SHORT COMMUNICATION

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF *TOMATO SPOTTED WILT VIRUS* ON LETTUCE IN JORDAN

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SUMMARY

Tomato spotted wilt virus (TSWV) was isolated from naturally infected lettuce grown in Jordan. This virus isolate (TSWV-J) was identified based on transmission studies, host range reaction, and serological tests. RT-PCR was used for the detection of TSWV in infected plant tissues. Total RNA isolated from infected lettuce plants was subjected to RT-PCR using primers specific to the nucleocapsid (N) gene of TSWV and the amplified 620 bp product was cloned and sequenced. Nucleotide sequence and phylogenetic analysis of the RT-PCR amplicon confirmed the Jordanian virus as an isolate of TSWV. Furthermore, the genetic diversity of TSWV in Jordan was investigated. The TSWV isolates shared high nucleotide sequence identity (93-99%) with those from other countries. To our knowledge, this is the first report of TSWV infection on lettuce in Jordan.

Key words: lettuce, ELISA, host range, RT-PCR, TSWV.

Lettuce (*Lactuca sativa* L.) is one of the most economically important vegetable crops in Jordan, where is grown on 1,590 ha with an annual production of nearly 39,753 tons (Anonymous, 2009). Diseases, especially those induced by viruses, are the major problems of lettuce, limiting production in Jordan and elsewhere (Al-Musa and Mansour, 1985; Moreno *et al*., 2004; Fletcher *et al*., 2005). A previous virus survey in Jordan had detected *Lettuce mosaic virus* (LMV) (Al-Musa and Mansour, 1985) on lettuce plants. However, in the 2008-2009 growing season, symptoms were observed on lettuce crops of the Jordan Valley, reminiscent of those induced by *Tomato spotted wilt virus* (TSWV) (genus *Tospovirus*, family *Bunyaviridae*), which had already been recorded in Jordan on tomato (Anfoka *et al*., 2006). TSWV is one of the most widespread and economically important pathogens (Yardimci and Kilic, 2009) and possesses one of the largest host range of any plant virus, with more 1090 plant species in over 100 families (Parrella *et al*., 1993; Hanssen *et al*., 2010). The virus is transmitted in a persistent manner and replicates in its thrips vectors, two properties that favour its dispersal (Ullman *et al*., 2002; Whitefield *et al*., 2005).

Since the areas cultivated with lettuce in Jordan are expanding and the information on lettuce viruses is very limited, a study was conducted to identify and partially characterize the causal agent of a severe lettuce disease frequently seen in lettuce crops in the Jordan Valley. In the course of this study, the sequence divergence of the N gene of the TSWV isolates from lettuce was investigated and compared with that of isolates from different geographical locations in the world.

Nineteen lettuce plants showing leaf curling, stunting, severe necrosis, and incomplete head formation were selected from lettuce fields in the Jordan Valley and tested for the presence of LMV, *Cucumber mosaic virus* (CMV), *Broad bean wilt virus* (BBWV) and TSWV by DAS-ELISA using commercially available kits (Bioreba, Switzerland).

Ten samples that were ELISA-negative for BBWV, CMV and LMV but positive for TSWV were tested by mechanical inoculation to *Chenopodium quinoa*, *Datura stramonium, Nicotiana benthamiana*, *N. glutinosa* and lettuce plants. Leaf tissues from symptomatic lettuce plants were triturated with mortar and pestle in 0.01 M potassium phosphate buffer pH 7.0, containing 0.01 M sodium sulfite and inoculated by rubbing the first true leaves of the above mentioned plants previously dusted with 600-mesh Carborundum. All lettuce plants inoculated with sap extracted from suspected TSWV-infected lettuce reacted with symptoms identical to those observed in the field. One TSWV isolate, denoted TSWV-J, obtained from an infected lettuce was selected as a representative of field isolates and used for host range and thrips transmission studies.

TSWV-J was mechanically inoculated onto five plants each of 16 herbaceous hosts of five botanical families. Five mock-inoculated plants of each species served as controls. All plants were grown in insect proof cages at 25°C under a light regime of 16 h (43-48 µmol m^{-2} s^{-1}). They were examined regularly for symptom de-

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velopment and were assayed for TSWV infection by ELISA and sap inoculations to *C. quinoa* and *D. stramonium*.

