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ANNOTATED SEQUENCE RECORD

# Full-length M and L RNA sequences of tospovirus isolate 2009-GZT, which causes necrotic ringspot on tomato in China

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**Abstract** The virus isolate 2009-GZT, collected from tomato in Guizhou province of China, was identified as a new member of the genus *Tospovirus* based on its S RNA sequence. Because its provisional name, "tomato necrotic spot virus" (TNSV), was identical to an already existing member of the genus *Ilarvirus*, 2009-GZT was renamed "tomato necrotic spot-associated virus" (TNSaV). In this study, the full-length sequences of the genomic M and L RNAs of TNSaV were determined and analyzed. The M RNA has 4,773 nucleotides (nt), encoding the NSm protein of 309 aa (34.4 kDa) in the viral (v) strand and the glycoprotein precursor (Gn/Gc) of 1123 aa (128 kDa) in the viral complementary (vc) strand. The NSm and Gn/Gc of TNSaV share the highest aa sequence identity (86.2 % and 86.9 %, respectively) with those of tomato zonate spot virus. The L RNA contains 8,908 nt and codes for the putative RNA-dependent RNA polymerase (RdRp) of 2885 aa (332 kDa) in the vc strand. The RdRp of TNSaV shares the highest aa sequence identity (85.2 %) with that of calla lily chlorotic spot virus (CCSV). Serological assays showed that TNSaV cross-reacts with rabbit antisera against watermelon silver mottle virus (WSMoV) NP and CCSV NP, indicating that TNSaV is a member of the WSMoV serogroup.

Tospoviruses cause significant losses of economically important crops worldwide [1]. *Tospovirus* is the only genus in the family *Bunyaviridae* that consists of plant-infecting viruses. In nature, tospoviruses are transmitted by thrips in a circular and propagative manner. Under experimental conditions, transmission can also be achieved through infected plant sap by mechanical rubbing. Particles of tospoviruses are quasi-spherical or pleomorphic, 80-120 nm in diameter. The tospoviral genome comprises three single-strand RNAs, designated as small (S), medium

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(M) and large (L) RNA based on their molecular weight. The S RNA encodes the structural nucleocapsid protein (NP) in the viral-complementary (vc) strand and the non-structural protein (NSs) in the viral (v) strand. NP is a structural protein that assembles the genomic RNAs into ribonucleoprotein complexes (RNPs) [2]. NSs is a gene-silencing suppressor and plays a key role in overcoming the effect of the resistance gene *Tsw* in tomato spotted wilt virus (TSWV)-resisitant pepper [3, 4]. The M RNA encodes the non-structural protein (NSm) in the v strand and the polyprotein containing the glycoproteins precursors (Gn/Gc) in the vc strand. NSm is involved in virus movement, and as an avirulence determinant responsive to the resistance gene *Sw-5* in TSWV-resistant tomato [5, 6]. Gn/Gc is embedded in the lipid envelope of virus particles to form the spikes that are critical for the infection of thrips [7]. The L RNA encodes an RNA-dependent RNA polymerase (RdRp) in the vc strand for virus replication [8].

In recent years, new tospoviruses infecting economically important crops have been reported worldwide. So far, more than 29 different tospoviruses have been reported [9–12]. In our previous study, a new tospovirus (isolate 2009-GZT) collected from tomato in Guizhou province, China, was identified based on the characterization of S RNA (accession no. KM355773), particle morphology, and plant host range, and it was provisionally named "tomato necrotic spot virus" [11]. However, we found that a new member of the genus *Ilarvirus* was named tomato necrotic spot virus (ToNSV) in the Ninth Report of the International Committee on Taxonomy of Viruses (ICTV) [9]. To avoid confusion, we renamed the tospovirus isolate 2009-GZT "tomato necrotic spot-associated virus" (TNSaV). In this study, the full-length sequences of the M and L RNAs of TNSaV isolate 2009-GZT were determined, and the molecular characterization and phylogenetic relationships of NSm, Gn/Gc and RdRp to those of other tospoviruses were analyzed.

The TNSaV isolate 2009-GZT was maintained in the systemic host *Nicotiana benthamiana* and the local-lesion host *Chenopodium quinoa* by mechanical inoculation as described previously [12]. The inoculated plants were cultivated under temperature-controlled greenhouse conditions (25 °C). TNSaV-infected leaves of *C. quinoa* were harvested 3 days post-inoculation (dpi), and total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primer pairs designed for amplification of M and L RNA segments are listed in Supplementary Table 1. Primer J13 (5′-CCCGGATCCAGAGCAAT-3′) was designed to bind to the highly conserved tospoviral 5′ and 3′ termini to facilitate RT-PCR amplification of the ends of the M and L segments. The cDNA was synthesized using EpiScript™ reverse transcriptase (Epicentre Technologies, Madison, USA). Amplified DNA fragments were cloned using a TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA). The positive clones were sequenced, and the nucleotide sequences of the M and L RNAs of TNSaV were assembled using DNAMAN software, version 5.0 (LynnonBiosoft, QC, Canada). The tospoviral sequences used for comparison were obtained from the GenBank database, and their accession numbers are listed in Supplementary Table 2. Phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replications in MEGA 6.0. The putative peptide cleavage sites, transmembrane domains, and N- and O-linked glycosylation sites of the Gn/Gc polyprotein were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) [13]. TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) [14], NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), and NetOGlyc4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/) [15].

