Tomato spotted wilt virus Isolates Giving Different Infection in Commercial *Capsicum annuum* Cultivars

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Tomato spotted wilt virus (TSWV)-infected *Capsicum annuum* plants were collected from open fields during June to July in 2010. The TSWV isolates were designated as Gneung, Ghang-Kjj, Gchang-Njc, Ghae, and Pap. The nucleotide sequence of the nucleocapsid protein (N) and movement protein (NSm) of the five isolates was determined. The pathogenicity of the five isolates was determined on 14 *C. annuum* cultivars two times by using mechanical inoculation. The five isolates induced different response: Both Gneung and Gchang-Kjj did not infect any of the cultivars in the 2nd trial, while Gchang-Njc, Ghae and Pap infected 11, 6 and 13 of 14 cultivars, respectively. The five isolates also were tested on *Solanum lycopersicum* breeding line TGC09-71 and three *Nicotiana* species. *S. lycopersicum* showed a similar response to the five isolates as did *C. annuum*. Both Gchang-Njc and Ghae infected systemically all three *Nicotiana* species tested. While both Pap and Gneung did not infect any of the *Nicotiana* species tested. In conclusion, five TSWV isolates induced different infection spectra in *C. annuum* cultivars, *Nicotiana* species and an *S. lycopersicum* breeding line. Amino acid sequence analysis of the NSm gene could not support or explain the different infection spectra of the five isolates. This study indicated that various isolates must be used as virus inocula for evaluation of *C. annuum* and *S. lycopersicum* cultivars in breeding programs for TSWV resistance.

Keywords : *Capsicum annuum*, pathogenicity, Tomato spotted wilt virus isolates

Tomato spotted wilt virus (TSWV) was first observed in Australia in 1915 (Brittlebank, 1919), and was later identified as a viral disease in 1930 (Samuel et al., 1930). TSWV has a wide host range of more than 800 species in 82 families and is spread throughout the world. In Korea, TSWV was first observed on paprika plants at Yesan,

Chungcheong Province in 2004 (Kim et al., 2004). At present, TSWV is widespread in *Capsicum annuum* crops in Korea.

TSWV have a tripartite RNA genome, composed of the large (L) segment, encoding an RNA-dependent RNA polymerase in negative sense; a medium (M) segment, encoding the NSm protein and the G1-G2 precursor glycoprotein in ambisense orientation; and the small (S) segment, encoding the NSs nonstructural protein and the Nucleocapsid (N) protein also in ambisense orientation (Moyer, 1999). The N protein encapsidates the virus RNA within the viral envelope (Richmond et al., 1998). NSm is involved in host range determination and symptomatology (Silva et al., 2001), as well as cell-to-cell movement (Kormelink et al., 1994).

Nine species of thrips are reported as vectors of TSWV (Gibbs, 1983). The Western flower thrips (*Frankliniella occidentalis*) is believed to be a major reason for the incidence of infection by TSWV increasing. However, as pesticides become less available, or less effective, other strategies are needed for crop protection. One such strategy is to use resistant cultivars. Thus, development of resistant cultivars is being conducted by using conventional and non-conventional methods (Cho et al., 1996; Prins and Goldbach, 1998). Accordingly, virus inoculum is needed to screen cultivars for resistance to TSWV. This study was conducted to determine the pathogenicity of TSWV isolates collected from open fields of *C. annuum* crops in Korea for use in screening of breeding lines for TSWV resistance.

C. annuum plants revealing yellowing and necrosis of the shoot tip were collected from the open fields of five places: at Gangneung (Gangwon Province), Gochang (Jeonbuk Province), and Gimhae (Gyeongnam Province) through June and July of 2010 (Table 1). The distance between Gangneung and Gochang is 350 km, between Gochang and Gimhae 230 km and between Gangneung and Gimhae 250 km. Five *C. annuum* plants collected from different regions were designated as TSWV-Gneung, TSWV-Ghae, TSWV-Gchang-Gchang-Kjj, TSWV-Gchang-Gchang-Njc and TSWV-Pap (Table 1). RT-PCR was used to confirm

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Table 1. Collection of *Tomato spotted wilt virus* isolates from the open fields of commercial *Capsicum annuum* and paprika plants

