encoded a 468 amino acid polypeptide with a predicted Mr of 52,446. Another ORF was located

near the 5' end of the viral complementary strand, initiating with the AUG codon at nucleotide 2,840 and terminating at the UAA stop codon at nucleotide 2,064 from the 5' end of the viral strand. This ORF could encode a protein of 258 amino acids with a predicted Mr of 28.8 kDa, which was the N protein based on the sequence comparison to the N proteins of other tospoviruses.

The ORFs coding for NSs and N proteins were separated by a 571-nt A+U-rich intergenic region (IGR). Size differences between the S-RNA molecules of different tospovirus isolates have been attributed to the variation in length of the intergenic region.

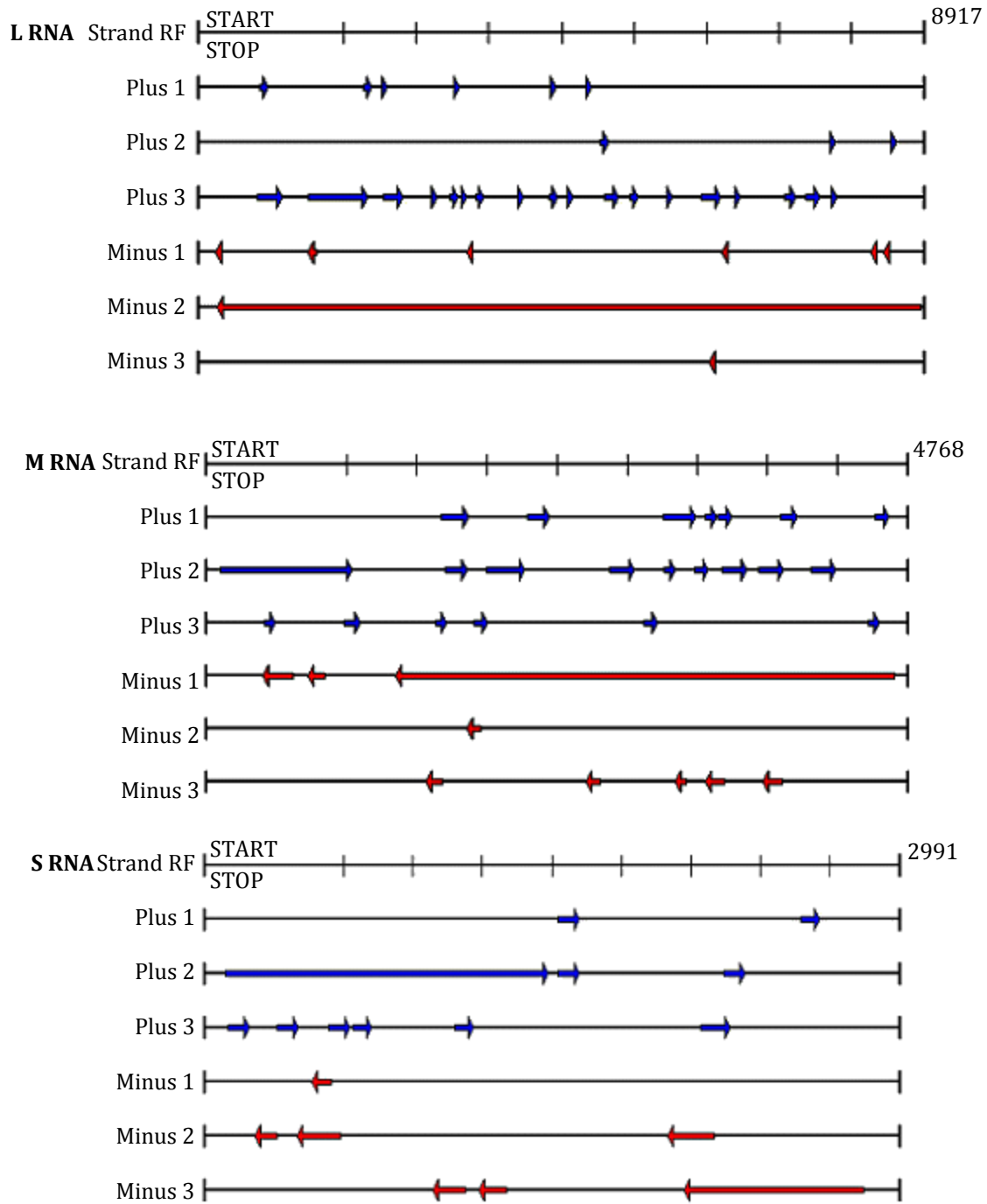


Figure 3. Distribution of open reading frame (ORF) in the three possible reading frames of the viral (a, 2, and 3) and viral complementary (-1, -2 and -3) L, M and S RNA strands of TSWV-KP.

