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Southern tomato virus: The link between the families *Totiviridae* and *Partitiviridae*☆

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ABSTRACT

A dsRNA virus with a genome of 3.5 kb was isolated from field and greenhouse-grown tomato plants of different cultivars and geographic locations in North America. Cloning and sequencing of the viral genome showed the presence of two partially overlapping open reading frames (ORFs), and a genomic organization resembling members of the family *Totiviridae* that comprises fungal and protozoan viruses, but not plant viruses. The 5′-proximal ORF codes for a 377 amino acid-long protein of unknown function, whereas the product of ORF2 contains typical motifs of an RNA-dependant RNA-polymerase and is likely expressed by a +1 ribosomal frame shift. Despite the similarity in the genome organization with members of the family *Totiviridae*, this virus shared very limited sequence homology with known totiviruses or with other viruses. Repeated attempts to detect the presence of an endophytic fungus as the possible host of the virus failed, supporting its phytoviral nature. The virus was efficiently transmitted by seed but not mechanically and/or by grafting. Phylogenetic analyses revealed that this virus, for which the name Southern tomato virus (STV) is proposed, belongs to a partitivirus-like lineage and represents a species of a new taxon of plant viruses.

1. Introduction

Extraction and analysis of double-stranded RNA (dsRNA) has proven to be a useful approach for studying virus-like diseases of unknown etiology (Dodds, 1993). It is based on the premise that healthy plants normally do not contain detectable amounts of high molecular weight dsRNA and, when present, dsRNA is considered an indicator of infections by ssRNA, ambi-sense or dsRNA viruses. Although the majority of plant viruses have ssRNA genomes, several families/genera of dsRNA viruses are known to infect plants, i.e. *Partitiviridae*, *Reoviridae* and *Endornavirus*.

DsRNA isolation has been employed in an attempt to identify the causal agent of three virus-like diseases reported in tomato. The first disorder, 'tomato decline', was reported in 1984 and it affected entire fields of fresh and processing tomato production in the Imperial Valley of California, USA. Affected plants of cultivars/hybrids 'UC-82', 'Celebrity', and 'Peto95' displayed virus-like symptoms consisting of general yellowing, decline, and poor fruit set. Although viral involvement was suspected, the causal agent had not been identified (Laemmlen et al., 1985). DsRNA analysis yielded a 3.5 kb dsRNA from cultivars 'UC-82' and 'Celebrity', but not from 'Peto95'. Attempts to transmit the putative causal agent from diseased tomato 'UC-82' to healthy plants of cv. 'Rutgers' by grafting failed (Valverde, unpublished data). In 2005, a disease called 'tomato little leaf' was observed in greenhouse-grown tomato plants in southwestern Mexico. Affected plants exhibited stunting of the growing tips, fruit discoloration, and reduced fruit size, resulting in lower than expected yield and reduced market quality (Fig. 1). Mature plants with a heavy fruit load often exhibited interveinal chlorosis with green vein banding. Various tests were conducted to determine if a virus was the causal agent. A dsRNA of about 3.5 kb was isolated from symptomatic tomato plants. In

☆ Sequences reported in this paper have been deposited in the NCBI GenBank database and have been assigned the accession numbers DQ361006, DQ361007, EU413670, and EF442780.

