Characterization of Tomato Necrotic Spot Virus, a Subgroup 1 Ilarvirus Causing Necrotic Foliar, Stem, and Fruit Symptoms in Tomatoes in the United States

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Abstract

The genomic, biological, and serological characterization of tomato necrotic spot virus (ToNSV), a virus first described infecting tomato in California, was completed. The complete genomic sequence identified ToNSV as a new subgroup 1 ilarvirus distinct from the previously described tomato-infecting ilarviruses. We identified ToNSV in Indiana in 2017 and 2018 and in Ohio in 2018. The coat protein coding region of the isolates from California, Indiana, and Ohio have 94 to 98% identity, while the same isolates had 99% amino acid identity.

ToNSV is serologically related to TSV, a subgroup 1 ilarvirus, and shows no serological relationship to ilarviruses in the other subgroups. In tomato, ToNSV caused symptoms of necrotic spots and flecks on leaves, necrotic streaking on stems, and necrotic spots and circular patterns on fruit resulting in a yield loss of 1 to 13%. These results indicate that ToNSV is a proposed new subgroup 1 ilarvirus causing a necrotic spotting disease of tomato observed in California, Indiana, and Ohio.

Tomato (*Solanum lycopersicum* L.) is the highest value vegetable crop with the second most acres harvested by commercial farms in the United States (National Agricultural Statistics Service 2018). California and Florida are the main states producing field grown processing tomatoes for canning (National Agricultural Statistics Service 2018). Locally produced vine-ripened fresh tomatoes fill the market during the summer months, while California and Florida are the major producers of vine-ripened or mature green tomatoes for the remaining time (Cook and Calvin 2005). Greenhouse grown fresh market tomatoes are, however, produced year-round (Cook and Calvin 2005). In 2017, the United States produced 13.8 million tons of tomatoes with 1.2 million tons utilized for the fresh market and 11 million tons utilized for processing with a total crop value of \$1.67 billion (National Agricultural Statistics Service 2018). This was 18% less (down from \$2 billion in 2016) than the previous year due to intense summer heat in California (National Agricultural Statistics Service 2018).

Tomatoes are susceptible to numerous insect pests and during production can be subject to a large amount of mechanical injury when transplanting, pruning, staking, and harvesting, which make the tomatoes at risk of viral disease outbreaks (Bai and Lindhout 2007; Zitter 2014). Tobacco streak virus (TSV), the type *Ilarvirus* species and a member of subgroup 1, has frequently been reported causing disease in tomato worldwide and was first identified in tomato in the United States in 1982 (Cupertino et al. 1984; Zitter 2014). Viruses belonging to the genus *Ilarvirus*, family *Bromoviridae*,

have been reported infecting woody and herbaceous plant species (Pallas et al. 2013). Based on serological relationship and nucleic acid sequence identity, ilarviruses are ordered into four subgroups (Bujarski et al. 2012). Ilarviruses have a tripartite genome of positive sense, single-stranded RNA encapsidated in quasi-spherical 26 to 36 nm diameter particles. RNA 1 (~3,400 nt), RNA 2 (~2,900 nt), and RNA 3 (~2,200 nt) encode four proteins: replicase (1a), RNA-dependent RNA polymerase (RdRp, 2a), movement protein (MP, 3a), and coat protein (CP, 3b) (Pallas et al. 2013). Subgroups 1 and 2 encode a fifth protein, a putative silencing suppressor (2b) (Bujarski et al. 2012; Shimura et al. 2013).

Other tomato infecting ilarviruses include the most recently described subgroup 2 ilarvirus, tomato necrotic streak virus (TomNSV), which was identified from fresh market tomatoes in Florida in 2013 (Adkins et al. 2015; Badillo-Vargas et al. 2016). Tomato isolates of Parietaria mottle virus (PMoV), a subgroup 1 ilarvirus, were first identified causing disease in tomatoes in Europe in the late 1990s and were subsequently found throughout tomato producing regions in Italy, France, Greece, and Spain (Aramburu 2001; Lisa et al. 1998; Marchoux et al. 1999; Ramasso et al. 1997; Roggero et al. 2000). Tomato necrotic spot virus (ToNSV), a proposed subgroup 1 ilarvirus, was identified causing disease in processing tomatoes in California in 2008 and was partially characterized (Batuman et al. 2009).

In 2017, Bratsch et al. identified ToNSV in fresh market tomatoes from a vegetable farmer in Indiana (Bratsch et al. 2018). Affected plants had foliar symptoms of necrotic spotting and flecking, stems had necrotic streaking, and fruit developed necrotic rings and patchy patterns (Fig. 1). ToNSV was subsequently identified in Indiana and Ohio in 2018. From 2017 to 2018, a disease incidence of <1 to 13% was observed. Fruit from infected Indiana and Ohio tomatoes were unmarketable due to the necrotic symptoms. The objectives of this study were to fully characterize ToNSV biologically, serologically, and genomically.