The results showed that the complete sequence of the M RNA of TNSaV (accession no. KT984753) has 4,773 nt and has 54-nt and 47-nt 5′- and 3′-untranslated regions (UTRs), respectively, and two open reading frames (ORFs), NSm and glycoprotein precursor (Gn/Gc), separated by a 372-nt AU-rich intergenic region (IGR). The NSm ORF ranges from nt 55 to nt 984 of the M RNA and encodes a protein of 309 aa (34.4 kDa). The TNSaV NSm gene shares higher nt (65.7 %-78.8 %) and aa (64.6 %-86.2 %) sequence identity with those of WSMoV serogroup members, including tomato zonate spot virus (TZSV), watermelon bud necrosis virus (WBNV), watermelon silver mottle virus (WSMoV), groundnut bud necrosis virus (GBNV), capsicum chlorosis virus (CaCV), and calla lily chlorotic spot virus (CCSV), than with members of the IYSV serogroup, including iris yellow spot virus (IYSV), tomato yellow ring virus (TYRV), polygonum ringspot virus (PoLRSV), and hippeastrum chlorotic ringspot virus (HCRV) (Supplementary Table 3). However, it shares lower nt (47.1 %-55.2 %) and aa (37.3 %-44.8) sequence identity with those of the TSWV serogroup (Table 3). As in other plant viruses, the consensus motifs of the "30 K" superfamily of virus movement proteins were found in the NSm protein of TNSaV (data not shown).

The Gn/Gc ORF extends from nt 4728 to 1354 of the M RNA of TNSaV on the vc-strand and encodes a precursor protein of 1123 aa (128 kDa). The Gn/Gc gene shares 66.8 %-77.3 % nt and 65.8 %-86.9 % aa sequence identity with those of TZSV, CCSV, GBNV, CaCV, WBNV, WSMoV, tomato necrotic ringspot virus (TNRV), PoLRSV, TYRV, IYSV, HCRV and melon yellow spot virus (MYSV) (Supplementary Table 3). The predicted protein domains of the TNSaV Gn/Gc are similar to those of several tospoviruses in the WSMoV serogroup [16, 17].