The identity values based on the amino acids and sequences of tospovirus N and NSs proteins were completely consistent with the known serological grouping.

The identities in the nucleotide and amino acid sequences of TSWV-KP N protein exhibited 38.2–96.4% and 19.9–98.4% identity with those of tospovirus

serogroups I–V. This value is in contrast to the 77.5–79.8% identity reported for serotype II, 54.5% identity observed with serotype III, 31.1% and 41.5% identity reported with serotype IV, and 19.9% identity observed with serotype V (Table 3). Thus, N protein TSWV-KP appears to represent the most closely related serotype I member of the genus Tospovirus (Fig. 7).

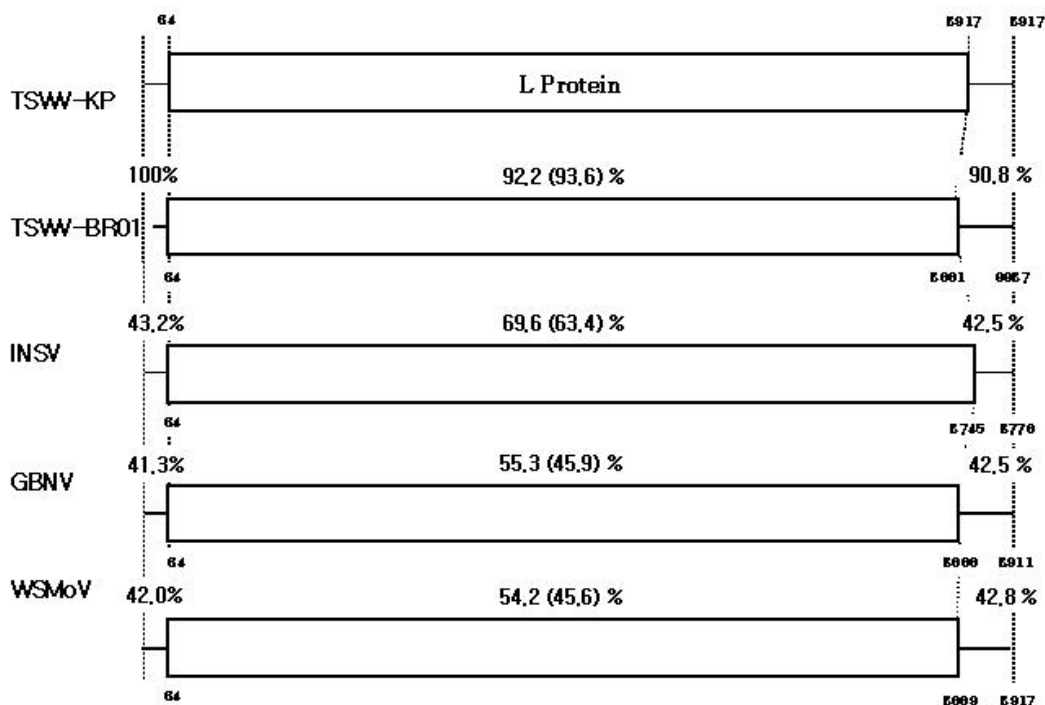


Figure 4. Comparison of the genomic L RNA molecules of TSWV-KP and other tospovirus (numbered from the 5' end of the vRNAs). The percentages refer to nucleotide and amino acid sequences homology between the various UTR and L protein domains.