TSWV isolates	Collection area	Host plants	Symptom	Collection date
TSWV-Gneung	Gangwondo Gangneung	<i>C. annuum</i> 'Supermanita'	Yellowing, shoot tip wilt, leaf wilt	06-28-2010
TSWV-Gchang-Gchang-Kjj	Jeonbuk Gochang Mu-jang-myeon	<i>C. annuum</i> 'Idangbaek'	shoot tip yellowing, leaf necrosis, fruit ring pattern necrosis	07-09-2010
TSWV-Gchang-Gchang-Njc	Jeonbuk Gochang Gong-um-myeon	<i>C. annuum</i> 'Chunhajaeil'	shoot tip yellowing, leaf and fruit necrosis	07-09-2010
TSWV-Ghae	Gyeongnam Gimhae	<i>C. annuum</i> 'Nockwang'	Double ring, yellowing, mottle, shoot tip wilt	06-20-2010
TSWV-Pap	Gangwondo Gangneung	'Paprika'	shoot tip yellowing	09-29-2009

that the five *C. annuum* plants were not infected by cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), pepper mild mottle virus (PMMoV) and pepper mottle virus (PepMoV), and they were singly infected by TSWV. The RT-PCR primers used were as follows: CMV forward: 5'-TTAACCACCCAACCTTTGTG-3', CMV reverse: 5'-TCGTCCTTTTGAATACACGAG-3'; TMV forward: 5'-CCGTAAAGGGAAAAATAGTAGTAG-3', TMV reverse: 5'-ACGTGCCTGCGGATGTA-3'; PMMoV forward: 5'-ATGGCTTACACAGTTTCCAGTG-3', PMMoV reverse: 5'-TTAAGGAGTTGTAGCCCAGGTG-3'; PepMoV forward: 5'-CACATCACCTTGGATGCTTG-3', PepMoV reverse: 5'-ATGCATTTGTACGGGAAGGT-3'; TSWV forward: 5'-TGTTGACACAAGGCAAAGAC-3', TSWV reverse: 5'-GCAAGTTCTGTGAGTTTTC-3'.

Each TSWV isolate was transmitted onto *Nicotiana rustica* plants by mechanical inoculation using 1×PBS (pH 7.2), for subsequent use as inocula for evaluation on *C. annuum* plants. Single infection of *N. rustica* plants with each corresponding TSWV isolate was confirmed by RT-PCR at two weeks after inoculation and the plants were maintained in an insect proof glasshouse. To avoid the generation of defective strains that might occur in successive mechanical passage (Resende et al., 1991), virus inocula plants were prepared every time for each experiments from the isolate source of *C. annuum* plants.

The pathogenicity of the five TSWV isolates was evaluated on 14 commercial *C. annuum* cultivars. To observe the effect of temperature on the response of *C. annuum* plants to infection with TSWV, two trials were conducted: on 31

Fig. 1. Evaluation of *Capsicum annuum* (upper panel) cultivars and *Solanum lycopersicum* breeding line TGC09-71 (lower panel) for resistance to TSWV isolates Gneung, Gchang-Njc, Gchang-Kjj, Ghae and Pap. In both upper and lower panels, plants positioned in the first, the second, the third, the fourth and the fifth rows from the top of each basket were inoculated with TSWV isolates Gneung, Gchang-Njc, Gchang-Kjj, Ghae and Pap, respectively. Virus was inoculated on Oct. 18, and virus was assayed on Oct. 27-29 by ELISA.

Table 2. *Tomato spotted wilt virus* isolates inducing differential responses in *Capsicum annuum* cultivars (1st trial)

TSWV isolates	Super-umchungna	Ryuckgangsungmunjang	Jungilpum	Bigthree	Docbulwang	Gonchowang	Nocwonfood	Nocrufood	Chungkwang	Saenggreen	Morningfood	Docyachungchung	Gumbit	Chonhajaeil
Gneung	0.067	0.078	0.056	0.079	0.067	0.066	0.083	0.077	0.074	0.079	0.067	0.089	0.065	0.099
Gchang-Kjj	3.565	0.087	0.065	0.089	0.056	3.347	0.083	0.077	0.074	0.079	0.067	0.089	0.065	0.099
Gchang-Njc	3.987	3.567	3.899	·	·	3.345	4.004	4.021	3.987	3.899	4.012	3.956	4.200	·
Ghae	2.950	3.553	4.120	2.788	4.012	2.908	4.150	2.897	4.092	2.615	3.970	2.981	3.082	3.035
Pap	2.478	2.218	3.169	0.049	0.051	1.308	1.299	·	0.047	0.761	1.215	1.186	3.056	·

^a Absorption at 405 nm by ELISA

· : not inoculated.

