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Characterization of *Tomato spotted wilt virus* from Paprika in Korea

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A *Tomato spotted wilt virus* (TSWV-KP) was isolated from Paprika (*Capsicum annuum* var. grossum) showing necrosis spot on the leaves and malformation of the fruit in Yesan, Korea. The virus infected *Chenopodium amaranticolor*, *C. quinoa*, *Petunia hybrida*, *Nicotiana glutinosa*, *Gomphrena globosa*, and *Physalis floridana*. Ten plants including tomato were observed to have systemic TWSV-KP infection. The virus produced necrosis or necrotic ring spots on the inoculated leaves and mosaic, vein necrosis or death on the upper leaves of *Datura stramonium*, *N. clevarandii*, *N. rustica*, and *N. tabacum* cvs. Thin sections of the infected leaf tissue contained spherical to oval particles, a characteristic of a *Tospovirus*. The virion contained three molecules of genomic RNAs, which were approximately 9.0, 4.9 and 3.0 kb. The 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.

Keywords : Nucleocapsid protein, paprika, *Tomato spotted wilt virus*, *Tospovirus*

Tomato spotted wilt virus (TSWV) belongs to the genus *Tospovirus* within the *Bunyaviridae* (Regenmortel et al., 2000). TSWV was first reported in tomato (Brittlebank, 1919). TSWV has a very broad host range estimated to be up to 900 plant species within 80 families and has a worldwide distribution (Goldbach and Peters, 1994). The virus causes severe yield losses (producing less valuable or unmarketable plant, fruit or flowers) in many economically important crops. It is transmitted exclusively by thrips in a propagative-circulative way (Wijkamp et al., 1993).

TSWV virions are spherical enveloped particles, about 80-110 nm in diameter. Nucleotide sequence and translational analyses have shown the large (L) genomic RNA of TSWV to be negative-stranded (de Haan et al., 1991), and the other two (S and M) RNAs to be ambisense (de Haan et al., 1990; Kormelink et al., 1992).

The L RNA (8.9 kb) is a negativeand encodes a 331.5 kDa putative viral polymerase. The M RNA (4.8 kb) encodes the precursor of the envelope glycoproteins G1 and G2 and the nonstructural protein NSm (Kormelink et al., 1994). The S RNA (2.9 kb) encodes a non-structural protein, NSs, and the structural nucleocapsid (N) protein.

Paprika has been cultivated since 1994 in Jeju Island, southern part of Korea. An economically important crop in the country, its production area has been increased to 134.5 ha in 2003. However, virus diseases are currently causing yield and quality losses of paprika fruit.

In a survey on virus diseases of pepper in 2003, a new undescribed virus was isolated from paprika fruit showing necrotic spot and malformation in a green house in Yesan, Korea. The isolate was identified as TSWV and then designated as TSWV-KP. This paper presents some characteristics of the virus isolate.

Materials and Methods

Virus isolation. Paprika plants showing symptoms of spotted wilt disease were collected from commercial green house in Yesan during the growing season of 2003. The virus was isolated and maintained by serial mechanical transmission in the *N. glutinosa* and *N. rustica*. The virus isolate was named as TSWV-KP.

Host range. The host range of the isolate was determined by mechanical inoculation using extracts from infected *N. rustica* in 0.1 M sodium phosphate buffer (pH 7.0), containing 0.01 M sodium sulfite, after dusting the leaves with 600 mesh carborundum powder. After inoculation, the tests plants were maintained for visual inspection of virus symptoms in the glass house at 25 ± 2°C for at least 3 weeks.

Electron microscopy. For ultra-thin section, systemically infected leaves were cut and fixed for 2 h in 3% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.2). The samples were washed 3 times in phosphate-buffered salts (PBS, pH 7.2) and post-fixed with 2% osmiumtetra oxide for 2 h at 4°C. After dehydration with an ascending series of 50%-100% ethanol, the samples were imbedded in LR-White medium and kept for polymerization at

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60°C for 24 h. Ultra-thin sections of 80 nm thickness were stained with 2% uranyl acetate and 0.5% lead citrate and rinsed with distilled water. The samples were examined by using a transmission microscope (LEO 906, ZEISS).

