



## First record of *Tomato chlorotic spot virus* in the USA

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### ABSTRACT

A tospovirus was identified in tomato plants from two counties in Florida by reverse transcription-PCR and sequencing of portions of the S, M and L genomic segments. The predicted amino-acid sequences of the N protein of PCR products from four plant samples were >96% identical to those of TCSV. Partial nucleic acid sequences of the L and M RNA were >97% identical to those reported for TCSV isolates. Extracts from field samples infected test plants and produced symptoms similar to those reported for TCSV. This is the first report of an isolate of TCSV in Florida and in the USA.

**Key words:** *Bunyaviridae*, *Solanum lycopersicum*, South Florida, Tospovirus.

Viruses in the genus *Tospovirus*, family *Bunyaviridae* are plant pathogens transmitted by thrips (*Thysanoptera: Thripidae*), with a tri-segmented ambisense RNA genome and the ability to replicate in thrips as well as plants. The diversity of the genus comprises 11 recognized species with a variable host range and geographic distribution, with an additional 15 proposed species (de Oliveira et al., 2012; Hassani-Mehraban et al., 2010; King et al., 2012). Species classification is based on serology, host range and nucleotide and predicted amino acid sequence of the N protein, which is located on the S RNA segment. Based in these criteria, tospoviruses have also been differentiated into American and Asiatic clades (de Oliveira et al., 2012; Hassani-Mehraban et al., 2010).

There are five tospoviruses reported from the USA: *Groundnut ring spot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), *Iris yellow spot virus* (IYSV), Soybean vein necrosis associated virus (SVNaV), and *Tomato spotted wilt virus* (TSWV) (Pappu et al., 2009; Webster et al., 2010; Zhou et al., 2011). The first three viruses belong to the American clade whereas IYSV belongs in the Asiatic clade. None of these viruses are native to North America and were likely introduced (Pappu et al., 2009). An isolate of GRSV, GRSV-L<sub>G</sub>M<sub>T</sub>S<sub>G</sub>, is a reassortant between GRSV and *Tomato chlorotic spot virus* (TCSV), and was reported from tomato in Florida in 2010 (Webster et al., 2011). This isolate is composed of the L and S RNA segments from GRSV but an M RNA more closely-related to that of TCSV than GRSV. GRSV-L<sub>G</sub>M<sub>T</sub>S<sub>G</sub> was proposed to have originated in an area where both TCSV and GRSV occur since neither virus had been reported from the USA at the time of the report (Webster et al., 2011).

During spring 2012, symptoms of systemic necrosis and leaf distortion were observed in tomato plants in fields

in the Florida counties of Miami-Dade and Lee (Figure 1). Fifteen and 12 plants were collected from each county and tested for the presence of tospoviruses. Eight and six plants from Miami-Dade and Lee Co., respectively, gave positive reactions in an enzyme-linked immunosorbent assay (ELISA) designed to detect GRSV and TCSV (SRA6100, AgDia, Inc. Elkhart, IN). To confirm the identity of the virus, the samples were assayed by reverse transcription-PCR and sap extract from selected samples were mechanically inoculated to test plants.

RNA was extracted using a Tri-Reagent procedure (Ambion Inc. Grand Island, NY) and two step reverse transcription-PCR was performed to amplify regions of the three RNA segments using one primer pair for each segment. For amplification of a region of the S RNA, a pair of primers, J13/UH1 (Cortez et al., 2001), was used which amplifies the N ORF and the intergenic region of many tospoviruses and generates different sized amplicons due to variations in the length of the intergenic region among tospovirus species. Amplification of a region of the M RNA was performed with the primer pair NS1/CLA1 (Eiras et al., 2001). Amplification of a region of the L RNA was performed with degenerate primer gL4510c (Cortez et al., 2001) and a TCSV sequence specific primer, JAP891 (5'ACTGGGTCCACTTTACTCACTT3'), which was designed for this study using an L RNA sequence of an isolate of TCSV (GenBank Acc. No. HQ700667) (Bertran et al., 2011). Samples were also tested with a pair of primers designed to specifically amplify a region of the TCSV S RNA (JAP885/JAP886, 5'CTCGGTTTTCTGCTTTTC3'/5'CGGACAGGCTGGAGAAATCG3'-vc) and two sets of primers specific for the GRSV S RNA, JAP887/JAP888 (5'CGTATCTGAGGATGTTGAGT3'/5'GCTAACTCCTTGTTCTTTTG3'-vc) and GRSV-N-v/GRSV-N-vc [12].