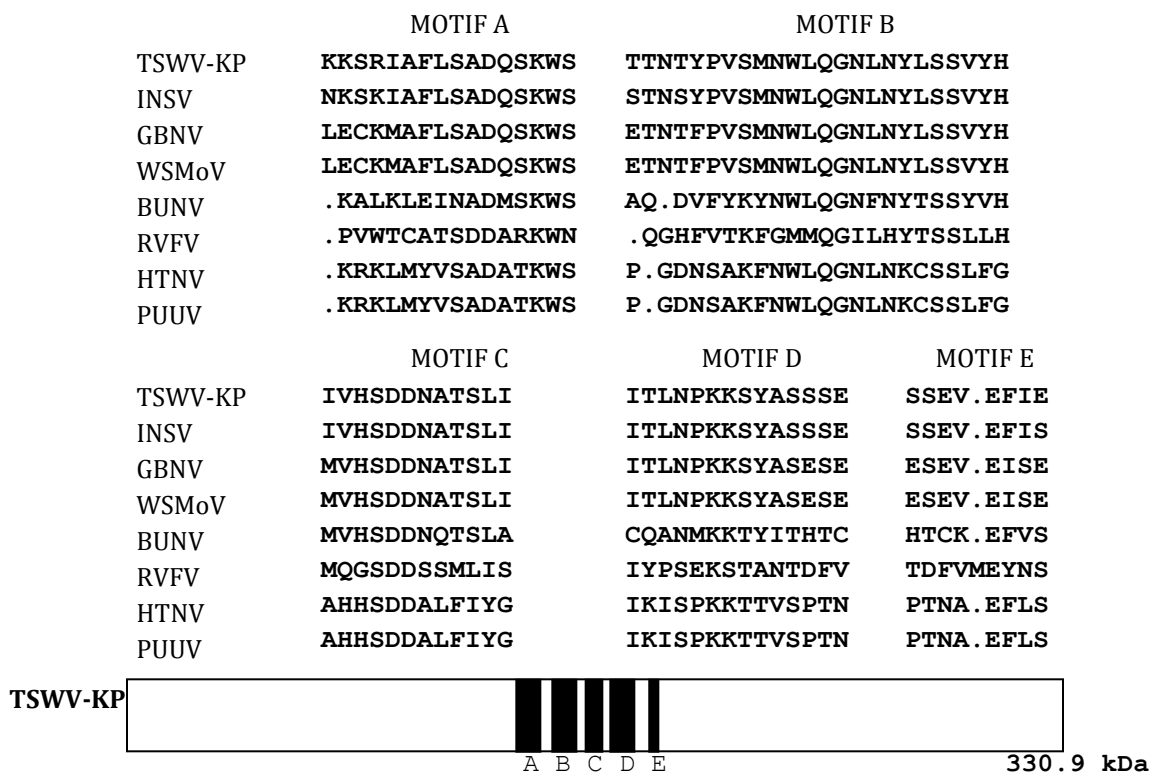


Fig. 5. Amino acid identity between *Bunyaviridae* L Proteins. INSV, NC_003625); GBNV, NC_003614; WSMoV, NC_3832; RVFV; NC_002043, (BUNV; NC_001925), Hantaan natavirus=6533 nt (HTNV; NC_005222) and Puumala hantavirus=6526 nt (PUUV; AY526217). Polymerase motif (A-E) are depicted as bars.