Isolation of viral dsRNAs. The isolation procedure of dsRNAs from the virus isolate infected *N. rustica* was similar to that of 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.

Purification of nucleocapsid. TSWV-KP was purified from the infected leaves of *N. rustica* by using the method of Satyanarayana et al. (1996). This was followed by additional centrifugation in 20-40% sucrose gradients prepared in CSE (0.05 M Citrate pH 7.6, containing 0.01 M sodium sulfite and 0.01 M EDTA, pH 8.0) buffer for 2.5 h at 33,000 rpm in a SW 50.2Ti rotor. The light-scattering zone was collected, diluted in CSE buffer. The virus was pelleted at 35,000 rpm for 2 h in a 50.2 Ti rotor. The purified virus was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a dissociated buffer at 30 mA for 4h. The gel was stained with Coomassie blue R-250.

Primers for amplifying the N gene. A specific primer pair derived from the TSWV isolate (GenBank No. NC_002051) was designed. The genome sense primer (5'ATGTCTAAGGTTAA-GCTCAC3') was derived from beginning of the N gene sequence and included were the first 20 bases of the coding region. The genome antisense primer (5'TCAAGCAAGTTCTGC GAGTT3') represented the last 20 bases of the N gene coding region.

RT-PCR and treatment of restriction enzyme. To facilitate synthesis of first strand cDNA, reverse transcription (RT) reaction was carried out with one cycle at 50°C for 45 min. The same reaction mixture was subjected to the following PCR profiles: 94? for 1 min, 50°C 1 min, and 72°C for 2 min. This was repeated for 35 cycles. The final profile consisted of 94°C for 1 min, 50°C for 1 min, and 72°C for 10 min. The amplified PCR product was digested with the restriction endonuclease *Eco*RI (TaKaRa Co.) and analyzed by electrophoresis in 1.5% agarose gel.

Cloning and sequencing of the N gene. The cDNA of the N gene of TSWV-KP was cloned into pGEM-T-Easy vector (Promega Co.). Nucleotide sequences were determined by using a BigDye DNA sequencing kit (Perkin-elmer Co.) on an ABI 377 DNA sequencer (PE Applied Biosystem). Sequence analysis and sequence comparisons with published sequences were done by using the DNASIS software package version 2.6 (Hitachi Co.). The BLAST program was used to identify related sequences available from the GenBank database (Table 2).

Results and Discussion

Symptoms. TSWV-infected paprika crops showed mosaic

Table 1. Biological reaction of *tomato spotted wilt virus*-KP isolate

Indicator plants	Host reaction[a]	
	TSWV-KP	TSWV-T_{TH}[b]
Chenopodium amaranticolor	NL/–	nt
C. quinoa	NL/–	N(pp)/–
Gomphrena globosa	CWL/–	NL/–
Physalis floridana	NL/–	NL, NR/TN, W, D
Datura stramonium	NR/NS	–/C, Mo
Nicotiana benthamiana	CS/M	CL, /C, LD, W, D
N. glutinosa	NL/–	NL/W, D
N. clevarandii	NS/NS	NL/Mo, W, N
N. rustica	NR/NS	CS/VC, M
N. tabacum cv. Xanthi nc.	NS/NS	nt
cv. White burley	NS/VN, LD	nt
cv. Ky-57	NS/VN, LD	nt
cv. Samsun	NS/VN, LD	NS/NS, NR, LD
Petunia × *hybrida*	NL/–	NL/–
LycBBpersicon esculentum		nt

[a]–: no infection, +: latent symptom, CL: chlorotic local infection, 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.
[b]Chatzivassiliou et al., 1996.