**FIGURE 1** - Symptoms of *Tomato chlorotic spot virus* in tomato plants from Miami-Dade and Lee Counties in Florida. **A.** Tomato plant (two weeks after transplant) in Miami-Dade Co. showing symptoms of necrosis and deformation, similar plants were dead within a week after this image was taken; **B.** Tomato leaf from an infected mature plant from Lee Co. showing necrotic spots and deformation; **C.** Fruit from an infected mature plant from Lee Co. showing necrotic spots, necrotic ringspots and deformation.

Six samples from Miami-Dade Co. and seven samples from Lee Co. yielded the expected size amplicons of 1408-1455 (S RNA), 878 (M RNA) and 1532 (L RNA) bp for primer pairs J13/UH1, NS1/CLA1, and gL4510c/JAP891, respectively. Primer pair J13/UH1 generated a single size amplicon from all 13 samples, suggesting that no other tospovirus was present in these samples. No sample amplified with primer pair JAP886/JAP887 or GRSV-N-v/GRSV-N-vc suggesting that GRSV was not present in any of these samples. All the samples that gave the expected size amplicon using primer pair J13/UH1 also amplified the expected size amplicon of 295 bp using the TCSV specific primer pair, JAP884/JAP885. Two samples from each county were selected and amplicons of the three segments from each of the four samples were cloned into pGEM-T-Easy vector (Promega Corp. Madison WI), sequenced, and the sequences compared to those in GenBank through Blastn and Blastp. Sequences generated were deposited in the GenBank database as accession numbers JX244195 through JX244206.

Sequences obtained using the S RNA primer pair, J13/UH1, were 96-99% identical with each other and were most closely related to variably sized sequences of the S RNA of TCSV. Sequences from Florida had nucleic acid sequence identities (NSI) of 97% with the N gene of TCSV-[BR-03] (GenBank Acc. No. S54325), which is the type isolate of TCSV (de Avila et al., 1993), and 98%

with a shorter segment (775 nt) of another isolate of TCSV from Brazil (GenBank Acc. No. AF282982) (Table 1). In contrast, the NSI of the isolates in this study were only 84% identical with the sequence of the N gene of a GRSV isolate from South Africa (GRSV-[95/0137], GenBank Acc. No. AF487517), 83% with a sequence from Brazil (GenBank Acc. No. AF251271), and 80% with GRSV-[L<sub>G</sub>M<sub>1</sub>S<sub>G</sub>] from Florida (Table 1). The NSI with other tospoviruses, including the recently proposed species *Pepper necrotic spot virus* (PNSV) (Torres et al., 2012), TWSV and INSV was 76% or lower.

The predicted amino acid sequences of the N protein of the sequences obtained in this study were almost identical to each other (Table 2). The percent of amino acid sequence identity (ASI) of these sequences with the respective protein sequences of TCSV ranged from 96 to 98%, in contrast to those of GRSV and TSWV isolates which ranged from 87 to 88% and 80 to 81%, respectively. Using the current criteria for taxonomy of the genus *Tospovirus*, which establishes 90% ASI in the N protein as the delimiting value for species demarcation (King et al., 2012), the virus identified in the samples would be considered isolates of TCSV.

Sequences obtained with primers for the M RNA had a NSI of 99% with each other. These sequences had an NSI of 99% with the equivalent region of GRSV-[L<sub>G</sub>M<sub>1</sub>S<sub>G</sub>] (GenBank Acc. No. HQ644141), followed by a NSI of 98% with two TCSV sequences reported from Brazil (GenBank

**TABLE 1** - Comparison of cloned sequences amplified from tomato plants from Miami-Dade and Lee Counties in Florida with the greatest nucleic acid sequence identity as determined by Blastn