Inoculation method: mechanical inoculation, inoculation period: Aug. 31, investigation period: Sept. 7–13, virus detection: ELISA.

Aug. (temperature inside the glasshouse: $35 \pm 3^\circ\text{C}$) and on 18 Oct. (temperature inside the glasshouse: $26 \pm 3^\circ\text{C}$). When two leaves of test plants were fully expanded, crude sap from the five TSWV isolates was rubbed onto the test plants. The crude sap was prepared in $1 \times$ PBS buffer (pH 7.2). The response was determined at 10 days after inoculation. Virus infection was confirmed by ELISA.

The response of 14 *C. annuum* cultivars to inoculation by the five TSWV isolates is shown in Fig. 1. In the first trial (31 Aug.), isolate Gneung did not infect any of the 14 *C. annuum* cultivars tested, while both isolates Gchang-Njc and Ghae gave systemic infection in all cultivars tested (Table 2). Isolates Gchang-Kjj and Pap showed different responses on the 14 cultivars. Isolate Gchang-Kjj infected only ‘Super-umchungna’ and ‘Gonchowang’ systemically. Isolate Pap infected nine cultivars tested, but not cvs. ‘Big three’, ‘Docbulwang’, or ‘Chungkwang’. Interestingly, symptom expression on Pap infected cultivars was delayed about for 4 to 5 days in comparison with those cultivars infected with Ghae, and most Pap infected plants showed necrotic double rings on the inoculated leaves (Fig. 2).

In the second trial (18 Oct.), both isolates Gneung and Gchang-Kjj did not infect any cultivars tested, while isolates Gchang-Njc, Ghae and Pap infected 11, six and 13 cultivars, respectively (Table 3). In general, in the second trial the tested cultivars showed more resistance to TSWV.

Fig. 2. Necrotic double rings formed on the inoculated leaves of *Capsicum annuum* cv. ‘Super-umchungna’ induced by infection with TSWV isolate Pap at 15 days post inoculation.

This phenomenon is consistent with the finding of Roggero et al. (2001), where TSWV resistance, determined by a dominant gene called *Tsw*, was broken at high temperature. In this study, the temperature inside the glasshouse in the 1st trial was higher than in the 2nd trial.

In the second trial, five TSWV isolates also were tested on *S. lycopersicum* segregation line TGC09-71. TGC09-71 is the progeny of crossing lines containing the *Tsw* gene, but

Table 3. *Tomato spotted wilt virus* isolates inducing differential response in *Capsicum annuum* cultivars (2nd trial)

TSWV isolates	Super-umchungna	Ryuckgangsungmunjang	Jungilpum	Bigthree	Docbulwang	Gonchowang	Nocwonfood	Nocrufood	Chungkwang	Saenggreen	Morningfood	Docyachungchung	Gumbit	Chonhajaeil
Gneung	0.078	0.088	0.073	0.082	0.086	0.076	0.072	0.077	0.075	0.099	0.087	0.099	0.072	0.123
Gchang-Kjj	0.086	0.079	0.082	0.071	0.081	0.095	0.099	0.101	0.072	0.082	0.083	0.087	0.087	0.080
Gchang-Njc	2.540	2.583	0.080	3.888	0.085	3.959	3.950	3.927	0.391	2.615	0.100	2.701	3.982	2.935
Ghae	0.225	3.907	0.105	0.124	3.059	2.589	2.489	0.115	3.001	0.120	0.088	3.495	0.102	0.087
Pap	1.606	1.645	3.040	3.022	1.408	0.409	0.187	1.821	1.312	1.241	1.545	1.732	2.380	2.378

^a Absorption at 405 nm by ELISA

Inoculation method: mechanical inoculation, inoculation period: Oct. 18, virus detection period and method: Oct. 27–29.