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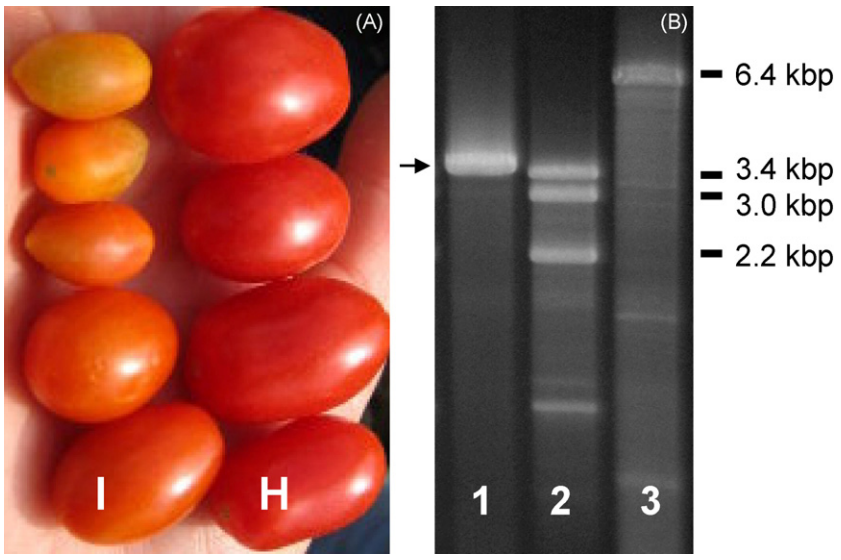

Fig. 1. (A) Discoloration and size reduction of the tomato fruits from Molina, Mexico (first row, I), alongside fruits from healthy tomato (second row, H). (B) Agarose gel electrophoresis of dsRNAs extracted from infected tomato MS-7 (lane 1, arrow) compared with replicative forms of *Peanut stunt virus* (lane 2) and *Tobacco mosaic virus* (lane 3) from infected tobacco plants. Molecular weights of reference dsRNAs are indicated.

the same year, tomato plants of an unknown cultivar showing general yellowing and poor growth were collected from a tomato field in northeastern Mississippi. As in the two former examples, attempts were made to determine the causal agent and a 3.5 kb dsRNA molecule was isolated.

Owing to the exchange of similar experimental results among the authors, it was evident that the same putative virus was being isolated from symptomatic tomato plants from different areas and tomato cultivars. Here we present evidence for a new type of dsRNA virus found in plants affected by tomato yellow stunt disease, a name used for the disorders observed in all three cases presented, and herein designate the virus as Southern tomato virus (STV).

2. Materials and methods

2.1. Plant materials

Plant materials used throughout this study included tomato accessions collected in greenhouses in Colima, Mexico (MT-1), an unknown tomato cultivar collected in northeastern Mississippi (designated MS-7) and cvs. 'UC-82', 'Peto95', and 'Celebrity' from California. Symptomatic tomato plants were tested by ELISA for the presence of *Tobacco mosaic virus*, *Cucumber mosaic virus*, *Tomato spotted wilt virus* and *Tomato mosaic virus*, by RT-PCR for potyviruses and by PCR for begomoviruses using genus-specific primers as described previously (Gibbs and Mackenzie, 1997; Rojas et al., 1993; Wyatt and Brown, 1996). In the case of the samples from Mexico, additional RT-PCR tests were performed in the Arizona laboratory to test for the recently described Tomato apex necrosis virus and Tomato torrado virus (Turina et al., 2007; Verbeek et al., 2007).

2.2. Double-stranded RNA (dsRNA) isolation and analysis

The dsRNAs were isolated from various sources of tomato leaf tissue by clarification with phenol and chromatography through CF-11 cellulose columns following the methods reported by Valverde et al. (1986) or Yoshikawa and Converse (1990). After selective digestions with DNase and RNase under high salt conditions, dsRNAs were analyzed by agarose and/or 6% polyacrylamide gel electrophoreses (PAGE) and their approximate molecular sizes were estimated by comparison with the dsRNA replicative forms of *Peanut stunt virus* and *Tobacco mosaic virus* as internal reference markers. A total of 26 different tomato genotypes were examined for dsRNA (Table 1).

2.3. Attempts to purify virions

Leaf tissue of greenhouse-grown, dsRNA-positive 'UC-82' was homogenized in a blender 1:1 (w/v) in 0.2 M potassium phosphate buffer, pH 7.0. After filtering through cheesecloth, the extract was mixed with chloroform/butanol (1:1), agitated for 15 min at room temperature, and clarified by low speed centrifugation (8000 × *g* for 15 min). The supernatant was centrifuged at 90,000 × *g* for 2 h

Table 1
Tomato genotypes tested for the presence of Southern tomato virus by dsRNA and/or RT-PCR (NT – not tested).