Genomic segment	Isolate (clone) <sup>1</sup>	Length <sup>2</sup> (nt)	TCSV-[BR-03] (S54325) 929 nt <sup>3</sup>	TCSV (AF282982) 775 nt	GRSV-[L <sub>G</sub> M <sub>T</sub> S <sub>G</sub> ] (HQ644140) 1250 nt	GRSV-[95/0137] (AF487517) 857 nt	PNSV (HE584762) 1234 nt
S RNA	T9-MD <sup>3</sup> (S2)	1428	97	98	80	84	76
	T11-MD (S7)	1454	97	98	80	84	76
	T18-H (S3)	1408	97	98	80	84	76
	T36 H (S4)	1455	97	98	80	84	76
M RNA	T9-MD (M3)	878	98	98	99	94	
	T11-MD (M2)	878	98	98	99	95	
	T18-H (M1)	878	98	98	99	95	
	T36-H (M2)	878	98	98	99	95	
L RNA	T9-MD (L2)	1532	98	98	83		
	T11-MD (L3)	1532	99	98	83		
	T18-H (L1)	1532	99	98	83		
	T36-H (L3)	1532	99	98	83		

<sup>1</sup>Isolates T9-MD and T11-MD were collected from Miami-Dade Co., Florida and samples T18-H and T36-H were collected from Lee Co., Florida. GenBank Acc. No.'s of clones used in this comparison are as follows: T9-MD (S2) (JX244195), T11-MD (S7) (JX244196), T18-H (S3) (JX244197), T36-H (S4) (JX244198), T9-MD (M3) (JX244199), T11-MD (M2) (JX244200), T18-H (M1) (JX244201), T36-H (M2) (JX244202), T9-MD (L2) (JX244203), T11-MD (L3) (JX244204), T18-H (L1) (JX244205), T36-H (L3) (JX244206).

<sup>2</sup>Number of nucleotides in the amplicon.

<sup>3</sup>This value reflects the size of the sequence used in this comparison which varied due to differences in the sequences available for comparison as well as variations in the size of the intergenic regions among the viruses.

**Table 2** - Percentage of amino acid sequence identity of the N protein of tospovirus isolates in this study with that of isolates of the most closely related tospoviruses based on BLASTp analysis

Sample ID No.	Percent amino acid sequence identity											
	T9-MD <sup>1</sup> (JX244195)		T11-MD (JX244196)		T18-MD (JX244197)		T36-H (JX244198)		Isolate or Virus (GenBank Accession No.)			
	TCSV	TCSV	TCSV	GRSV	GRSV	GRSV	GRSV	GRSV	GRSV	PNSV (HE584763)	TSWV (CAC01284)	TSWV (CBX24340)
T9-MD <sup>1</sup>	100	99.6	99.2	99.2	98.4	95.7	87.9	87.2	87.2	84.4	81.3	79.8
T11-MD			99.6	99.6	98.8	96.1	88.3	87.5	87.5	84.8	81.7	80.2
T18-H				99.2	98.4	95.7	87.9	87.2	87.2	84.4	81.3	79.8
T36-H					98.4	95.7	87.9	87.2	87.2	84.4	81.3	79.8

<sup>1</sup>Isolates T9-MD and T11-MD were collected from Miami-Dade Co., Florida and samples T18-H and T36-H were collected from Lee Co., Florida.

Acc. No. AF282983 and AF213674) (Table 1). The next most similar were isolates of GRSV reported from South Africa (GRSV-[SA] GenBank Acc. No. AF213673) and Brazil (GenBank Acc. No. AF213673, AF513220) with an NSI of 94-95% (Table 1). The translated sequence, 255 aa of the NSm protein had 100% ASI with the equivalent sequence of GRSV-[L<sub>G</sub>M<sub>T</sub>S<sub>G</sub>], 99% ASI with the two TCSV isolates, and 98% and 96% ASI with GRSV isolates from South Africa and Brazil, respectively.