Table 4. Evaluation of *S. lycopersicum* breeding line TGC09-71 for resistance to TSWV isolates TSWV-Gneung, TSWV-Gchang-Kjj, TSWV-Gchang-Njc, TSWV-Ghae and TSWV-Pap

TSWV isolates	No. of plants infected/inoculated (ELISA+)	Symptom rating ^a			
		0	1	2	3
Gneung	0/12	12	0	0	0
Gchang-Kjj	1/12	11	0	1	0
Gchang-Njc	6/12	6	0	0	6
Ghae	7/12	5	0	0	7
Pap	10/12	2	0	0	10

Inoculation period: Oct. 18, investigation period: Oct. 27–29, Virus infection was determined by ELISA.

^aSymptom rating 0: Not infected, 1: infect without symptom, 2: mild mosaic, 3: stunting and mosaic.

Table 5. Response of three *Nicotiana* species on infection of TSWV

TSWV isolates	<i>N. debneyi</i>	<i>N. benthamiana</i>	<i>N. occidentalis</i>
TSWV-Gneung	-/- ^a	-/-	-/-
TSWV-Gchang-Kjj	-/-	Mo/Mo	Mo/Mo
TSWV-Gchang-Njc	Ne/SNe	Mo/Mo	Ye/Mo
TSWV-Ghae	YS/Mo+ye	Mo/Mo	Ye/Mo
TSWV-Pap	-/-	-/-	-/-

Inoculation period: Oct. 18, investigation period: Oct. 27–29, Virus infection was determined by ELISA.

^ainoculated leaf/upper leaf

Ne: necrosis, SNe: severe necrosis, YS: yellow spot, Mo+ye: mottle with yellowing, Ye: yellowing, Mo: mottle.

it is susceptible as a heterozygote. TGC09-71 plants also showed similar response to the five TSWV isolates as did *C. annuum* (Table 4). Isolate Gneung did not infect any of the plants and isolate Gchang-Kjj infected only one plant, while isolates Gchang-Njc, Ghae and Pap infected six, seven and 10 of 12 plants, respectively.

In the test of three *Nicotiana* species, both isolates Gchang-Njc and Ghae infected *N. debneyi*, *N. occidentalis* and *N. benthamiana* systemically. Isolate Gchang-Kjj infected only *N. benthamiana* systemically (Table 5). Both isolates Pap and Gneung isolates did not infect any of the three *Nicotiana* species.

In conclusion, five TSWV isolates induced different infection spectra in *C. annuum* cultivars, *Nicotiana* species and an *S. lycopersicum* breeding line. This study indicated that various isolates must be used as virus inocula for evaluation of *C. annuum* and *S. lycopersicum* cultivars in breeding programs for TSWV resistance.

The sequencing of the N and NSm genes was done to assess the degree of similarity or difference in the genes likely to contribute to the differences in host range or pathogenicity, as well as the degree of variation between isolates of TSWV and others obtained in previous studies. RNA was isolated from leaf tissue using RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The RT-PCR method and primers were the same as in a previous report (Chung et al., 2006). The nucleotide sequences of the N and NSm genes of the isolates Gneung, Gchang-Kjj, Gchang-Njc, Ghae and Pap were determined using the ABI Prism™ Terminator Cycle Sequencing

Fig. 3. Phylogenetic relationship of amino-acid sequence of Nucleocapsid (N) gene among Korean TSWV isolates including TSWV-Gneung, TSWV-Gchang-Kjj, TSWV-Gchang-Njc, TSWV-Ghae and TSWV-Pap. The scale beneath the tree represents the distance between amino-acid sequences. NCBI GenBank accession numbers are as follows: Ch: DQ453158; KAbal: EF195225; KACTm: EF195226; KALxe: EF195227; KAPo: EF195228; KATm: EF195229; KDRP: EF195230; KP: AB190819; Gchang-Kjj: HQ260980; Gchang-Njc: HQ260981; Nwon: HQ260982; Pap: JF730744; Ghae: HQ260977; Gneung: JF730743; Jmj: HQ260979.