Results of host range studies showed that TSWV-J has a wide experimental host range. All inoculated plants, but not the controls, became infected and showed symptoms 4 to 14 days post inoculation. TSWV infection was confirmed by ELISA in all plants and back indexing on *C. quinoa* and *D. stramonium*. Whereas only chlorotic/necrotic local lesions were produced in *C. amaranticolor*, *C. quinoa*, *Cucumis melo*, *C. sativus*, *Gomphrena globosa* and *Petunia hybrida,* all the other indicators reacted with local and systemic symptoms consisting of chlorotic mottling, mosaic, yellowing, veinal necrosis, malformation, stunting, necrotic spots and ringspots, wilting and death of the plants (Table 1).

Frankliniella occidentalis Pergande, one of the major thrips species found in Jordan and the most important vector of TSWV (Ullman *et al*., 2002; Whitefield *et al*., 2005), was tested for its ability to transmit TSWV-J. Adult thrips were collected from citrus flowers and introduced onto TSWV-infected *D. stramonium* plants at the four-leaf stage, kept in an insect proof cage. Two weeks later, 10 healthy plants of lettuce, pepper and tomato were transferred to the same cage and observed for symptom appearance. Lettuce, pepper and tomato plants not exposed to thrips served as controls. TSWV-J infection was confirmed by ELISA.

Typical TSWV symptoms developed in all lettuce, pepper and tomato plants following exposure to *F. occidentalis* that had access to TSWV-J-infected *D. stramonium* plants, whereas none of the 10 control plants became infected. TSWV was detected by ELISA in all symptomatic plants. In addition to biological and serological assays, RT-PCR was used successfully for virus detection. It was conducted according to Adkins and Rosskopf (2002), using primers designed to amplify a fragment (620 bp) of the virus nucleocapsid (N) gene: 5'- GCTGGAGCTAAGTATAGCAGC-3' and 5'-CACAAGGCAAAGACCTTGAG-3' (Operon Technolo-

Table 1. Reaction of host plant species to infection with *Tomato spotted wilt virus*, TSWV-J from lettuce.

Plant family and species	Symptoms produced[a]		Back indexing[b] ELISA
	Inoculated leaves	Non inoculated leaves	
Amaranthaceae			
Gomphrena globosa	NS	-	-
Chenopodiaceae			
Chenopodium amaranticolor	CLL	-	-
C. quinoa	CLL	-	-
Compositae			
Lactuca sativa cv. Paris Island cos	-	MM, NS, SN	+
Cucurbitaceae			
Cucumis melo cv. Ananas	NS	-	-
C. sativus cv. Beit Alpha	CS	-	-
Solanaceae	NS, PD, VN	MM, NS, S	+
Capsicum annum cv. Anaheim Chilli	NS		
Datura stramonium	NS	CS, LD, MM	+
Lycopersicon esculentum cv. Marmande		LB, MM, NS, S, VN	+
Nicotiana benthamiana	NS	LD, MM	+
N. glutinosa	NS		
N. occidentalis	NS	C	+
N. megalosiphon	NS	LB, LD, NS	+
N. tabacum cv. White Burley	NS	Y	+
Petunia hybrida	NS	LD, NS,VN	+
Solanum melogena cv. Black Beauty	NS	-	-
		LD	+

[a] C = chlorosis, CLL = chlorotic local lesions, CS = chlorotic spots, LB = leaf bronzing, LD = leaf death, MM = mosaic and mottling, NS = necrotic spots, PD = premature drop, S = stunting, SN = severe necrosis, VN = vein necrosis, Y = yellowing.
[b] + = virus detected, - = virus not detected.

gies, USA). RT-PCR products were analyzed in 1.0% (w/v) agarose gel electrophoresis in Tris-acetate-EDTA buffer and visualized after ethidium bromide staining with a UV transilluminator. RT-PCR products were recovered and purified using Wizard DNA Clean-up System kit (Promega, USA) and ligated to pGEM-T Easy (Promega, USA). Plasmids were transformed into *Escherichia coli* JM109 according to the manufacturer's instructions. Recombinant colonies were screened and their plasmids were isolated using the Pure Yield Plasmid Miniprep System kit (Promega, USA), digested with *Eco*RI (New England Biolabs, USA) to determine insert size and analyzed using agarose gel electrophoresis. Nucleotide sequences were obtained by Macrogen (Korea) from two cDNA clones using a 3730 XL DNA automated Sequencer and were analyzed using sequence analysis and data management software from Invitrogen, Vector NTI Advance™ 10 (InforMax, USA).