Materials and Methods

Virus source. An isolate of ToNSV was obtained from two symptomatic high tunnel grown hybrid beefsteak type tomatoes ('New Girl' and 'Big Dena') in Delaware County, Indiana in 2017 (Bratsch et al. 2018). This isolate will be referred to as ToNSV-Indiana. Symptomatic samples were also obtained from the same Indiana farm in 2018. Another isolate of ToNSV was identified from one

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symptomatic plant obtained from a greenhouse growing red cherry tomatoes in Fulton County, Ohio in 2018. This isolate will be referred to as ToNSV-Ohio. Two asymptomatic plants from Ohio were tested by reverse transcription polymerase chain reaction (RT-PCR) and were negative for ToNSV. In both Indiana and Ohio, symptomatic plants were randomly distributed throughout the growing space. All samples were obtained from the Purdue University Plant and Pest Diagnostic Laboratory.

Sap transmission. Transmission from symptomatic ToNSV-Indiana infected plants to three healthy 6-week-old tomato (*S. lycopersicum*

L. Burpee's 'Big Boy', seed obtained from Burpee, U.S.A.) plants was completed by mechanical inoculation using symptomatic ToNSV-Indiana positive leaves homogenized in 20 mM sodium phosphate buffer (pH 7.0) with 0.5% 2-mercaptoethanol. Sap was rubbed onto three leaves of each tomato plant, which had been previously dusted with 600 mesh silicon carbide. Three control tomato plants (also Burpee's 'Big Boy') were mock inoculated with buffer using the same procedure. All plants were confirmed to be virus-free prior to inoculation by using Agdia ImmunoStrips to test for tobacco mosaic virus (TMV) and by RT-PCR using the ToNSV CP detection primers described below. Plants

Fig. 1. Symptoms caused by tomato necrotic spot virus infection in hybrid tomato (A-C) and cherry tomato (D-F). Foliage and stems (A-B, D-E) show typical necrotic spots, streaks, and petiole necrosis, while fruit (C, F, top and bottom) have a range of necrotic ring patterns from mild to severe. A-C (top) from Bratsch et al. (2018).

were kept in a greenhouse at 22–25°C 16/8-h day/night photoperiod. Plants were monitored weekly for 8 weeks for virus-like symptoms, and each plant was tested by RT-PCR using the ToNSV CP detection primers described below to confirm infection.

Sequence analysis, genome organization, and phylogenetic analysis. Total RNA was extracted from ToNSV-Indiana infected symptomatic tomato leaves collected from the farm in 2017 using RNeasy Plant Mini Kit; Qiagen, U.S.A. The RNA was sent to Seq-Matic, CA for cDNA library construction and high-throughput sequencing (HTS) on an Illumina NextSeq platform. Sequence assembly was performed on CLC Genomic Workbench 11.01, and the resulting contigs were compared with a custom virus database. Sequence mapping and sequencing depth were determined by CLC Workbench.

To determine the complete genome sequence of ToNSV-Indiana, additional primers were designed from conserved subgroup 1 ilarvirus sequences identified through alignment in MEGA7 for the 5' and 3' ends. Sequences used for alignment and 5' or 3' genome end primer design included: PMoV (AY496068.1, AY496069.1, U35145.1), TSV (U80934.1, U75538.1, X00435.1), strawberry necrotic shock virus (DQ318818.3, AY743591.2, AY363228.2), bacopa chlorosis virus (FJ607140.1, FJ607141.1, FJ607142.1), and blackberry chlorotic ringspot virus (BCRV) (NC_011553.1, DQ091194.2, DQ091195.2). The primers designed to the 5' or 3' ends were used with primers anchored in the NGS contigs. Total RNA was again extracted from symptomatic 'New Girl' and 'Big Dena' tomato leaves collected from the Indiana farm in 2017 (RNeasy Plant Mini Kit; Qiagen, U.S.A.) and used in a one-step RT-PCR (Illustra Ready-to-Go RT-PCR Beads; GE Healthcare Life Sciences, U.S.A.) using primers documented in Supplementary Table S1. Amplicons of the expected sizes for each reaction (5' RNA 1, 2, 3, and 3' RNA 1, 2, 3 for both tomato varieties) were obtained, purified (PureLink Quick PCR Purification Kit, Invitrogen, U.S.A.), ligated, and cloned (pGEM-T Easy Vector System; Promega, U.S.A.). Four to six clones from each transformation event were Sanger sequenced (MCLAB; California), and the ends were assembled with the sequences obtained by HTS.