Table 2. Percentage sequence identities between the N Protein genes of *Tomato spotted wilt virus*-KP and other *Tospoviruses*

Virus	Identity (%) of N proteins		GenBank Accession number	Reference
	Nucleotide	Amino acid		
TSWV-KP	–	–	AB175809	In this study
TSWV-O	97.2	97.7	AB088385	Takeda et al. (unpublished)
TSWV-J	96.9	98.1	AB010997	Tsuda et al. (1994)
TSWV-C	96.5	98.5	AB 038341	Kato and Hanada (2000)
WSMV	32.2	25.9	NC_003843	Yeh et al. (1990)
MYSV	28.8	22.8	AB038343	Kato and Hanada (2000)
INSV	48.4	51.7	NC_003624	de Haan et al. (1992)
IYSV	31.5	26.3	AB121026	Doi, M. (2003)
GBNV	32.3	25.1	NC_003619	Satyanarayana et al. (1996)

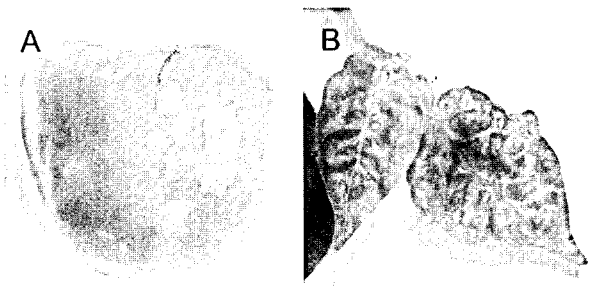

Fig. 1. Symptoms on paprika fruit (**A**) and leaves (**B**) naturally infected with TSWV-KP.

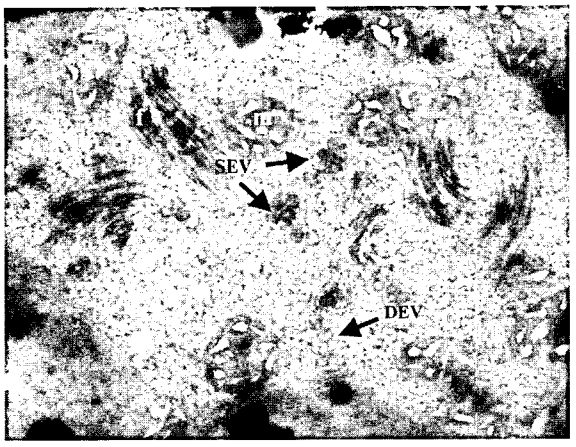

Fig. 2. Formation of single enveloped virion (SEV) by fusion of DEV. Clustered SEV inside membranes, as found in TSWV infections of *Capsicum annuum*. The DEV at the bottom of the image. f, filamentous inclusions and m, mitochondria. Bars, 200 nm.

and sometimes necrotic spot on the upper leaves. Infected fruits had generally large chlorotic ring or necrotic spots and were not marketable anymore (Figure 1A and 1B).

The reaction of indicator plants. Inoculated leaf of *N. rustica* indicated necrotic lesions with chlorotic rings then systemic mosaic and necrosis spot appeared. Chlorotic local lesions on inoculated leaf of *N. benthamiana* appeared in 4-5 days after inoculation, followed by yellowing. *Petunia hybrida* and *Gomphrena globosa* showed necrotic local and white necrotic local lesions on inoculated leaves in 3-4 days after inoculation, respectively. *N. tabacum* cv. White burley and cv. Ky-57 showed local lesions on the inoculated leaves and systemic symptoms. Finally, the plants died with severe necrosis (Table 1).

Electron microscopy. In ultrathin section of the tissue of TSWV-infected *Capsicum annuum*, TSW viral particles accumulated in the cistae of endoplasmic recticulem (ER), and single enveloped virion (SEV), in cytoplasm. SEV concentration in leaf tissue was rich and double envelope virion (DEV), as well as, fiber and fiber structures could also be found in most part of the cytoplasm of the diseased plant (Fig. 2), as reported previously (Ie, 1971). Flexuous filaments display a diverse appearance, which include loose scattered fibers in the cyroplasm, thumbprint formation, crosscut formation, and bundles of long parallel filaments (Fig. 3).