Table 6. The amino-acid sequence percent identity of the N gene among TSWV Korean isolates

TSWV	Ch	KABal	KACTm	KALxe	KAPo	KATm	KDRP	KP	Gchang-Kjj	Gchang-Njc	Nwon	Pap	Ghae	Gneung	Jmi
Ch		100.0	94.6	93.8	94.6	95.0	100.0	94.2	98.1	91.1	93.4	91.9	95.8	92.3	91.9
KaBal			94.6	93.8	94.6	95.0	100.0	94.2	98.1	91.1	93.4	91.9	95.8	92.3	91.9
KACTm				99.2	100.0	99.6	94.6	98.8	92.7	93.4	93.1	94.2	92.7	94.6	91.1
KALxe					99.2	98.8	93.8	98.1	91.9	92.7	92.3	93.4	91.9	93.8	90.3
KAPo						99.6	94.6	98.8	92.7	93.4	93.1	94.2	92.7	94.6	91.1
KATm							95.0	99.2	93.1	93.8	93.4	94.6	93.1	95.0	91.5
KDRP								94.2	98.1	91.1	93.4	91.9	95.8	92.3	91.9
KP									93.8	94.6	93.4	95.4	93.8	95.0	91.5
Gchang-Kjj										90.7	92.3	91.5	95.4	91.1	90.7
Gchang-Njc											89.6	99.2	90.7	98.8	88.4
Nwon												90.3	92.3	90.7	93.4
Pap													91.5	99.6	89.2
Ghae														91.1	90.7
Gneung															89.6

NCBI GenBank accession numbers are as follows: Ch: DQ453158; KABal: EF195225; KACTm: EF195226; KALxe: EF195227; KAPo: EF195228; KATm: EF195229; KDRP: EF195230; KP: AB190819; Gchang-Kjj: HQ260980; Gchang-Njc: HQ260981; Nwon: HQ260982; Pap: JF730744; Ghae: HQ260977; Gneung: JF730743; Jmj: HQ260979.

Ready Reaction Kit and ABI Prism 377 Genetic Analyzer (Perkin Elmer, USA), and was registered under the GenBank accession numbers JF730743, HQ260977, HQ260980, HQ260981 and JF730744 for the N gene, and AB650469, AB663306, AB650470, AB650467 and AB650471 for the Nsm gene, respectively.

The amino-acid sequences of the N gene of the five TSWV isolates were compared to previously reported N gene amino-acid sequences using the Clustal W method of DNASTar ver. 7. They were grouped into five cluster (Fig. 3): The first cluster contained five Korean isolates including AB190819-KP, a TSWV isolate firstly reported in Korea in 2004; the second cluster consisted of isolates Gchang-Njc, Pap and Gneung; the third cluster contained Gchang-Kjj, Ghae and three other Korean isolates; the fourth and the fifth clusters consisted of isolates Nwon and Jmj alone, respectively. All isolates belonging to the first

cluster were the ones isolated in 2005 (Cho et al., 2005; Cho et al., 2009) and were more than 98.1% identical (Table 6). The percent identity of the N gene amino-acid sequences between first cluster TSWV Korean isolate KP and each of the isolates Gchang-Kjj, Gchang-Njc, Nwon (unpublished), Pap, Ghae, Gneung and Jmi (unpublished), isolated in 2010 or 2009 (Table 1), ranged from 91.5 to 95.4% (Table 6). Among the recently collected isolates, Gchang-Kjj showed the closest relationship with 2005 isolates KDRP and KABal (Cho et al., 2005; Cho et al., 2009) and a 2006 isolate Ch (Chung et al., 2006), 98.1% identity in each case (Fig. 3, Table 6). Based on this result, the nucleotide sequence of the N gene of TSWV Korean isolates has notably varied over the last 7 years since the first emergence of TSWV in Korea. Accordingly, since viruses vary or evolve abruptly, virus inocula used for the evaluation of cultivars for resistance to TSWV should be

Fig. 4. Phylogenetic relationship of amino-acid sequence of movement protein (NSm) gene among 5 Korean TSWV isolates TSWV-Gneung, TSWV-Gchang-Kjj, TSWV-Gchang-Njc, TSWV-Ghae and TSWV-Pap. The scale beneath the tree represents the distance between sequences.