Genotype	dsRNA	STV RT-PCR	Seedlings tested (infected/total)
Better Boy	+	+	3/10
Bonny Best	–	NT	0/5
Celebrity	+	+	5/5
Duke	–	–	0/5
Early Girl	–	NT	0/5
Fantastic	–	NT	0/10
Fireball	–	–	0/10
H-100	–	–	0/5
Hybrid Fresh Market	+	NT	2/5
Jackpot	–	NT	0/5
Loica	+	+	8/10
Marglobe	–	NT	0/5
Ozark Pink	–	–	0/5
Peto95	–	NT	0/10
Pink Red	–	NT	0/5
Plantaco	–	NT	0/10
Quilquil	–	–	0/5
Rutgers	–	–	0/10
Saturn	–	NT	0/5
Super Beefsteak	–	–	0/10
Sweet Heart	+	+	6/10
Sunny	+	NT	7/10
Traveler 76	–	NT	0/5
Trust	+	+	8/10
UC-82	+	+	5/5
Venus	–	–	0/10
Yellow Stuffer	–	NT	0/5

and the resulting pellet was resuspended in 0.02 M potassium phosphate buffer, pH 7.0. Alternatively, polyethylene glycol (MW 6000) was added to the supernatant to a final concentration of 8%, followed by alternate low- and high-speed centrifugations. Pellets were resuspended in 0.02 M phosphate buffer pH 7.0, negatively stained with 2% uranyl acetate and viewed with a JEOL JEM 100CXII electron microscope.

2.4. cDNA cloning and sequencing

Purified dsRNAs from 'UC-82', MT-1, and MS-7 were used as template for cDNA synthesis following the procedures described by Tzanetakis et al. (2005a) and Halgren et al. (2007). Selected plasmids were sequenced at Macrogen Inc. (Seoul, South Korea), MWG Biotech (High Point, NC) or the Life Science Biotechnology Institute of Mississippi State University. After identification of putative viral sequences using BLASTp, the missing regions of the virus genome were obtained as described by Tzanetakis et al. (2005b). Rapid amplification of cDNA ends (RACE) was used to determine the sequences of 5′ and 3′ viral termini. To verify the identity of the 5′ nucleotide (nt), tailing with two different nucleotides was performed as described by Tzanetakis and Martin (2004). Genome sequences deposited in GenBank represent at least a 4× nucleotide coverage. Sequence analysis was performed using DNAStar software (Lasergene, Madison, WI). A search for conserved domains was performed using conserved domain database (CDD) (Marchler-Bauer et al., 2007). Sequence comparisons and phylogenetic analysis were carried out using ClustalW (Thompson et al., 1994) using the default parameters and generated trees were visualized with the TreeView program (Page, 1996).