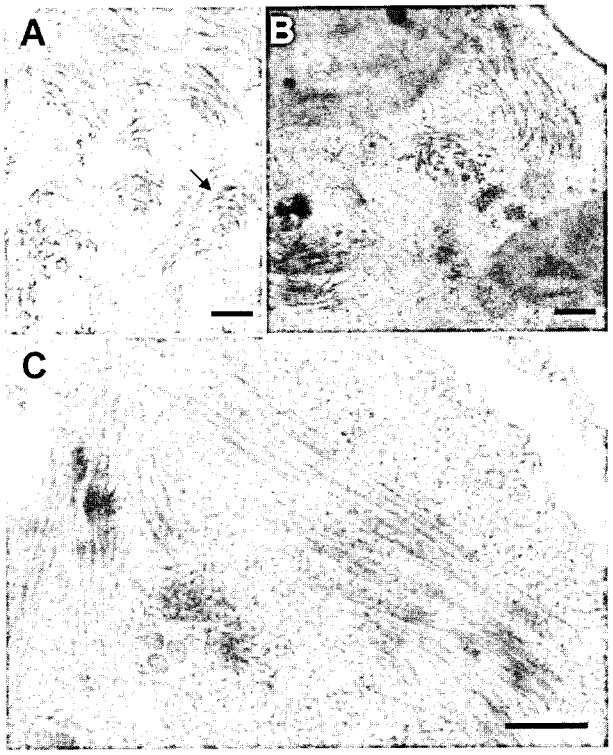

Fig. 3. Flexible filamentous inclusions (F) produced by TSWV. Thumbprint formation (arrows) (**A**), cross cutted formation (**B**), and bundles of long parallel filaments (**C**). Bars. 300 nm.

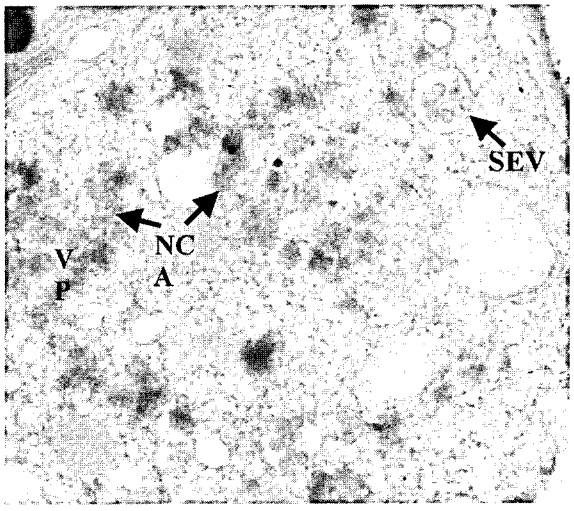

Fig. 4. Localization of tospovirus N protein in cytoplasmic inclusion. SEV, single enveloped virion; NCA, nucleocapsid aggregates; VP, viroplasm. Bars, 300 nm.

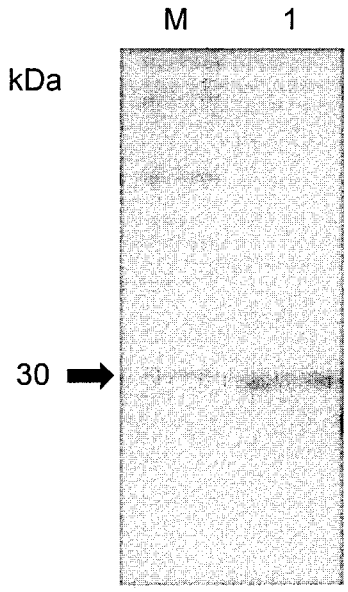

Fig. 5. Estimation for molecular weight of N protein of TSWV-KP by SDS-PAGE. Lane M, Standard protein for SDS-PAGE (Novagen); and 1, The purified nucleocapsid protein of TSWV-KP.

