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CHARACTERIZATION OF A NEW TOMATO SPOTTED WILT VIRUS ISOLATES FOUND IN *HIPPEASTRUM HYBRIDUM* (HORT.) PLANTS IN POLAND

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ABSTRACT

Two Tomato spotted wilt virus (TSWV) isolates H1 and H2 found in *Hippeastrum hybridum* plants were characterized based on biological, serological, and molecular properties. Virus isolates showed differences in symptom expression – H1 isolate displayed severe necrotic spots and patterns, whereas mild mosaic symptoms were observed on H2-infected *H. hybridum* plants. Both TSWV isolates showed comparable reactivity with TSWV-specific antibodies and they induced similar symptoms on herbaceous indicator plants, but some differences between these isolates were detected at the nucleotide sequence level of genomic S and M ssRNAs segment fragments. The nucleotide sequences encoding nucleocapsid (N) and nonstructural (NSs and NSm) proteins showed 98.2%, 97.5%, and 96.5% identity, respectively. Phylogenetic analysis of N and NSs sequences conducted for tested isolates and 31 TSWV isolates included for comparison revealed that H1 and H2 isolates fell into the same cluster and they were grouped together with isolates found previously in different vegetables, ornamentals, and weeds. When NSm ORF was analyzed, the tested isolates formed a separate cluster: H1 isolate showed the highest affinity with TSWV isolates infecting chrysanthemum and pepper plants, whereas H2 isolate was most closely related to other virus isolates found in sweet pepper and tomatoes. These results indicate that both isolates were reassortants between different virus isolates, and represented two novel genetic patterns of TSWV.

Key words: TSWV, properties, phylogenetic analysis, reassortment

INTRODUCTION

Tomato spotted wilt virus (TSWV) is a member of the *Tospovirus* genus in the family *Bunyaviridae* (Goldbach & Peters 1996). The virus genome consists of three negative or ambisense ssRNAs designated S (~2.9 kb), M (~4.8 kb), and L (~8.9 kb). The ambisense S RNA encodes a 52.4-kDa nonstructural protein (NSs) acting as a suppressor of gene silencing in the viral (v) sense and the 29-kDa nucleocapsid (N) protein in the viral complementary (vc) sense (de Haan et al. 1990). The M RNA encodes a 33.6-kDa viral movement protein (NSm) in the v sense and a 127.4-kDa precursor to G1 and G2 (envelope membrane glicoproteins) in the vc sense (Kormelink et al. 1992a). The 8.9-kb L RNA has a completely negative sense and contains a single ORF in the vc sense encoding the 331-kDa putative RNA-dependent RNA polymerase (Kormelink et al. 1992b).

TSWV is transmitted from one plant to another by several thrips species (Thysanoptera: Thripidae). Only larval thrips can acquire TSWV, while both larval and adult thrips transmit the virus (Riley et al. 2011). The TSWV host range exceeds 1100 plants species, including several horticultural and agronomic crops across temperate, subtropical, and tropical regions of the world. Major crops susceptible to TSWV infection are tomato, pepper, lettuce, potato, papaya, peanut, tobacco, and chrysanthemum (German et al. 1992).

In Poland, TSWV has been known since 1950s as a pathogen of tobacco and tomato grown in the open fields (Zawirska et al. 1983). In 1990s,

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the outbreak of TSWV was reported on 37 greenhouse crops, which was related to introduction of an effective virus vector *Frankiniella occidentalis* (Kamińska 1992, 1994; Kamińska & Korbin 1994). The most severely affected species were tomato, sweet pepper, gerbera, and chrysanthemum (Kamińska 1992). Over the last decade, TSWV has occurred in ornamental crops sporadically; however, since 2012, exacerbation of TSWV-associated symptoms on chrysanthemum in Polish nurseries and plantations has been recorded and number of virus findings increases substantially every year (Berniak 2014). On *Hippeastrum* plants, TSWV was detected for the first time in 1989 (Kobyłko & Bach 1989). Plants affected with this isolate showed severe leaf mosaic with sharply delimited pale yellow and dark green spots. A survey of *Hippeastrum* plants for virus diseases done in 1990s confirmed the presence of TSWV in this ornamental plant species in Poland. However, the virus was detected sporadically and its influence on plants growth was small (Kamińska 1992). Virus isolates found previously in *H. hybridum* in Poland were characterized based on results of bio-indexing and *in vitro* properties in infected plant sap, but there is no reports on other properties and characteristics of these TSWV isolates.