The RT-PCR successfully amplified a DNA fragment of the expected size from TSWV-infected lettuce, but not from the healthy controls. Sequence analysis of the TSWV-J clone (GenBank accession No. HQ630626) revealed 99% identity with the corresponding N gene of a number of TSWV isolates.

The phylogenetic relationship of TSWV-J and additional 10 isolates recovered in 2011 from separate let-

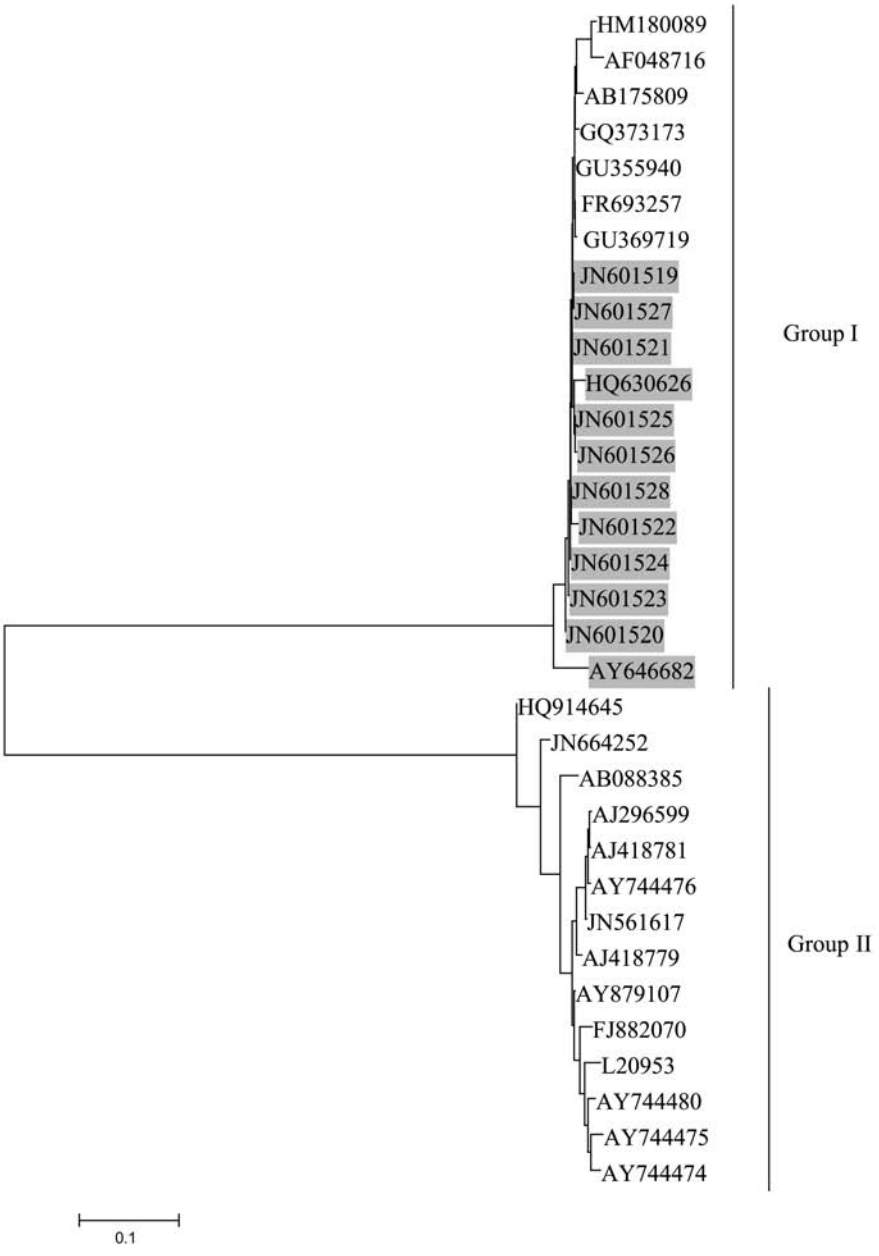

Fig. 1. Phylogenetic relationships between TSWV Jordanian isolates and a selection of other known TSWV isolates based on the partial nucleotide sequence of the N-gene (620 bp). Tree was constructed with MEGA2. The bar represents substitution per site. Jordanian isolates are shaded in grey.

tuce fields, was also studied in comparison with sequences of other strains retrieved from GenBank. All Jordanian isolates were preliminarly tested for confirming their TSWV nature by DAS-ELISA and RT-PCR and the amplified products were cloned and sequenced as described previously. Sequences were aligned with ClustalX (Thompson *et al*., 1997), neighbour-joining (NJ) phylogenies, based on Kimura 2-parameter distance matrix were generated by MEGA2 software (Kumar *et al*., 2001) and bootstrap confidence limits were obtained by 1000 replicates.