The phylogenetic relationship of ToNSV-Indiana to other members of the genus *Ilarvirus* were deduced using MEGA 7.0 for sequence alignment (Kumar et al. 2016). Phylogenetic analysis was completed using the maximum-likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model in IQ-TREE version 1.6.6 (Nguyen et al. 2015). Phylogenetic trees were constructed based on alignments of complete amino acid sequences of replicase (P1), RdRp (P2), movement protein (MP), and coat protein (CP). The coat protein nucleotide sequence was analyzed by MEGA 7.0 with the Kimura 2-parameter model to estimate the overall mean distance and pairwise distance of the ToNSV isolates.

Detection. The farm in Indiana, which in 2017 had ToNSV-positive tomatoes, subsequently grew tomato plants with virus-like symptoms in 2018. Two of these symptomatic plants were collected and tested by Agdia ImmunoStrips for: Impatiens necrotic spot virus (INSV), Pepino mosaic virus (PepMV), the genus *Potyvirus* (POTY), tobacco mosaic virus (TMV), and tomato spotted wilt virus (TSWV). Furthermore, in 2018 a commercial tomato grower in Ohio had symptomatic tomatoes, one of which was tested by Agdia ImmunoStrips for the same viruses plus *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). Total RNA was extracted from the leaves of the two symptomatic Indiana tomato plants and from the leaves of one symptomatic and two asymptomatic tomato plants collected from a farm in Ohio in 2018. RNA was also extracted from the leaves of one healthy control plant (Burpee's 'Big Boy') (RNeasy Plant Mini Kit; Qiagen, U.S.A.). All RNAs were used in a one-step RT-PCR (Illustra Ready-to-Go RT-PCR Beads; GE Healthcare Life Sciences, U.S.A.) with 5 µM of primers designed from the ToNSV-Indiana isolate HTS contig corresponding to RNA 3 flanking the CP open reading frame (ORF). These primers were: ToNSV_CP_For (5'-GTGAACCTTCGTGAAGTCCACC-3'), and ToNSV_CP_Rev (5'-CTCCGTCTTGATCGGAGTCAT-3').

The cycling conditions were 42°C, 45 min; 95°C, 5 min; 39 cycles of 95°C, 30 s; 56°C, 30 s; 72°C, 30 s; followed by 72°C for 10 min.

No products were obtained from the asymptomatic Ohio tomato plants and healthy tomato Burpee's 'Big Boy' plants. Positive reactions with amplicons of 854 nts were obtained from the two Indiana 2018 samples and the Ohio 2018 sample. The amplicons were purified (PureLink Quick PCR Purification Kit, Invitrogen, U.S.A.), ligated, and cloned (pGEM-T Easy Vector System; Promega, U.S.A.). Five clones from all three positive reactions were Sanger sequenced (MCLAB; U.S.A.) and trimmed to the CP ORF of 615 nts.

Serology. Serological relationships between ToNSV and other ilarviruses were investigated using commercially available ELISA kits, as directed by the manufacturer, for subgroup 1–4 ilarviruses including TSV (triple antibody sandwich ELISA), asparagus virus 2 (AV-2) (antigen coated plate ELISA), apple mosaic virus (ApMV) (double antibody sandwich ELISA), and prune dwarf virus (PDV) (double antibody sandwich ELISA) (Agdia, U.S.A.). Positive controls included: TSV provided by Ben Lockhart (University of Minnesota); and AV-2, ApMV, PDV, and American plum line pattern virus (APLPV) all obtained from Agdia, U.S.A.

Symptomatic ToNSV-Indiana 2018 and ToNSV-Ohio 2018 leaf tissue, confirmed to be positive by RT-PCR above, was tested by ELISA at a 1:10 (wt/vol) dilution in extraction buffer as directed by the kits. The negative control was tomato leaf tissue that had tested negative for ToNSV by RT-PCR. Duplicate wells were used for each sample. Absorbance at 405 nm was measured using a Bio-Rad model 2550 EIA reader after 1–1.5 h. Background absorbance readings were not removed from sample absorbance values. Samples with an absorbance value 2.5 times the negative control were considered positive. Absorbance readings for duplicate wells were averaged.

Results

Sap transmission. Inoculated tomato plants were confirmed positive for ToNSV by RT-PCR detection of the ToNSV coat protein. No RT-PCR products were obtained from the mock-inoculated control plants.

Symptoms of ToNSV in inoculated tomatoes resembled those observed in the initial samples positive for ToNSV from Indiana and Ohio as observed in Figure 1. Foliar symptoms included necrotic spots, flecks, and line patterns. Terminal branches expressed the strongest symptoms with some becoming entirely necrotic. Stem symptoms included necrotic streaking. Fruit varied in the intensity and amount of surface area covered with symptoms, which included necrotic concentric rings, circles, and patches.