The present paper describes some biological and serological properties, as well as molecular analyses and phylogenetic relationships of two TSWV isolates showing differences in symptom expression in *Hippeastrum hybridum* plants.

MATERIALS AND METHODS

Plant source material and virus isolates. Naturally infected *Hippeastrum hybridum* plants showing different virus-like symptoms were observed during the inspection of three commercial ornamental plants farms located in central Poland in 2014. Twenty diseased plants were collected and maintained in the greenhouse of Research Institute of Horticulture, Skierniewice. Two representative virus isolates H1 and H2 with differential severity of disease expression on *H. hybridum* plants were selected and further characterized. These isolates were mechanically transmitted using inocula prepared by grinding of infected leaves in cold 0.02 M phosphate buffer (pH 7.6) with 1% sodium sulfite. For biological test, the following herbaceous plant species were used: *Chenopodium quinoa*, *Cucumis sativus* cv. Kronos, *Nicotiana rustica*, *N. clevelandii*, *N. glutinosa*, *N. tabacum* cv. Samsun, *Petunia hybrida* cv. Pink Beauty and *Tropaeolum majus*. All tested plant species were grown in environmentally controlled greenhouse at 20-25 °C with supplementary lighting. Test plants were observed up to four weeks after inoculation.

Virus indexing. Samples of leaves of naturally infected *H. hybridum* and experimentally inoculated herbaceous test plants were tested for the presence of four viruses: Tomato spotted wilt virus (TSWV), Cucumber mosaic virus (CMV), Impatiens necrotic spot virus (INSV), and Hippeastrum mosaic virus (HiMV), using ELISA or RT-PCR assays.

Commercial ELISA kits containing immunoglobulines and alkaline phosphatase conjugates against TSWV were obtained from Loewe Biochemica, Germany (cat. no. 07501) and DSMZ, Germany (cat. nos. AS-105 and AS-106/1+116/1). The TSWV-specific IO kit produced in Research Institute of Horticulture, Poland (Kamińska & Korbin 1994), was included in the research.

ELISA sets against CMV and INSV were obtained from Loewe Biochemica, Germany (cat. nos. 07108C and 07505C, respectively). Detection of HiMV was carried out by RT-PCR employing HiMV-specific primers described by Malandraki et al. (2015). All tests were carried out according to the manufacturers' instructions.

Isolation of nucleic acids and amplification of TSWV genome segments by RT-PCR. Total nucleic acids (TNA) were isolated from approximately 300 mg of virus infected and healthy leaves by using the silica capture method described originally by Boom et al. (1990) and adapted to the diagnosis of plant viruses by Malinowski (1997). One microliter of TNA was used for reverse transcription-polymerase chain reaction (RT-PCR) in total volume of 10 µl. Amplification was performed using Titan One Tube RT-PCR System (Roche Diagnostics, Poland) according to manufacturer's recommendation. Specific oligonucleotides used in RT-PCRs were: SIs, SIa, SIIs, and SIIs (Naidu et al. 2008)

for amplification of NSs coding region, MIs and MIa (Naidu et al. 2008) for amplification of NSm ORF, and TSW1, TSW2 (Dietzgen et al. 2005) for amplification of N gene fragments. In order to amplify the complete nucleocapsid protein gene, additional primers were designed. These included a reverse primer S2262R (5'-ATGACACCAGAGAAGCCTTAGG-3') and two forward primers S1036F (5'-CACACAAACYATGTCTTACTTGG-3') and S2518F (5'-AGCTATCAAGCCTTCTGAAGGTC-3').

The PCR products (5 µl) were separated on 1% TBE-agarose gels and visualized under UV light after staining with ethidium bromide.