Amplicon sequences of the L RNA (primers gL4510c/JAP891) had NSIs of 99% to each other and 98-99% with a portion of the coding sequence for the RNA-dependent RNA polymerase (RdRp) of TCSV (GenBank Acc. No. HQ700667) [1] (Table 1). These sequences had only 83% NSI with the equivalent region of the L RNA from GRSV-[L<sub>G</sub>M<sub>T</sub>S<sub>G</sub>] (GenBank Acc. No. HQ644142) (Table 1). The NSI of the amplicon sequences had lower similarities with the L RNA of other tospoviruses; 79-80% NSI with isolates of TSWV (GenBank Acc. No. HM581934, NC\_002052, AB190813) and 76% NSI with INSV (GenBank Acc. No. X93218) (data not shown). A predicted amino acid sequence of 510 nt, which corresponds to a portion of the *Bunyaviridae* RdRp domain, had 99%, 96%, 90-91% and 80% of ASI with those of TCSV, GRSV-[L<sub>G</sub>M<sub>T</sub>S<sub>G</sub>], TSWV, and INSV (GenBank Acc. No. HQ700667, HQ644142, HM581934, X93218, respectively). These values indicate that the sequences obtained from field samples using L RNA primers gL4510c/JAP891 are more similar to isolates of TCSV than to any other tospovirus including GRSV-[L<sub>G</sub>M<sub>T</sub>S<sub>G</sub>].

To obtain biological information on the virus isolates, two samples from Miami-Dade Co. which tested positive for TCSV by ELISA and reverse transcription-PCR using primer pairs J13/UH1, NS1/CLA1, gL4510c/JAP891 and JAP884/JAP885 were mechanically inoculated to pepper (*Capsicum annuum* L. 'California Wonder') using a buffer composed of 0.1 M sodium/potassium phosphate and 0.01 M sodium sulfite, pH 7.4. Large chlorotic ring spots (which later developed into necrotic ring spots) appeared on the inoculated leaves eight days post inoculation. Systemic symptoms of leaf distortion and curling and veinal chlorosis appeared within nine days. A chlorotic lesion from pepper was collected and inoculated to pepper seedlings, *Datura stramonium* L., and *Nicotiana glutinosa* L. Pepper plants inoculated with the local lesion produced symptoms identical to the originally inoculated plant, while *D. stramonium* and *N. glutinosa* plants exhibited necrotic lesions on the inoculated leaf four days after inoculation. Seven days after inoculation, systemic symptoms of necrotic spots, leaf distortion and leaf curling appeared on plants of *D. stramonium*. All inoculated symptomatic plants tested positive by reverse transcription-PCR with primers JAP884/JAP885 and the GRSV/TCSV ELISA. Additionally, direct sequencing of reverse transcription-PCR products for S, M, and L RNA (primer pairs gL4510c/JAP891, NS1/CLA1 and J13/UH1, respectively) from pepper and *D. stramonium*

plants yielded sequences 100% identical to the clones from the tomato field samples.

TCSV was not detected in every field plant with necrotic symptoms. The lack of detection of a tospovirus in every necrotic sample was most likely due to the poor quality of the plant tissue. We have found that nucleic acid extractions of necrotic tissue contain plant compounds associated with the necrosis (ie tanins etc.) which can interfere with reverse transcription PCR.

The symptoms observed in tomato plants from the field, combined with ELISA results and sequence analyses of twelve amplicons obtained from four field samples using six primer pairs are consistent with the presence of TCSV in two locations in Florida. The symptoms in tomato in the field and in the test plants (*C. annuum*, *D. stramonium*, *N. glutinosa*) are consistent with those reported for a TCSV isolate from Brazil (de Avila et al., 1993). The sequences of all amplicons had greater than 97% NSI with sequences of TCSV isolates. The predicted amino acid sequence of the N protein of the isolates in this study had greater than 95% ASI with TCSV isolates. While the M RNA of TCSV has been reported previously as part of the genome of the reassortant virus, GRSV-[L<sub>G</sub>M<sub>T</sub>S<sub>G</sub>] (Webster et al., 2011), no evidence for the presence of any segment of GRSV was found in these samples using reverse transcription-PCR and three primer pairs. These data support the conclusion that an isolate of TCSV is present in Florida and in the USA.

It is not clear how or when TCSV was introduced into Florida. Several TCSV vector species have existed in Florida for some time (Frantz & Fasulo, 1997). However, the dark form of *Frankliniella schultzei*, demonstrated in one study to be the most efficient vector of TCSV, has only recently become a pest in vegetable crops in South Florida (Wijkamp et al., 1995; Kakkar et al., 2012). It is possible that TCSV is a recent introduction possibly on an unknown ornamental host. Miami-Dade Co. produces a very wide range of ornamental crops. It is also possible that TCSV has been in Florida for some time, and only recently become apparent due to the increase in populations of *F. schultzei*.

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