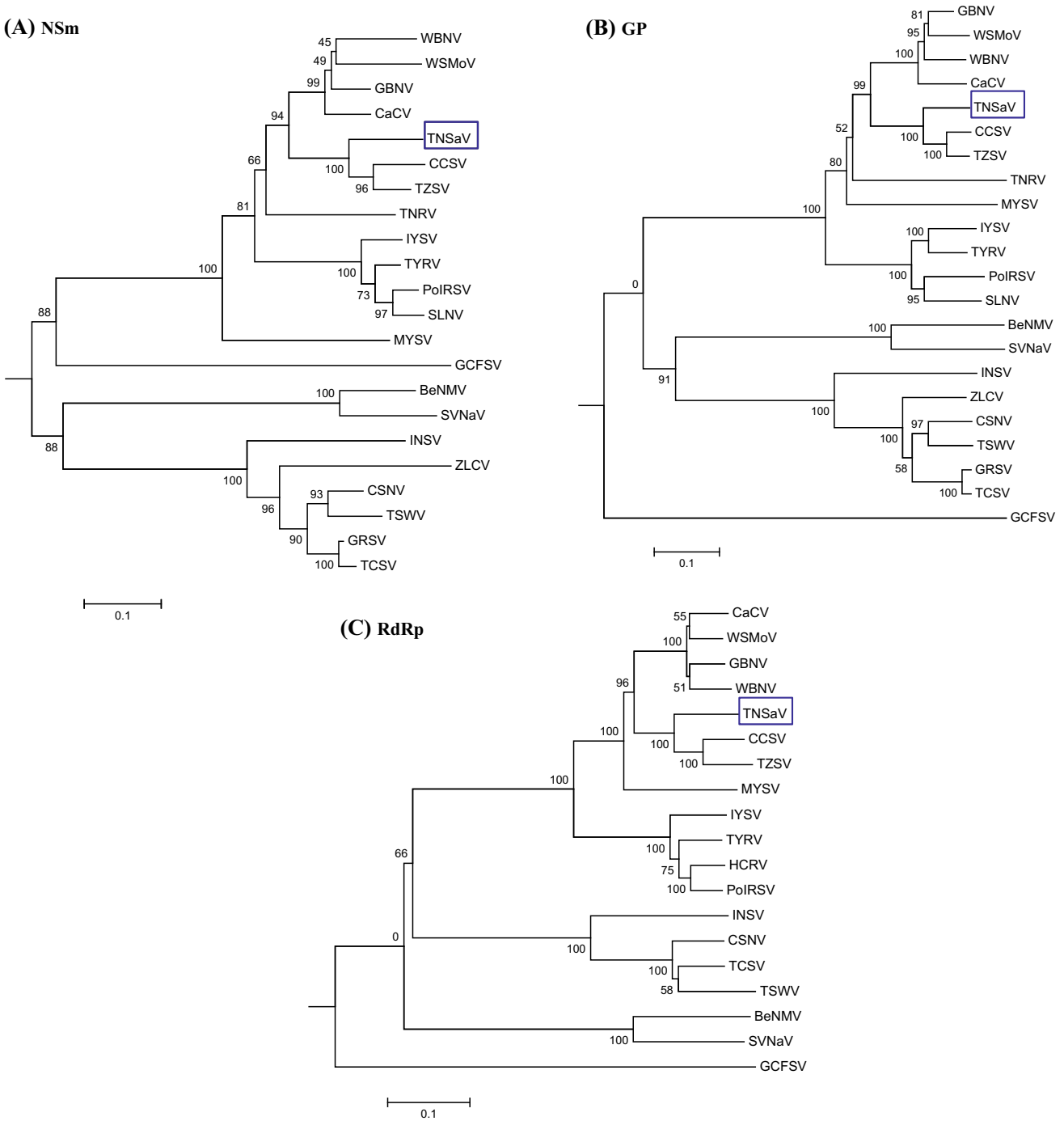


**Fig. 1** Phylogenetic trees of tospoviruses based on the amino acid sequences of the NSm protein (A), glycoprotein (Gn/Gc) (B) and RdRp (C). The analyses were conducted using MEGA6.0 software. Trees were constructed by the neighbor-joining method based on 1,000 bootstrap replicates

The TNSaV Gn/Gc possesses a signal peptide cleavage site located at aa 23/24 (SEV/FL) and an internal peptide cleavage site at aa 436/437 (PSSMA/IK), at which the glycoprotein precursor is separated into Gn (47.7 kDa) and Gc (77.5 kDa) proteins. The Gn/Gc of TNSaV has five transmembrane domains, two possible O-linked *N*-acetyl-galactosamine glycosylation sites and four N-linked glycosylation sites. The Gn protein of TNSaV as well as all members of the WSMoV and IYSV serogroups lacks the RGD motif that is found in members of the TSWV serogroup.

The TNSaV L RNA (accession no. KT984754) is 8,908 nt in length and consists of a 221-nt 5′-UTR and a 27-nt 3′-UTR, as well as a large ORF extending from nt 29 to 8686

in the vc strand, encoding a 2885-aa (332-kDa) RdRp. The RdRp gene of TNSaV shares high nt (71.9 %-76.2 %) and aa (77.9 %-85.2 %) sequence identity with those of WSMoV-serogroup tospoviruses, including TZSV, CCSV, GBNV, CaCV, WBNV, and WSMoV, followed by the IYSV serogroup (67.2 %-70.7 % nt sequence identity and 68.5 %-75.2 % aa sequence identity) and the TSWV serogroup (54.2 %-54.8 % nt sequence identity and 44.7 %-47.0 % aa sequence identity) in Supplementary Table 3. The RdRps of all viruses in the family *Bunyaviridae* have been shown to share six characteristic motifs [18–21] that are also present in the TNSaV RdRp, including DxxKWS (motif A), QGxxxxxSS (motif B), SDD (motif C), K (motif D), EFxSE (motif E) and KxQxxxxxR (motif F) (Supplementary Fig. 1). Phylogenetic relationships based on NSm, Gn/Gc and RdRp indicate that TNSaV is closely related to CCSV, TZSV and WSMoV (Fig. 1).

The serological assays showed that TNSaV can positively react with rabbit antisera against WSMoV NP, CCSV NP and TYRV NP, but not with rabbit antisera against groundnut chlorotic fan-spot virus (GCFSV) NP and TSWV NP (Supplementary Fig. 2). Phylogenetic analysis of all tospoviral proteins, NP, NSs, NSm, Gn/Gc and RdRp, revealed that TNSaV is mostly closely to TZSV and CCSV. These results indicate that TNSaV is closely related to members of the WSMoV serogroup.

**Compliance with ethical standards**

I have read and have abided by the statement of ethical standards for manuscripts submitted to Archives of Virology.

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**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no conflict of interest.

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