Sequence analyses. Sequencing of amplicons was performed using the same primers as for RT-PCRs in AbiPrism 3100 Genetic Analyzer apparatus (Applied Biosystems, USA), in Maria Skłodowska Memorial Cancer Center and Institute of Oncology, Warsaw, Poland. The assembled nucleotide sequences were analyzed and their identities were determined using Lasergene v. 7.1 software package (DNASTAR, USA). A comparison of obtained cDNA fragments with sequences available in GenBank was accomplished using BLAST algorithm (http: http://www.ncbi.nlm.nih.gov:80/BLAST/). For phylogenetic analysis, Lasergene v. 7.1 software was used to perform sequence editing and compilation. The neighbor-joining method implemented in MEGA version 5.0.5 (Tamura et al. 2011) was used to infer the tree topologies based on nucleotide sequences. Branch support was calculated with 1000 bootstrap replicates. The corresponding nucleotide sequences of 31 other TSWV isolates (for which both S and M segment nucleotide sequences were available) were included in the study and are indicated by GenBank accession numbers in Table 1.

RESULTS

Symptoms. *H. hybridum* plants harboring H1 or H2 isolates showed different leaf symptoms. Plant infected with H1 isolate initially displayed single elliptic chlorotic spots and rings (Fig. 1a). Along with the plant growth increase, symptoms became more severe – yellow rings turned into necrotic line patterns and they have spread on entire leaves (Fig. 1b). The plant infected with H2 isolate exhibited mild light-green to yellow mosaic symptoms on leaves throughout the growing season (Fig. 1c).

Biological test. Symptoms indicating TSWV infection occurred in all species of herbaceous plants infected with isolates H1 or H2. These symptoms were similar and they included:

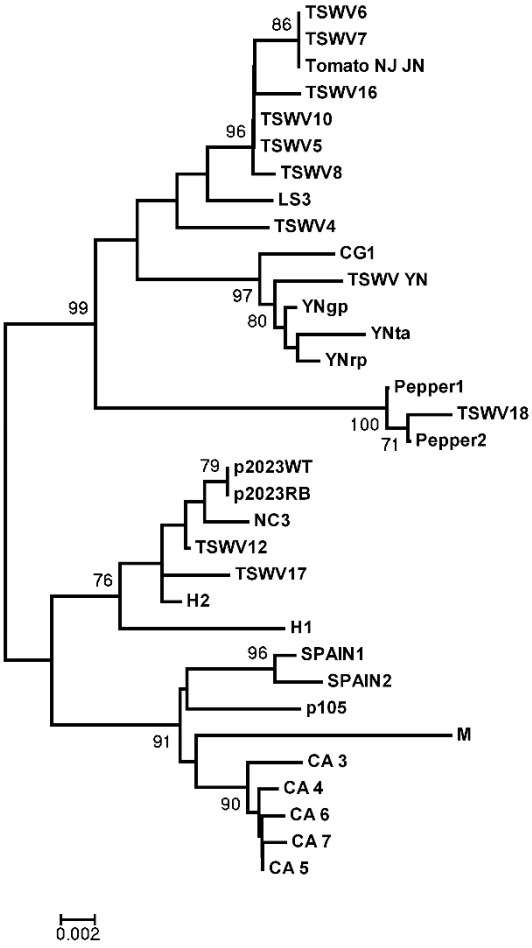
- on *C. quinoa* plants – chlorotic local lesion followed by systemic lesion
- on *Nicotiana* spp. – local necrotic spots; systemic symptoms included chlorotic patterns and leaves deformation
- on *P. hybrida* plants – local, extensive necrotic patches; no systemic symptoms
- on *C. sativus* – local small chlorotic spots; no systemic symptoms
- on *T. majus* – no local symptoms; systemic chlorotic spots and patterns.