Viroplasm (VP), characterized as amorphous medium-density material, was seen very often in association with nucleocapsid aggregates (NCA), which was much more dense (Fig. 4), as reported previously (Marjolein et al., 1999).

N protein analysis. Nucleocapsid of TSWV-KP was purified from *N. rustica* that showed typical tospovirus symptoms. The preparation, analyzed by SDS-PAGE, gave a single protein band (Fig. 5). N protein of the TSWV -N, M, and P isolates had a molecular weight of 30 kDa; while that of the W and K isolates was 32 kDa (Tsuda et al., 1996). Molecular size pattern of TSWV-KP was similar to that of the Japanese isolate of N, M and P of TSWV.

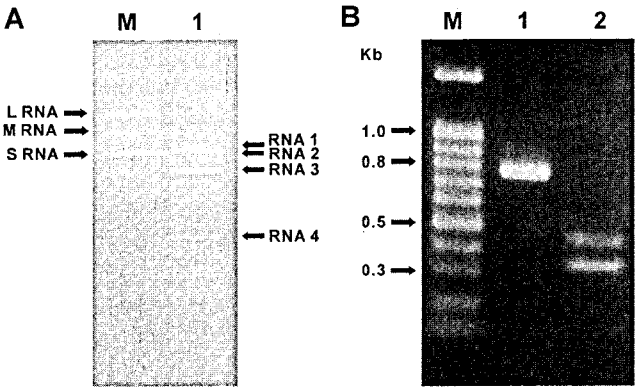

Fig. 6. DsRNA profiles of *Tomato spotted wilt virus*-KP extracted from infected *N. rustica* (A) and RFLP analysis of RT-PCR amplified products of TSWV-KP (B). Photo (A) Lane 1, TSWV-KP dsRNAs; 2, CMV-Mf dsRNAs. Photo (B) Lane M, 1 kb DNA ladder; 1, the amplified DNA product of TSWV-KP; and 2, the DNA digested with *Eco*RI.

Isolation of dsRNAs. The total dsRNA segments extracted from leaves of *N. rustica* infected with TSWV-KP were analyzed by electrophoresis in 0.8% agarose gel. A typical dsRNA virus is shown in Figure 6A (lane 1). The ds RNAs extracted from *Cucumber mosaic virus* (CMV) infected tabacco was used as markers (Fig. 6A, lane 2). The sizes of the three dsRNAs of TSWV-KP were estimated at 9.0 kb, 4.9 kb, and 3.0 kb. According to the molecular size of the dsRNAs (Tsuda et al., 1992), they were considered as the replication forms of the genomic S, M, and L RNA, respectively.

RT-PCR amplification and restriction enzyme digestion. Using the primers designed in the experiment, it was possible to detect specifically TSWV isolate. RT-PCR, performed with total RNA extracted from *N. rustica* infected with TSWV-KP, yielded a single product of 777 bp, the correct size predicted for the product of these primers. When the DNA product of TSWV-KP gene was digested with *Eco*RI, two fragments of the DNA product were 327 bp and 450 bp in molecular size (Fig. 6B, lane 2).

Sequence analysis of N gene of TSWV-KP. The N gene was 777 bases long and can potentially code for 259 amino-acid protein. The sequence has been deposited in the GenBank/EMBL databases under accession number AB175809. The N gene of TSWV-KP showed 96.5-97.2% and 97.7-98.5% identities to the three different TSWV strains (Table 2) at the nucleotide and amino acid, respectively.

This result is the first report of the *Tospovirus* infecting paprika in Korea. This paper provides information on the composition of the N gene and the biological properties of TSWV-KP for detection and identification of the virus.

Because of the ubiquitous presence of thrips, and their year-round reproduction in most cultivated areas, our findings suggest that many of the crops colonized by thrips are also susceptible to *tospovirus*. To improve disease control, we require monitoring and improved detection methods for the presence of viruliferous thrips at an early stage of infection.

Acknowledgments

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