Sequence analysis, genome organization, and phylogenetic analysis. After trimming for quality, HTS of ToNSV-Indiana generated over 23 million sequencing reads. These reads assembled to 39,535 contigs, three of which showed significant similarity to the tripartite genome of ilarviruses. These contigs were identified as RNA 1, RNA 2, and RNA 3, with preliminary lengths of

Fig. 2. Genome organization of tomato necrotic spot virus. Three genomic RNA segments are shown as solid lines, with the nucleotides indicated at the 5' and 3' termini. Predicted open reading frames are shown as boxes containing protein names and nucleotides (shown above or below) indicating start and stop codons. RNA 1 encodes 1a (replicase). RNA 2 is bicistronic and encodes 2a (RNA-dependent RNA polymerase) and 2b (silencing suppressor). RNA 3 is also bicistronic and encodes a movement protein (MP) and coat protein (CP).

3,516, 2,825, and 2,326 nts, respectively. Reads were mapped to RNA 1 (2,313,506), RNA 2 (2,645,569), and RNA 3 (2,101,656), with an average nt depth per position of 49,888, 68,898, and 68,522, respectively. After Sanger resequencing, the genome was determined to be 3,519 (RNA 1), 2,903 (RNA 2), and 2,290 (RNA 3) nts long (Fig. 2) and were deposited in GenBank as accession numbers MH780154, MH780155, and MH780156. At the whole-genome level, ToNSV-Indiana exhibits high sequence identities to members of subgroup 1 ilarviruses: PMoV, 74–82%, ageratum latent virus, 60–73%, and BCRV, 59–68% (Supplementary Table S2).

RNAs 1, 2, and 3 have a genetic structure consistent with other members of the ilarvirus genus. RNA 1 contains a single ORF

encoding a replicase (protein 1A) of 1,099 amino acids with a viral methyltransferase domain (pfam01660) from 69–413 and an RNA helicase domain (pfam01443) from 811–1066. RNA 2 was bicistronic: ORF2a encodes a putative RNA-dependent RNA polymerase (RdRp, protein2a) of 819 amino acids and includes the RdRp domain (RdRp_2, pfam00978) from aa 260–696 and the conserved ilarvirus domain ASDDSLI (aa 564–571) (O'Reilly and Kao 1998). ORF2b has 217 residues corresponding to protein 2b, which is unique to ilarvirus subgroups 1 and 2 and is predicted to be involved in virus movement and gene silencing (Pallas et al. 2013). RNA 3 is also bicistronic, encoding a movement protein (MP, 3a) of 299 aa, which includes the *Bromovirus* movement protein domain (c103270) from

Fig. 3. Phylogenetic analysis of tomato necrotic spot virus with accepted and proposed members of the genus *Ilarvirus* with amino acid sequences of **A**, replicase; **B**, RNA-dependent RNA polymerase; **C**, movement protein; **D**, coat protein. Cucurbit mosaic virus (*Cucurbitavirus*) was used as an outgroup. Tomato necrotic spot virus is indicated with a white box grouping within subgroup 1 ilarviruses, indicated with a gray box. Phylogenetic trees were constructed using the maximum likelihood algorithm. Bootstrap values (1,000 iterations) $\geq 60\%$ are shown. The scale bars correspond to substitutions per amino acid site. Accession numbers for all sequences are divided by subgroup. Subgroup 1: Ageratum latent virus (YP_008470969.1, YP_008470970.1, YP_008470972.1, YP_008470973.1); Blackberry chlorotic ringspot virus (YP_002308569.1, YP_002308570.1, YP_002308572.1, YP_002308573.1); Parietaria mottle virus (YP_006446.1, YP_006447.1, YP_006463.1, YP_006464.1); PMoV-Tomato (CP only, ALA50824.1); Privet ringspot virus (YP_009165996.1, YP_009165997.1, YP_009165999.1, YP_009166000.1); Strawberry necrotic shock virus (YP_941474.2, YP_941472.1, YP_941470.1, YP_941471.1); Tomato necrotic spot virus-Yolo (N/A, N/A, N/A, ACI96015.1); ToNSV-Ohio (N/A, N/A, N/A, MK036502.1); Tobacco streak virus (NP_620772.1, NP_620768.1, NP_620773.1, NP_620774.1). Subgroup 2: Asparagus virus 2 (YP_002455928.1, YP_002455929.1, YP_002455926.1, YP_002455927.1); Citrus leaf rugose virus (NP_613282.1, NP_613281.1, NP_613279.1, NP_613280.1); Citrus variegation virus (YP_001285482.1, YP_001285483.1, YP_001285480.1, YP_001285481.1); Elm mottle virus (NP_619576.1, NP_619575.1, NP_619577.1, NP_619578.1); Spinach latent virus (NP_620677.1, NP_620678.1, NP_620680.1, NP_620681.1); Tomato necrotic streak virus (AMN16528.1, AMN16529.1, AMN16531.1, AMN16532.1); Tulare apple mosaic virus (NP_620753.1, NP_620754.1, NP_620756.1, NP_620757.1). Subgroup 3: Apple mosaic virus (NP_604469.1, NP_604470.1, NP_604484.1, NP_604485.1); Blueberry shock virus (YP_008519304.1, YP_008519305.1, YP_008519306.1, YP_008519307.1); Lilac leaf chlorosis virus (YP_009104367.1, YP_009104368.1, YP_009104372.1, YP_009104373.1); Prunus necrotic ringspot virus (NP_733823.1, NP_733824.1, NP_733825.1, NP_733826.1). Subgroup 4: Fragaria chiloensis latent virus (YP_164801.1, YP_164802.1, YP_164804.1, YP_164805.1); Prune dwarf virus (YP_611154.1, YP_611151.1, YP_611152.1, YP_611153.1). No relationship to existing groups: American plum line pattern virus (NP_602312.1, NP_602313.1, NP_602314.1, NP_602315.1); Humulus japonicus latent virus (YP_054422.1, YP_054423.1, YP_054424.1, YP_054425.1). Outgroup: Cucurbit mosaic virus (NP_049323.1, NP_049324.1, NP_040776.1, NP_040777.1).