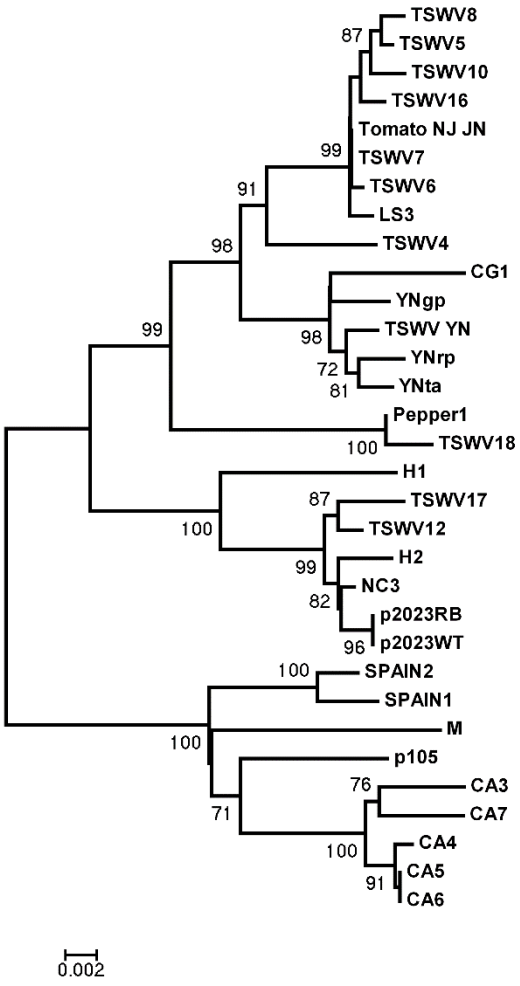
Fig. 1. Leaves of *Hippeastrum hybridum* plants naturally infected with Tomato spotted wilt virus: primary (A) and later (B) symptoms associated with H1 isolate infection; (C) symptoms on the plant infected by H2 isolate.

Table 1. TSWV isolates used for sequence analyses

Isolate name	GenBank accession number		Original host	Geographical location
	S segment	M segment		
TSWV-YN	JF960235	JF960236	tomato	China
M	AY870391	AY870390	ND*	USA
TSWV-4	KC261949	KC261948	pepper	South Korea
TSWV-5	KC261952	KC261951	water chickweed	South Korea
TSWV-6	KC261955	KC261954	chickweed	South Korea
TSWV-7	KC261958	KC261957	pepper	South Korea
TSWV-8	KC261961	KC261960	Indian lettuce	South Korea
TSWV-10	KC261964	KC261963	water chickweed	South Korea
TSWV-12	KC261967	KC261966	lettuce	South Korea
TSWV-16	KC261970	KC261969	tomato	South Korea
TSWV-17	KC261973	KC261972	chickweed	South Korea
TSWV-18	KC261976	KC261975	chrysanthemum	South Korea
Pepper1 CY-CN	HM581939	HM581938	pepper	South Korea
Pepper2 CY-CN	HM581942	HM581941	pepper	South Korea
LS3	KM076653	KM076652	Siberian motherwort	South Korea
CG-1	JN664252	JN664253	lettuce	China
CA-3	AY744470	AY744481	chrysanthemum	USA, CA
CA-4	AY744471	AY744482	chrysanthemum	USA, CA
CA-5	AY744472	AY744483	chrysanthemum	USA, CA
CA-6	AY744473	AY744484	chrysanthemum	USA, CA
CA-7	AY744474	AY744485	dahlia	USA, CA
SPAIN-1	AY744479	AY744492	tomato	Spain
SPAIN-2	AY744480	AY744493	tomato	Spain
p105	DQ376178	KJ575621	pepper	Italy
p202/3RB	HQ830186	HQ830185	pepper	Italy
p202/3WT	HQ830187	HQ830188	pepper	Italy
Tomato NJ-JN	HM851936	HM581935	tomato	South Korea
YNgp	KM657116	KM657119	pepper	China
YNta	KM657115	KM657118	tobacco	China
YNrp	KM657114	KM657117	pepper	China
NC-3	AY744478	AY744486	dahlia	USA, NC
H1	KU308368 KU308376	KU308372	hippeastrum	Poland
H2	KU308369 KU308377	KU308373	hippeastrum	Poland