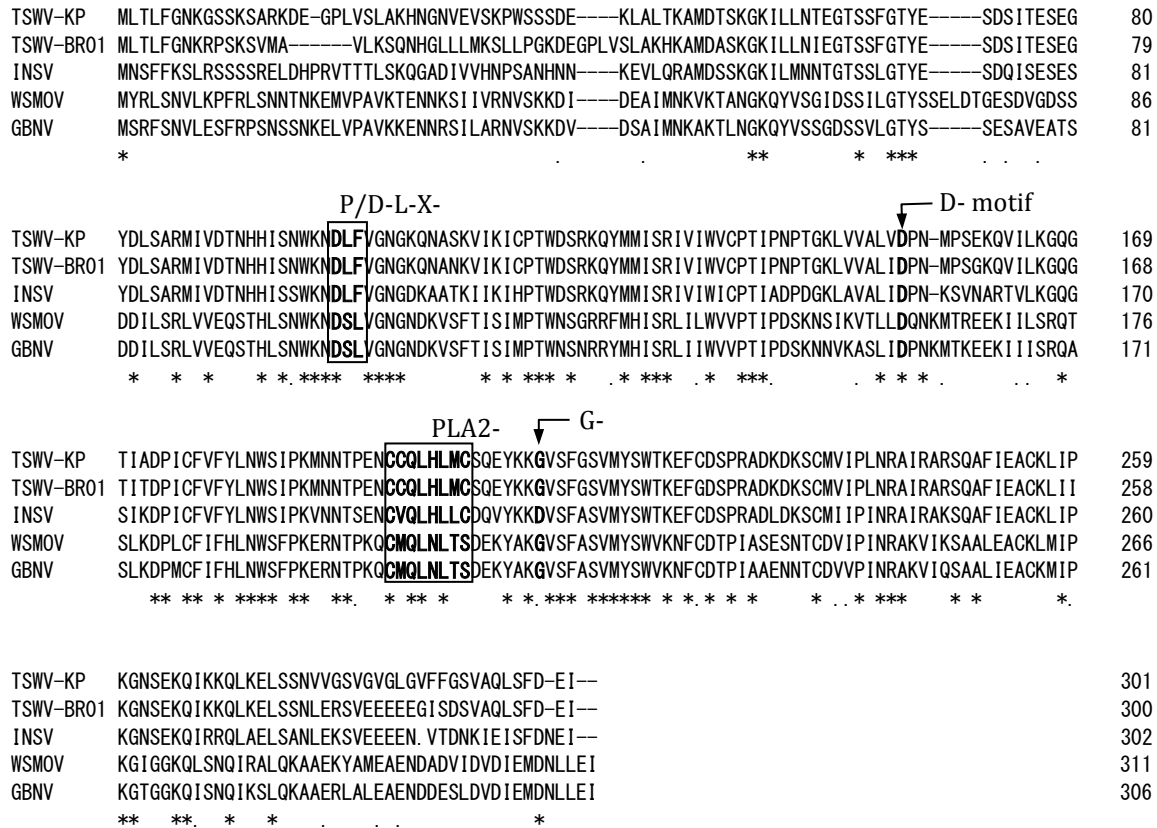


Figure 6. Multiple alignment of the NSm protein sequence of the five tospovirus isolates. The alignment was done with the DNAMAN program. Dashes represent gaps introduced to reach optimal alignment. Perfectly conserved residues are indicated by an asterisk and highly conserved residues are indicated by dot. Data from TSWV-BR01 [NC_002050]; INSV [NC_003616]; WSMoV [NC_003841]; GBNV [NC_003620].

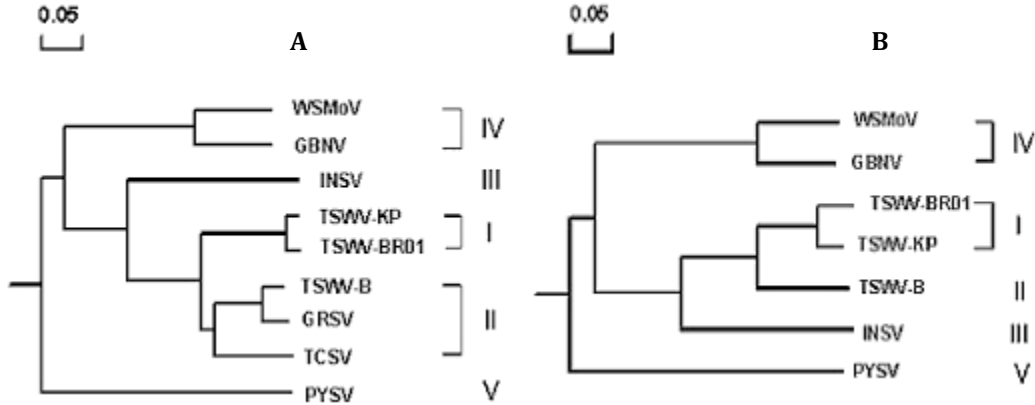


Figure 7. Phylogenetic relationship among various tospovirus isolates based on the amino acid sequences of the N protein (A) and NSs proteins (B). The scale at the top of the figure indicate genetic distance. Roman number represented the serogroups from I to V of Tospovirus genus.

The TSWV-KP N protein consisted of 258 amino acids, which were 3, 16, and 17 amino acids shorter than those for the INSV [NC_003624; 261 amino acids], GBNV [NC_003919; 275 amino acids], and WSMoV [NC_003843; 274 amino acids], respectively. However, it was 12 amino acids longer than that of the PYSV

[AF013994; 246 amino acids]. The N protein of TSWV-KP, however, showed lower identity with those of other serotype III–V tospoviruses, whereas, a higher consensus in amino acid residues of the C terminal region for 60 amino acids was observed between serotypes I and II.

The TSWV-KP NSs protein sequence revealed only 34.4–93.1% identity for the nucleotides and 13.3–87.9% identity for the amino acid sequences with the members of serogroups I–V (Table 2). The TSWV-KP NSs protein

was 3, 18, 28, and 28 amino acids longer than the respective proteins of TSWV-BR01, INSV, GBNV, and WSMoV, whereas the PYSV NSs protein was 13 amino acids longer than that of TSWV-KP.