aa 6-275 and a coat protein (CP) of 204 aa including the conserved ilarvirus coat protein domain (pfam01787) from 3-203.

Phylogenetic trees were constructed based on alignments of complete amino acid sequences of P1, P2, MP, and CP and showed that ToNSV was consistently placed in the same clade with ilarvirus subgroup 1 (Fig. 3 A–D).

The overall mean evolutionary Kimura distance estimate for the CP nucleotide sequence of the three ToNSV isolates, including two identified in this report (ToNSV-Indiana and ToNSV-Ohio) and one previously identified in California (ToNSV-Yolo), was 0.038. The pairwise Kimura distance estimation of the CP nucleotide sequence was 0.020 for ToNSV-Yolo versus ToNSV-Indiana, 0.060 for ToNSV-Yolo versus ToNSV-Ohio, and 0.064 for ToNSV-Indiana versus ToNSV-Ohio.

Detection. The two symptomatic samples from Indiana tested negative for INSV, PepMV, potyvirus-group test, TMV, and TSWV using Agdia ImmunoStrips. A commercial greenhouse tomato grower in Ohio had one symptomatic tomato that also tested negative for these viruses plus Cmm. Symptomatic plants from both sites tested positive for ToNSV using primers designed to amplify an 854 bp region of the ToNSV genome containing the CP. No product was obtained from healthy controls (Burpee's 'Big Boy') or from asymptomatic plants from Ohio. The CP sequence from Ohio was deposited in GenBank as accession number MK036502.1.

The CP nucleotide sequences of the tomatoes sampled in both 2017 and 2018 from the Indiana farm were 100% identical. ToNSV-Indiana has 98% nucleotide identity (nt) and 99% amino acid (aa) identity to the Californian Yolo County ToNSV isolate (ToNSV-Yolo). ToNSV-Ohio, obtained from a red cherry tomato in Ohio, has 94% nt identity and 99% aa identity to both ToNSV-Yolo and ToNSV-Indiana.

Serology. To determine the serological relationship between ToNSV and *Ilarvirus* species, samples of both ToNSV-Indiana and ToNSV-Ohio infected tomato leaves were compared with TSV, AV-2, ApMV, and PDV in ELISA (Table 1). These four viruses are members of subgroups 1 to 4, respectively, in the genus *Ilarvirus*. ToNSV-Indiana and ToNSV-Ohio infected tomato leaves reacted weakly with anti-TSV antisera only (Table 1). These results suggest that ToNSV is serologically distinct from other ilarviruses but shares the most similarity to the subgroup 1 ilarvirus TSV.

Discussion

In this study, we report the complete genome sequence of a newly discovered ilarvirus, provisionally named ToNSV, initially reported (by others) infecting tomatoes in California and subsequently identified in Indiana and Ohio. The genomic organization of ToNSV is typical of members of the genus *Ilarvirus* (Fig. 2) and specifically of subgroup 1 ilarviruses as shown by nucleotide and amino acid sequence comparison and phylogenetic analysis (Fig. 3). ToNSV joins three other ilarviruses (TSV and PMoV-Tomato; both in subgroup 1 and TomNSV in subgroup 2) reported causing disease in tomato.