* ND – no data





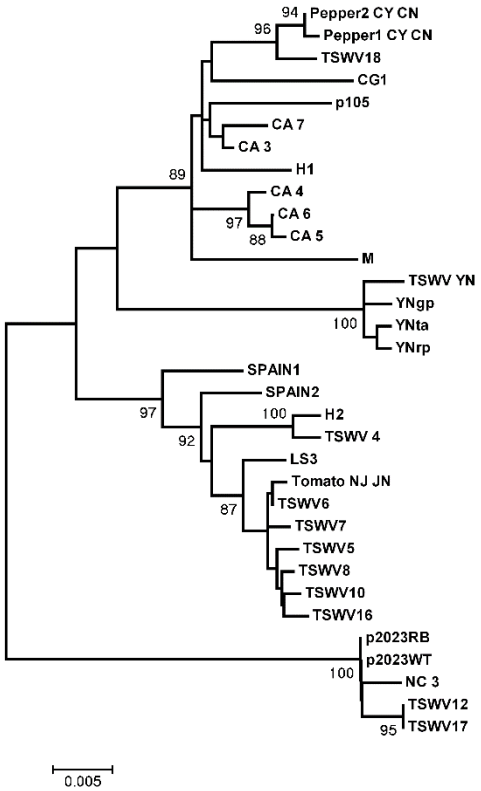


Fig. 2. Phylogenic analysis of the nucleotide sequences of the N (A), NSs (B) and NSm coding region (C) of TSWV isolates. The trees were created by the neighbor-joining method using the MEGA 5.0.5 package. The percentages of bootstrap replicates, based on 1000 bootstrap samples, are placed at the tree nodes (only values above 70%). The bar scale indicates the rate of substitution per base.

Virus detection. All of the tested *H. hybridum* and inoculated herbaceous plant samples reacted with antibodies 07501 against TSWV. The serological properties of the selected virus isolates H1 and H2 were further analyzed with three other TSWV-specific ELISA kits (AS-105, AS-106/1+116/1 and IO). The virus isolates reacted with all antibodies against TSWV used in the experiment. No significant differences were observed in absorption readings obtained in the DAS- or TAS-ELISA assays for both virus isolates, which indicates that that H1 and H2 isolates display similar serological characteristics.

Neither CMV, INSV nor HiMV were detected in the tested plants.

Sequence identity between TSWV isolates. Fragments of TSWV M and S RNA segments, containing the complete nucleotide sequences of coding regions of the nucleocapsid (N), movement (NSm), and nonstructural (NSs) proteins, have been deposited in GenBank under accession numbers: KU308368, KU308372, KU308376 for H1 isolate and KU308369, KU308373, and KU308377 for the H2 isolate. The sizes were identical for the corresponding three ORFs of the two TSWV isolates H1 and H2. The full nucleocapsid protein ORF was 777 bp, NSm ORF 909 bp, and NSs ORF 1404 bp long and they encoded a 258 aa, 302 aa, and 467 aa proteins, respectively.

A detailed analysis of the three ORFs revealed a relatively high nucleotide sequence identity between H1 and H2 isolates. The tested isolates shared 98.2% identity of N ORF sequences, 97.5% of NSs coding region, and 96.5% nucleotide sequence identity of NSm ORF. The percentage of nt identities between H1, H2, and 31 other TSWV isolates (Table 1) retrieved from GenBank and included for comparison ranged from 94.2% to 99.6% (data not shown).

Phylogenetic analysis. The results of phylogenetic analysis of three genomic regions between *Hippeastrum*-infecting and other TSWV isolates are shown in Fig. 2. For N and NSs genes, tested H1 and H2 isolates formed the independent clades with TSWV isolates found in pepper, lettuce, dahlia, and weeds (GenBank accession numbers HQ830186, HQ830187, KC261967, AY744478, KC261972), supported by 68% (for N ORF) and 99% (for NSs ORF) bootstrap values (Fig. 2 a, b). Interestingly, analysis of NSm ORF revealed that H1 and H2 isolates fell into two distinct clusters, and showed affinity to a completely different TSWV isolates (Fig 2c). Sequence of H1 isolate fell into the clade composed mainly with TSWV isolates affecting chrysanthemum and pepper plants (acc. nos. AY744481-84, KC261975, HM581938, HM581941, KJ575621). The bootstrap values of branches dividing these isolates into subclusters were below 50%, indicating that subdivisions were generally not well supported. H2 isolate was most closely related to isolate TSWV4 found in sweet pepper, and somehow distantly related to virus isolates affecting tomato and weeds (AY744492-93, KC261951, KC261969 and others).