Table 3. Identities of nucleotide sequence (nt) and amino acid (aa) of TSWV-KP S RNA and those of other tospoviruses^a

Virus	5'UTR	NSs		IGR	N		3'UTR
		nt	aa		nt	aa	
TSWV-BR01	96.6	93.1	87.9	89.2	96.4	98.4	92.2
TSWV-B	-	79.3	81.3	-	77.5	79.8	-
TCSV	-	-	-	-	76.6	77.5	-
GRSV	-	-	-	-	76.9	78.7	-
INSV	41.0	61.4	56.7	56.7	57.6	54.5	45.5
GBNV	46.2	43.0	45.7	45.7	45.9	31.1	58.6
WSMoV	49.2	42.2	49.2	49.2	46.1	31.5	52.9
PYSV	52.6	34.4	35.9	-	38.2	19.9	72.2

^aThe following sequences were obtained from NCBI GenBank database. TSWV-BR01 [NC_002051], TSWV-B [L12048], TCSV [S54325], GRSV [S54327], INSV [NC_003624], GBNV [NC_003619], WSMoV [NC_003843], PYSV [AF013994].

DISCUSSION

TSWV is polyphagous on a great number of mostly herbaceous hosts. The host range is estimated to comprise up to 900 plant species within 80 families and has a worldwide distribution (Goldbach and Petter 1994). The virus causes severe yield losses in many economically important crops. However, TSWV has not been reported in pepper fields in Korea.

In this study, a strain of TSWV, named TSWV-KP, was isolated for the first time from a diseased paprika, and its properties were characterized. TSWV-KP was highly similar to the known TSWV isolates (de Avila *et al.*, 1992; Iwaki *et al.*, 1984; Kameyi-Iwaki *et al.*, 1988; Law *et al.*, 1990; Maiss *et al.*, 1991; Yen *et al.*, 1992). Host reactions to TSWV-KP are summarized in Table 1. Symptoms generally included local necrotic lesions, systemic mottling, leaf distortions, and stunting. Comparisons of the symptomatology of TSWV-KP and two other isolates (Chatzivassiliou 1996; Pang 1993) showed that TSWV-KP differed from TSWV-T_{TH} and TSWV-B, in that TSWV-KP induced only local lesions on *N. glutinosa*, which is a systemically infected host of TSWV-T_{TH} and TSWV-B. Unlike TSWV-B, which systemically infected *Petunia hybrida*, TSWV-KP did not systemically infect *P. hybrida* and only induced local lesions on the inoculated leaf. Tospoviruses can establish widespread systemic infections in the plants. They invade the root (Ie 1964; Kitajima 1965), stem

(Peters *et al.*, 1991), leaves (Best and Palk 1964; Ie 1964; Kitajima 1965; Milne and de Zoeten 1967), and anthers (Ie 1973). Many of the cytopathological differences reported between tospovirus isolates relate to differences in virion maturation. Plants infected with non-defective isolates typically contain masses of single enveloped (“mature”) virions clustered within swollen cisternae of the endoplasmic reticulum (ER) (Ie 1964, 1971; Kitajima 1965; Milne and de Zoeten 1967).

Some tospovirus isolates are morphologically defective or partially defective; infections of these isolates contain primarily unenveloped nucleocapsids (Ie 1982; Verkleij and Peters 1983; Resende *et al.*, 1991 a, b; Urban *et al.*, 1991; Kitajima *et al.*, 1992b). Electron microscopical studies of TSWV have demonstrated that viral particles accumulate in the cisternae of the ER in the cytoplasm of infected cells (Fig. 1-B). Ultrastructural investigations of tissues infected by tospoviruses have shown that mature infections typically contain SEVs within membranous cisternae (Ie 1964, 1971; Kitajima 1965; Milne and Zoeten 1967; Francki and Grivell 1970; Milne 1970).

Time-course studies have revealed the presence of early DEVs (Milne 1970). The composition of proteins in the outer membrane has not been established, and it has been interpreted as a second viral envelope (Milne 1970) or as the membrane of a Golgi vesicle (Kitajima *et al.*, 1992a). Outer envelopes are thought to fuse with host membranes and with each other, which release

mature SEV into expanding cisternal spaces (Milne, 1970; Kitajima *et al.*, 1992a). Fibers and fibrous structures can be found; however, the presence of filamentous inclusions in infected tissue is considerably variable, which has led to speculation that their development might depend on the stage of infection and the host (Kitajima *et al.*, 1992a).