Sequences of ToNSV replicase, RdRp, 2b, and movement protein genes showed the highest amino acid identity to PMoV (90, 81, 48, and 82%, respectively) (NC_005848.1, NC_005849.1, NC_005854.1), while the CP gene showed the highest amino acid identity to ToNSV-Yolo (99%) (FJ236810.1). Although there are currently no delineated levels of sequence similarity to distinguish *Ilarvirus* spp. or subgroups, all phylogenetic analysis results in this study support placement of ToNSV in subgroup 1 of the genus *Ilarvirus*. This conclusion is supported by the serological data, where a weak relationship between ToNSV and subgroup 1 TSV was observed.

ToNSV was transmitted by mechanical inoculation from infected tomato leaves to healthy tomatoes consistent with that reported by Batuman et al. (2011). ToNSV can be sap-transmitted to and cause symptoms in tomato (necrotic spots, streaks, circular necrotic fruit patterns), *Chenopodium quinoa* and *C. amaranticolor* (stunted growth, leaf curl, and necrosis), *N. benthamiana* (necrotic leaf and stem lesions), *N. tabacum* 'Havana' and 'Turkish' (stunting, necrotic ringspots, and recovery for 'Havana' but not 'Turkish'), and *Datura stramonium* (mild mottle and ringspots in new leaves, recovery), but

no symptoms were observed in common bean, pumpkin, pepper, and *N. glutinosa* plants (Batuman et al. 2011). Field crops of symptomatic onions, peppers, and lettuce have also been shown to be infected with ToNSV (Batuman et al. 2009, 2011; Gilbertson and Batuman 2013). Pollen mediated thrips transmission of many ilarviruses, including ToNSV, has been reported (Gilbertson et al. 2015). The mechanism is a nonpersistent transmission of virus particles on or in infected pollen that is introduced into plants through mechanical injury caused by feeding insects (Aramburu et al. 2010; Bujarski et al. 2012; Greber et al. 1991; Kaiser et al. 1982; Sdoodee and Teakle 1987, 1993). ToNSV has been detected in pollen collected from ToNSV-infected plants (Gilbertson et al. 2015). In California, the appearance of ToNSV has been associated with large populations of western flower thrips, *Frankliniella occidentalis* (Batuman et al. 2011; Gilbertson et al. 2015). In this study, the presence of ToNSV-Indiana was also associated with high levels of thrips on the tomato plants in both 2017 and 2018. ToNSV-Ohio, isolated from cherry tomato by sequencing the entire CP gene, was identified from a greenhouse with minimal insect infestation, suggesting ToNSV may be seed transmitted. Varying rates of seed transmission of ilarviruses in tomatoes have been reported ranging from 0.52% for TSV (Sdoodee and Teakle 1988) to 0.33% for TomNSV (Badillo-Vargas et al. 2016). PMoV can be transmitted by seed in *Parietaria officinalis* plants at a rate of 36% but has not been shown to be seed transmitted in tomato (Aramburu et al. 2010; Ramasso et al. 1997). In both Indiana and Ohio, a very low rate and sporadic distribution of ToNSV infection was reported across the crops. Further experiments are required to determine if ToNSV is seed transmitted in tomatoes or other crops, perhaps at a rate less than 1%, as observed with other tomato infecting ilarviruses.

The economic impact of ToNSV at the Indiana and Ohio farms used in this study was mainly due to plant loss from the destructive control method of roguing symptomatic plants (Indiana 2017, 2018) and of roguing symptomatic plants plus neighboring healthy-looking plants (Ohio 2018). Roguing is done to prevent the spread of the virus and viruliferous thrips. In all three incidences, it was not possible for the farmer to replace infected plants due to the methods of production and limited growing season. In addition to roguing, in Indiana in 2017 and 2018, the farmer elected to use nonplanned insecticide applications to reduce their high thrips population. The overall economic impact of ToNSV in these farms was a reduction of around 1 to 13% of their expected yield. In addition, the farms experienced increased expenditure for the labor needed to identify and pull symptomatic and neighboring plants, and due to nonplanned insecticide applications.

Comparative sequence analysis of the coat protein (CP) gene from the three isolates of ToNSV revealed nucleotide changes at 42 positions between nucleotides 11-522. The CP nucleotide identity between isolates ranged from 94% (ToNSV-Indiana versus

Table 1. Serological relationship of tomato necrotic spot virus and the four ilarvirus subgroups in indirect plate trapped antigen ELISA

Antigen	Subgroup	Antivirus antisera, absorbance at 405 nm ^a			
		TSV	AV-2	ApMV	PDV
TSV	1	3.000	0.052	0.117	0.024
AV-2	2	0.056	1.005	0.001	0.013
ApMV	3	0.044	0.035	0.705	0.025
PDV	4	0.089	0.023	0.005	0.605
APLPV	Not grouped	0.067	0.047	0.009	0.017
ToNSV-Indiana leaf	1, proposed	0.199	0.063	0.027	0.023
ToNSV-Ohio leaf	1, proposed	0.106	0.059	0.012	0.019
Healthy leaf		0.057	0.056	0.002	0.020
Blank		0.004	0.020	0	0.002

^a Average absorbances of two wells for each sample measured after 1 h incubation for AV-2, ApMV, and PDV and 1.5 h incubation for TSV. Blank is average of 8 wells. Positive controls are highlighted with a dark gray box while ToNSV samples that reacted are highlighted with a light gray box.