The details of the sequences used in the analysis are shown in Table 2. Our study showed 96-99% identity in the N gene sequences of TSWV isolates from naturally infected lettuce from the Jordan Valley. Although all TSWV isolates used in the comparison revealed a nucleotide sequence identity greater than 93%, a distinct evolutionary clustering pattern was observed (Fig. 1). The phylogenetic tree based on N gene sequences revealed two distinct groups of isolates. All those from Jordan belonged to group I, together with isolates from other countries (France, Georgia, Italy, Korea, Montenegro, Serbia, Taiwan), whereas all American isolates were comprised in group II, with isolates from Australia, Brazil, Bulgaria, China, Germany, Czech Republic, Japan, Spain and Syria. The observed clustering pattern is in accordance with the findings of Sivparsad and Gubba (2008), who tentatively designated the two clusters as "European" and "American". On the other hand, the comparison of the TSWV isolates sequenced in this study and the Jordanian strain from tomato (Anfoka *et al*., 2006) revealed 93% nucleotide identity, which is indicative of a genetic differentiation between tomato and lettuce isolates. Further experiments are needed to investigate the variability of TSWV populations that will be useful in the formulation of control strategies using genetic engineering approaches (Tsompana *et al*., 2005). To this aim, a project has been initiat-

Table 2. GenBank accession numbers, host and geographic origin of *Tomato spotted wilt virus* isolates used in phylogenetic analysis.

GeneBank accession no.	Host	Origin	Reference
FR693257	*Stellaria media*	France	Tentchev *et al*., 2011
GU355940	*Primula* sp.	Montenegro	Unpublished
GU369719	Lettuce	Italy	Unpublished
AY744476	Dahlia	North Carolina	Tsompana *et al*., 2005
AJ418781	*Lysimachia* sp.	Germany	Heinze *et al*., 2003
GQ373173	Tobacco	Serbia	Stankovic *et al*., 2011
AY879107	Tomato	Australia	Unpublished
AJ418779	Tomato greenhouse	Bulgaria	Heinze *et al*., 2003
AB175809	Paprika	Korea	Unpublished
FJ882070	Potato	Texas	Crosslin *et al*., 2009
HM180089	Pepper	Taiwan	Unpublished
AB088385	-	Japan	Takeda *et al*., 2002
AY646682	Tomato	Jordan	Anfoka *et al*., 2006
AJ296599	-	Czech Republic	Heinze *et al*., 2001
AY744480	Tomato	Spain	Tsompana *et al*., 2005
AY744474	Dahlia	California	Tsompana *et al*., 2005
AY744475	Falso lulo	Colorado	Tsompana *et al*., 2005
L20953	Tobacco	Hawaii	Pang *et al*., 1992
AF048716	Pepper	Georgia	Unpublished
HQ914645	Lettuce	Brazil	De Haan *et al*., 1989
JN664252	Lettuce	China	Unpublished
JN561617	Pepper	Syria	Unpublished
HQ630626	Lettuce	Jordan	This study
JN601519	Lettuce	Jordan	This study
JN601520	Lettuce	Jordan	This study
JN601521	Lettuce	Jordan	This study
JN601522	Lettuce	Jordan	This study
JN601523	Lettuce	Jordan	This study
JN601524	Lettuce	Jordan	This study
JN601525	Lettuce	Jordan	This study
JN601526	Lettuce	Jordan	This study
JN601527	Lettuce	Jordan	This study
JN601528	Lettuce	Jordan	This study

-: host unknown

ed to determine the genetic diversity among TSWV isolates from different crops in Jordan by sequencing parts of the three RNA genome segments.

Based on positive ELISA reactions, symptomatology and phylogenetic patterns, it can be concluded that TSWV is one of the viruses detrimental to lettuce in the Jordan Valley. The warm dry weather characterizing most growing areas has facilitated the increase of thrips populations which, together with the recurring planting of susceptible crops, appear to favour TSWV outbreaks (Cho *et al*., 1987). Further studies are currentlly underway to understand the epidemiology of TSWV in the Jordan Valley in order to develop feasible control strategies.

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