A large portion of the nucleocapsid protein produced during an infection is not used for producing particles but appears to be stored in large nucleocapsid aggregate (NCA) clusters. Cytoplasmic accumulations that contain N protein have been described in different terms as follows: dense band (Kitajima 1965), amorphous densely stained material (Milne 1970), amorphous masses (Ie 1982; Verkleij and Peters 1983), nucleocapsid aggregates (Verkleij and Peters 1983; Peters *et al.*, 1991; de Avila 1992; Kitajima *et al.*, 1992a; Lawson *et al.*, 1993; Kormelink *et al.*, 1994), dense aggregates (Resende *et al.*, 1991a), viroplasm (Francki and Grivell 1970; Milne 1970; Francki *et al.*, 1985; Kitajima *et al.*, 1992a), and dense masses (de Avila *et al.*, 1990; Peters *et al.*, 1991).

Determination of the complete nucleotide sequence of TSWV-KP L-RNA demonstrates that TSWV is a negative-strand RNA virus. The present nucleotide sequence data confirms the previous conclusion, derived from the S-RNA sequence, that TSWV should be considered as a member of *Bunyaviridae* (de Hann *et al.*, 1990).

Indeed, at the ICTV meeting during the Eighth International Congress of Virology in Berlin (1990), TSWV was accepted as the first member of the newly created genus, *Tospovirus*, within *Bunyaviridae*. The TSWV-KP L-RNA segment comprises 8,917 nucleotides, which is significantly longer than the L-RNAs of Bunyamwera (6,875 nt) and Hantaan viruses (6,530 nt) (Elliott 1989; Schmaljohn 1990).

Additional domains may be present in the gene product of TSWV L-RNA, which may reflect the adaptation of this bunyavirus to plants. TSWV-KP L-RNA contains a complementary end that is 60–64 nucleotides in length. Previously, TSWV-BR-01 strains L-RNA could be folded into a stable panhandle structure, which may be involved in the appearance of circular nucleocapsids in virus particles (Peters *et al.*, 1991), as also found for *Bunyaviridae* (Raju and Kolakofsky 1989).

Moreover, these terminal sequences would play an important role in genome transcription and replication, since they contain the initiation signals for encapsidation

and RNA synthesis (Krug *et al.*, 1989; Parvin *et al.*, 1989). TSWV-KP L-RNA contains a single ORF in the viral complementary sense, corresponding to a protein with a predicted Mr of 330.9 kDa. The identities in the nucleotides and amino acid sequences of the L protein exhibited 54.2–92.2% and 45.6–93.6% identity with other tospoviruses, respectively (Fig. 4).

Analysis of viral RNA species in infected plants cells indicates that this ORF is expressed by the formation of an mRNA of approximately genome length. The predicted 330.9-kDa protein encoded by TSWV-KP L-RNA most probably corresponds to the viral polymerase. Comparisons of RNA polymerases from tospoviruses as well as Bunyamwera, Rift Valley fever, Hantaan, and Puumala viruses reveal the presence of amino acid sequence motifs that are present in all polymerases showing RNA template specificity and most likely form the active sites for RNA synthesis (Poch *et al.*, 1989).

Recently, the core polymerase regions (motifs A–E) have been suggested to be directly involved in enzyme activity, based on the superimposition on to the known three-dimensional structure of HIV reverse transcriptase (Muller *et al.*, 1994). The nucleotide sequence of TSWV-KP M-RNA showed an ambisense arrangement typical for tospoviruses. The M-RNA encodes in the viral sense a nonstructural protein called NSm (301 aa); and in the viral complementary sense, a precursor of the two glycoproteins G1 and G2, which encompasses 1,134 amino acids. Between the M-RNA-encoded protein genes, a 266-nucleotide non-coding intergenic region is present. It contains a stretch rich in adenine residues followed by a stretch rich in uracil residues, thereby enabling the formation of the stable hairpin structure.

As previously discussed for other tospoviruses, this structure could be involved in the termination of transcription of subgenomic mRNAs (Kormelink *et al.*, 1991). A conserved sequence found at the top of the tospovirus M-RNA hairpin indicates a possible function in transcription termination (Cortez *et al.*, 2002). Whether the structural features are a requirement for the termination of transcription remains to be investigated.