ToNSV-Ohio) to 98% (ToNSV-Indiana versus ToNSV-Yolo). The amino acid identity between ToNSV isolates was 99%. Only three of these nucleotide differences between isolates are nonsynonymous, resulting in three amino acid substitutions near the amino terminal region of the coat protein (aa 4, 47, and 88). The first amino acid substitution at aa 4 is a conservative substitution from asparagine (ToNSV-Yolo and ToNSV-Ohio) to serine (ToNSV-Indiana). At aa 47 there is a radical substitution from serine (ToNSV-Yolo and ToNSV-Indiana) to proline (ToNSV-Ohio). The final substitution at aa 88 is another conservative substitution from serine (ToNSV-Yolo and ToNSV-Indiana) to asparagine (ToNSV-Ohio).

The overall genetic diversity (evolutionary distance) of the CP nucleotide sequences from these three ToNSV isolates is 0.038. The average evolutionary distance between isolates of PMoV-Tomato has been identified as 0.047, and was completed with seven Spanish isolates infecting tomato (Galipienso et al. 2008). The diversity observed with only three isolates of ToNSV is similar to PMoV, with larger differences observed at the pairwise level. The largest pairwise distance was found between ToNSV-Ohio with a rate of 0.060 in comparison with ToNSV-Yolo and a rate of 0.064 with ToNSV-Indiana. ToNSV-Indiana and ToNSV-Yolo had the lowest diversity with a rate of 0.020. The nucleotide diversity observed with ToNSV CP sequences suggest ToNSV may have been present throughout the United States for some time or been introduced from more than one source.

Although ToNSV has previously only been reported from California, this report provides evidence that ToNSV is distributed more widely in the United States and is infecting processing tomatoes, hybrid fresh market tomatoes, and cherry tomatoes. It has been suggested that ToNSV is an indigenous virus naturally infecting a perennial plant host and that it is spread to tomatoes when high levels of *F. occidentalis* are present (Gilbertson et al. 2015). Additional research is needed on this potentially emerging virus. Future studies should determine the incidence of ToNSV within the wider United States and North American production areas and clarify the roles of both seed transmission and insect vectors and alternate hosts in the disease epidemiology.