DISCUSSION

A few viruses have been reported worldwide to cause foliar symptoms in *Hippeastrum* sp. plants. An irregular light and dark green mosaic patterns are usually associated with *Hippeastrum mosaic virus* (HiMV) (Duarte et al. 2009; Raj et al. 2009) whereas yellow concentric ring and line patterns are associated with Cucumber mosaic virus (CMV) or Tomato spotted wilt virus (TSWV) (Derks 1995). In this report, we presented characteristics of two virus isolates associated with different symptoms expression on *H. hybridum* plants. The plant naturally infected with H1 isolate showed severe chlorotic, and then necrotic patterns, similar to those previously described on TSWV-affected *Hippeastrum* plants (Derks 1995; Kobyłko & Bach 1989). In contrast, mild mosaic symptoms observed on H2-infected plants indicated HiMV infection. However, despite differences of observed disease symptoms, both tested plants were found to be infected with TSWV alone. It has been reported that this virus display a considerable degree of biological diversity – symptoms of TSWV infection differ among hosts and can be variable in a single host species (Qiu et al. 1998; Mandal et al. 2006).

Both tested TSWV isolates showed comparable serological properties and they induced similar symptoms on herbaceous indicator plants, but some differences between these isolates were detected at nucleotide sequence level of genomic S and M RNA segment fragments. To date, no sequence information on

TSWV found in *Hippeastrum* sp. plants has been reported, thus we cannot conclude whereas these differences are characteristic for TSWV isolates affecting *H. hybridum*.

Generally, sequence identity of all virus isolates compared in this study was relatively high, which is typical for TSWV. Genetic diversity of TSWV isolates collected from diverse plant species and different geographical localizations has been the subject of intense study in recent years. Most of these studies, utilizing the N gene sequence to assess genetic variability, revealed that this genome fragment is highly conserved among TSWV isolates worldwide (de Ávila et al. 1993; Dietzgen et al. 2005; Pappu et al. 1998).

In our research, we analyzed genetic diversity of TSWV isolates based on three ORFs, and we observed that the NSm ORF sequence identity was slightly lower (96.5%) in relation to two other tested N and NSs ORFs (98.2% and 97.5%, respectively). Moreover, the cluster dendrogram of NSm sequences was distinct from those obtained for N and NSs sequences. It was demonstrated that two analyzed genome segments showed affinity to different TSWV isolates, thus, collectively, phylogenetic analyses indicated that H1 and H2 isolates might be a reassortants between different TSWV isolates. Reassortment (genetic shift) is considered to be an important mechanism for generating genetic variation of TSWV isolates (Qiu et al. 1998). Since the virus occurs in plants as a heterogeneous mixture of variants, during coinfection, isolates can exchange genome segments with one another, and this mechanism allows the virus to adapt rapidly to new hosts and environments (Moyer 1999). It has been reported that the S RNA segments of certain isolates are preferentially exchanged (Qiu et al. 1998). However, our results indicate that reassortment can also occur between M RNA segments.

It remains unclear whether observed molecular differences have an impact on the variability of symptoms observed on *H. hybridum* plants. Literature data suggest that determinants responsible for symptoms development are located on the S RNA segment (Margaria et al. 2007). The NSs protein located in this segment, interfering with the antiviral RNAi or RNA silencing response in plants and arthropod cells, has been identified as a virulence factor in both plant host and insect vector. However, in our research, the analyzed S RNA segment regions were similar for tested H1 and H2 isolates. Thus, it is more likely that the NSm protein located on the M segment can be involved in disease symptoms expression in *H. hybridum* plants. This protein has been shown to display several activities during viral infection in the plant host, including cell-to-cell movement and long distance movement (Eifan et al. 2013), but its involvement in disease symptom development also has been suggested (Prins et al. 1997). Overall, these data suggest that unique genomic regions of TSWV are involved in disease symptom expression, thus further studies are needed to examine differences in gene functions and virulence determinants between virus isolates.

In conclusion, this is the first report of sequence analysis of TSWV isolates found in *H. hybridum* plants. Described isolates can be putative reassortants, and they represent a unique genetic pattern of TSWV.

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