Mushegian and Koonin (1993) aligned the sequences of movement proteins encoded by several plant viruses and grouped viruses according to the homology of the corresponding movement proteins. The largest plant virus movement protein group was denoted the “30K superfamily,” since most of the members encode a

movement protein of ~30 kDa molecular mass. The “30K superfamily” comprises movement proteins, which contain only one conserved region of ~30 amino acids. This region includes an aspartate residue (“D-motif”) which is almost invariable among all the “30K superfamily” viruses, preceded by hydrophobic amino acids. NSm amino acid sequences available so far present the “D-motif” along with other residues typically conserved among members of the “30K superfamily” proteins (Melcher 2000), as the “P/D-L-X motif” and the “G-residue” (Fig. 6).

The adaptation of tospoviruses to plants as hosts has been made possible with the inclusion of gene coding for the potential viral movement (NSm) protein. This has the capacity to modify plasmodesmata that simultaneously transports the viral entity through these channels (Storms *et al.*, 1995). As this gene is of major importance for the infection cycle in plant tissues, it is likely that the determinants for the host range of tospoviruses reside within this protein. In this respect, it is interesting to note that within the currently available NSm protein sequences, a highly diverging N-terminal sequence of about 60 amino acids is present, which very well could play a role in this, reflecting the different host ranges observed for many tospoviruses.

Sequence analysis revealed that the complete nucleotide sequence of the TSWV-KP S-RNA comprised 2,991 nucleotides, i.e., 75 nucleotides longer than that of the S-RNA of TSWV-BR01. This difference was mainly attributed to the elongated intergenic region of the TSWV-KP S-RNA. The 5' and 3' extreme ends of the TSWV-KP S-RNA were conserved and complementary, except a mismatch at nucleotide position 9.

The same mismatch was previously observed in the S-RNA of TSWV-CNPH1 (de Haan *et al.*, 1989; de Haan *et al.*, 1990) and in animal viruses (Bouloy 1991; Elliot 1990) in the family *Bunyaviridae*. The significance of this discrepancy is unknown. Nevertheless, conservations of the terminal sequences among tospoviruses and of the mismatch within the family *Bunyaviridae* indicated their possible roles in replication and encapsidation (Bouloy 1991; Elliot 1990).

From the sequence, it can be deduced that the TSWV-KP S-RNA has an ambisense gene arrangement (Fig. 3). The two ORFs were separated by an intergenic region of 571 nucleotides longer than its counterparts in TSWV-BR01. The complex and stable hairpin structure that potentially could be generated by internal base pairing

of U-rich stretches with A-rich stretches of the intergenic region appears to be similar to those produced in TSWV-BR01, with regard to both structure and stability.

It has been suggested that this internally base-paired structure acts as a transcription termination signal (Bouloy 1991). Traditionally, the classification of *Bunyaviridae* has been based mainly on serology, which results in a categorization of these viruses into serogroups and serotypes (Elliott 1990). Following this classification, and using the antigenic properties of the N protein, three serogroups (I to III) have thus far been recognized within *Tospoviruses*, a genus of *Bunyaviridae* (Francki *et al.*, 1991).

The International Committee on Taxonomy of Virus Taxonomy finds it important to define criteria to delineate species and to avoid the use of less discriminative concepts such as serogroups and serotypes. Serogroup IV members—PBNV and WSMoV—are serologically distinct from the members of serogroups I, II, and III (Adam *et al.*, 1993).

The N protein of TSWV (serogroup I) showed 77–78% sequence identity with GRSV and TCSV (serogroup II) (de Avila *et al.*, 1993), 55% sequence identity with INSV (serogroup III) (de Haan *et al.*, 1992), and 33–35% sequence identity with PBNV and WSMV (serogroup IV) (Yeh and Chang 1995).

Recently, PYSV was separated into a distinct species and proposed to be included in a newly established serogroup of the genus *Tospovirus* based on lack of serological cross reactivity and nucleic acid hybridization with TSWV, INSV, and PBNV (Satyanarayana *et al.*, 1996). The results of a clustering analysis of the aligned sequences of N and NSs proteins of tospoviruses are shown in Fig. 7. The phenograms suggest that TSWV-KP should be included into serogroup I based on N or NSs protein sequences.

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