Literature Cited

- Adkins, S., Baker, C. A., Badillo-Vargas, I. E., Frantz, G., Mellinger, H. C., Roe, N., and Funderburk, J. E. 2015. Necrotic streak disease of tomato in Florida caused by a new ilarvirus species related to Tulare apple mosaic virus. *New Dis. Rep.* 31:16.
- Aramburu, J. 2001. First report of *Parietaria mottle virus* on tomato in Spain. *Plant Dis.* 85:1210.
- Aramburu, J., Galipienso, L., Aparicio, F., Soler, S., and López, C. 2010. Mode of transmission of *Parietaria mottle virus*. *J. Plant Pathol.* 92:679-684.
- Badillo-Vargas, I. E., Baker, C. A., Turechek, W. W., Frantz, G., Mellinger, H. C., Funderburk, J. E., and Adkins, S. 2016. Genomic and biological characterization of Tomato necrotic streak virus, a novel subgroup 2 ilarvirus infecting tomato in Florida. *Plant Dis.* 100:1046-1053.
- Bai, Y., and Lindhout, P. 2007. Domestication and breeding of tomatoes: What have we gained and what can we gain in the future? *Ann. Bot.* 100:1085-1094.
- Batuman, O., Chen, L. F., and Gilbertson, R. L. 2011. Characterization of Tomato necrotic spot virus (ToNSV), a new ilarvirus species infecting processing tomatoes in the Central Valley of California. (Abstr.) *Phytopathology* 101:S13.
- Batuman, O., Miyao, G., Kuo, Y.-W., Chen, L.-F., Davis, R. M., and Gilbertson, R. L. 2009. An outbreak of a necrosis disease of tomato in California in 2008 was caused by a new ilarvirus species related to *Parietaria mottle virus*. *Plant Dis.* 93:546.
- Bratsch, S., Creswell, T., and Ruhl, G. 2018. First report of tomato necrotic spot virus infecting tomato in Indiana. *Plant Health Prog.* 19:224-225.
- Bujarski, J., Figlerowicz, M., Gallitelli, D., Roossinck, M. J., and Scott, S. W. 2012. Family Bromoviridae. Pages 965-976 in: *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on*
- Taxonomy of Viruses*. A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz, eds. Elsevier Academic Press, San Diego, CA.
- Cook, R. L., and Calvin, L. 2005. Greenhouse tomatoes change the dynamics of the North American fresh tomato industry. No. 3. U.S. Department of Agriculture, Economic Research Service.
- Cupertino, F. P., Grogan, R. G., Petersen, L. J., and Kimble, K. A. 1984. Tobacco streak virus infection of tomato and some natural weed hosts in California. *Plant Dis.* 68:331-333.
- Galipienso, L., del Carmen Herranz, M., López, C., Pallás, V., and Aramburu, J. 2008. Sequence analysis within the RNA 3 of seven Spanish tomato isolates of *Parietaria mottle virus* (PMoV-T) reveals important structural differences with the *parietaria* isolates (PMoV). *Eur. J. Plant Pathol.* 120:125-135.
- Gilbertson, R. L., and Batuman, O. 2013. Emerging viral and other diseases of processing tomatoes: biology, diagnosis and management. *Acta Hortic.* 971: 35-48.
- Gilbertson, R. L., Batuman, O., Webster, C. G., and Adkins, S. 2015. Role of the insect supervectors *Bemisia tabaci* and *Frankliniella occidentalis* in the emergence and global spread of plant viruses. *Annu. Rev. Virol.* 2: 67-93.
- Greber, R. S., Klose, M. J., Teakle, D. S., and Milne, J. R. 1991. High incidence of *Tobacco streak virus* in tobacco and its transmission by *Microcephalothrips abdominalis* and pollen from *Ageratum houstonianum*. *Plant Dis.* 75:450-452.
- Kaiser, W. J., Wyatt, S. D., and Pesho, G. R. 1982. Natural hosts and vectors of *Tobacco streak virus* in Eastern Washington. *Phytopathology* 72:1508-1512.
- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:1870-1874.
- Lisa, V., Ramasso, E., Ciuffo, M., and Roggero, P. 1998. Tomato apical necrosis caused by a strain of *Parietaria mottle* ilarvirus. Pages 290-291 in: *Proc. 9th Conference of the ISHS. VVWG, Recent Advances in Vegetable Virus Research*. Turin.
- Marchoux, G., Parella, G., Gebre-Selassie, K., and Ganglon, P. 1999. Identification de deux ilarvirus sur tomate dans le sud de la France. *Phytoma* 522: 53-55.
- National Agricultural Statistics Service. 2018. USDA Vegetables 2017 Summary. 7, 11, 109. <http://archive.fo/HfaL5>
- Nguyen, L. T., Schmidt, H. A., von Haeseler, A., and Minh, B. Q. 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32:268-274.
- O'Reilly, E. K., and Kao, C. C. 1998. Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. *Virology* 252: 287-303.
- Pallas, V., Aparicio, V., Herranz, M. C., Sanchez-Navarro, J. A., and Scott, S. W. 2013. The molecular biology of ilarviruses. *Adv. Virus Res.* 87:139-181.
- Ramasso, E., Roggero, P., Dellavalle, G., and Lisa, V. 1997. Necrosi apicale del pomodoro causata da un ilarvirus. *Inf. Fitopatol.* 47:71-77.
- Roggero, P., Ciuffo, M., Katis, N., Alioto, D., Crescenzi, A., Parella, G., and Gallitelli, D. 2000. Necrotic disease in tomatoes in Greece and south Italy caused by tomato strain of *Parietaria mottle virus*. *J. Plant Pathol.* 82:159.
- Sdoodee, R., and Teakle, D. S. 1987. Transmission of *Tobacco streak virus* by *Thrips tabaci*: A new method of plant virus transmission. *Plant Pathol.* 36: 377-380.
- Sdoodee, R., and Teakle, D. S. 1988. Seed and pollen transmission of *Tobacco streak virus* in tomato (*Lycopersicon esculentum* cv. Grosse Lisse). *Aust. J. Agric. Res.* 39:469-474.
- Sdoodee, R., and Teakle, D. S. 1993. Studies on the mechanism of transmission of pollen-associated tobacco streak ilarvirus virus by *Thrips tabaci*. *Plant Pathol.* 42:88-92.
- Shimura, H., Masuta, C., Yoshida, N., Sueda, K., and Suzuki, M. 2013. The 2b protein of *Asparagus virus 2* functions as an RNA silencing suppressor against systemic silencing to prove functional synteny with related cucumoviruses. *Virology* 442:180-188.
- Zitter, T. A. 2014. Diseases caused by viruses. Pages 71-79 in: *Compendium of Tomato Diseases and Pests*, 2nd ed. J. B. Jones, T. A. Zitter, T. M. Momol, and S. A. Miller, eds. American Phytopathological Society, St. Paul, MN.