Gneung	MLTLFGNKRPSKSAGKDEGPLVSLAKHNGNVEVSKPWSSSDEKLALTKAMDTSGKILLNAEGTSSFGTYESDSITESEG	80
Gchang-Kjj	MLTFFSNKGSSKSAGKDEGPLVSLAKHNGNVEVSKPWSSSDEKLALTKAMDTSGKILLNTEGTSSFGTYESDSITESEG	80
Gchang-Njc	MLTXFGNKGSSKSXRKDEGPLVSLAKHNGNVEVSKPWSSSDEKLALTKAMDTSGKILLNTEGTSSFGTYESDSITESEG	80
Ghae	MLTFFSNKGSSKSAGKDEGPLVSLAKHNGNVEVSKPWSSSDEKLALTKAMDTSGKILLNTEGTSSFGTYESDSITESEG	80
Pap	MLTLFGNKGSSKSTRKDEGPLVSLAKHNGNVEVSKPWSSSDEKLALTKAMDTSGKILLNTEGTSSFGTYESDSITESEG	80
Gneung	YDLSARMIVDTNHHISNWKNDLFVGNKGQNANKVIKICPTWDSRKQYMMISRIVIWCPTIPNPTGKLVVALVDPNMSSE	160
Gchang-Kjj	YDLSARMIVDTNHHISNWKNDLFVGNKGQNANKVIKICPTWDSRKQYMMISRIVIWCPTIPNPTGKLVVALVDPNMSSE	160
Gchang-Njc	YDLSARMIVDTNHHISNWKNDLFVGNKGQNANKVIKICPTWDSRKQYMMISRIVIWCPTIPNPTGKLVVALVDPNMSSE	160
Ghae	YDLSARMIVDTNHHISNWKNDLSVGNKGQNANKVIKICPTWDSRKQYMMISRIVIWCPTIPNPTGKLVVALVDPNMSSE	160
Pap	YDLSARMIVDTNHHISNWKNDLFVGNKGQNANKVIKICPTWDSRKQYMMISRIVIWCPTIPNPTGKLVVALVDPNMSSE	160
Gneung	KQVILKGQGTITDPICFVYFLNWSIPKMNNTPENCCQLHLMCSQEYXKGVSFSGVMYSWTKEFCDSPRADKDKSCMVIPL	240
Gchang-Kjj	KQVILKGQGTITDPICFVYFLNWSIPKINNTPENCCQLHLMCSQEYKGVSFSGVMYSWTKEFCDSPRADKDKNCVVIPL	240
Gchang-Njc	KQVILKGQGTITDPICFVYFLNWSIPKMNNTPENCCQLHLMCSQEYKGVSFSGVMYSWTKEFCDSPRADKDKSCMVIPL	240
Ghae	KQVILKGQGTITDPICFVYFLNWSIPKINNTPESCCQLHLMCNQEYKGVSFSGVMYSWTKEFCDSPRADKDKSCVVIPL	240
Pap	KQVILKGQGTITDPICFVYFLNWSIPKMNNTPENCCQLHLMCSQEYKRVSFSGVMYSWTKEFCDSPRADKDKSCMVIPL	240
Gneung	NRAIRARSQAFIEACKLIIPKGNSEKQIKKQLKELSSNLSRVSEEEEEGISDSVAQLSFDEI	302
Gchang-Kjj	NRAIRARSQAFIEACKLIIPKGNSEKQIKKQLKELSSNLSRVSEEEEEGISDSVAQLSFDEI	302
Gchang-Njc	NRAIRARSQAFIEACKLIIPKGNSEKQIKKQLKELSSNLSRVSEEEEEGISDSVAQLSFDEI	302
Ghae	NRAIRARSQAFIEACKLIIPKGNSEKQIKKQLKELSSNLSRVSEEEEGEGISDSVAQLSFDEI	302
Pap	NRAIRARSQAFIEACKLIIPKGNSEKQIKKQLKELSSNLSRVSEEEEEGISDSVAQLSFDEI	302

Fig. 5. Alignment of *Tomato spotted wilt virus* NSm amino-acid sequences. The alignment was carried out by the Clustal W method of DNASTar ver. 7

renewed.

Phylogenetic analysis of the amino-acid sequence of the NSm gene of the five TSWV isolates was conducted using the Clustal W method of DNASTar ver. 7. Isolates Pap and Gchang-Njc were clustered, while the others were more distantly related (Fig. 4). Amino-acid sequence analysis of the NSm gene could not support or explain the different infection spectra of the five TSWV isolates in the present study. Further detailed studies are needed to determine which motifs are involved in host range determination or symptomatology.

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