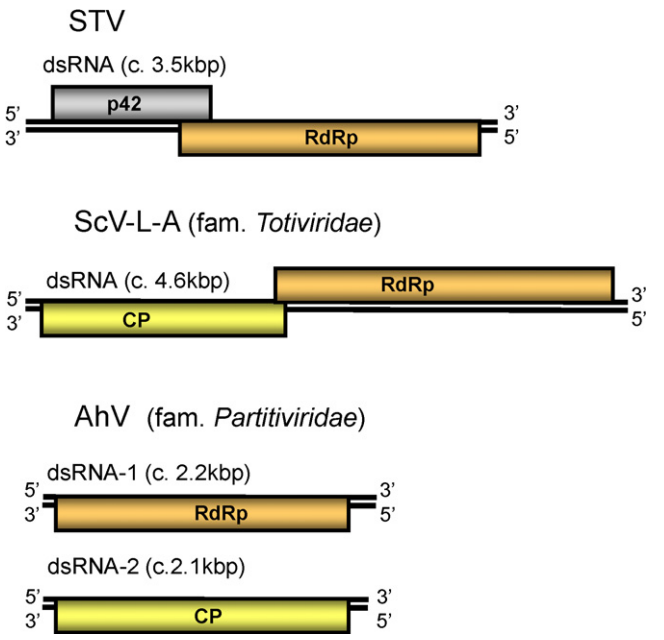

Fig. 2. Diagrammatic representation of genome organization of STV and representative members of family *Totiviridae* and *Partitiviridae*. Proteins with the same function are indicated by the same color.

```
Conf: 976763200358764467541025677899999985467887154056655555677763
Pred: CCCCCCCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHCCCHHHHHHHHHHHHHH
  AA: MAGVGGSAAGRVPNAANVPLTAKEKERTVMREIVEIGETFVELGIDKRYFQRTTYVSHML
              10        20        30        40        50        60

Conf: 525899999973574466766776779989898763053099999887555202430355
Pred: CCHHHHHHHHHHCCCCHHHHHHHHHHHHHHHHHHHHHHCCCHHHHHHHHHHHHCCCCHHHH
  AA: LPNQYFKLLKQFKGKTAEELDLALGAAVAHGVLRSMRGITFKKFFDFLNWVKTKEGKDAL
              70        80        90       100       110       120

Conf: 555889988761777511899999998753233332111100122156799899999997
Pred: HHHHHHHHHHHCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
  AA: GETMYAQKLEQKGRGDFSIAEVALLHCFETQRNDMLRDEKDVRLKAEEEIADLQRKIVKR
             130       140       150       160       170       180

Conf: 886677665410450035654075422003246787752382230135641279999999
Pred: HHHHHHHHHHHCCCCHHHHHHCCCCCCCCCCCHHHHHHHHCCHHHCCCCCCCHHHHHHHHH
  AA: REKLEEDLIATKSNYEPVSRYVGLSDYELNCKCWSLYQQFNPDKVTAGAKPTRKQVKEAF
             190       200       210       220       230       240

Conf: 999999752001456653872478998631011045764421377899988886035322
Pred: HHHHHHHHHHHHHHHHHCCCHHHHHHHHHHHHCCHHHHCCCHHHHHHHHHHHHHCCCCC
  AA: DMYAEFVARTNRLEFLKHGNVKDELQAFINAKILGYEQLGDKGKSRYLRRQLAAMGVEMD
             250       260       270       280       290       300

Conf: 10011015665315578763227886730000478987433465320012124122123 4
Pred: CCCCCCHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCEEECCCHHHCCC
  AA: HEVESEDEEEAAEEPAGGEAEEGEGDDPLQATVGDNPDGEARGEEDTAVQVASPAVTRNR
             310       320       330       340       350       360

Conf: 66666426230101279
Pred: CCCCCCCCCCCHHCCCC
  AA: GRGRGRVTPLSQRKDRG
             370
```

Fig. 3. Predicted secondary structure of the putative protein encoded by the ORF1 of STV generated by the PsiPred software. Note almost complete absence of β-strand structures (represented with a letter E and boxed). Pred: predicted secondary structure; H: helix; E: strand; C: coil; AA: target amino acid sequence; Conf: confidence.

(A)

```
                    4
VCV                 LDWSRFDKRAYFPLLRKIMYTVKSFLTFEEGYVPTHAAPNHPQWNQDKTDKLERLWLWTL 60
WCCV-1              LDWSRFDKRAYFPLLRRILYTVKTFLTFEEGYVPTHAAPTHPQWSQENIDRLERLWLWTL 60
HaV                 IDWSRFDKRAYFWLIRKIFIRTRQFFDFENGYTPTKDYP----FSPTHPDKLQALWEWTI 56
ZbV-Z               ADYSKFDSSVPACVVRKAFQIFKELFICFSIILP------------------LLFMILI 41
BCV-3               LDWSSFDSSVTRFEINCAFNLLKERIEFPNEETELAFELSR------------------- 41
PCV                 LDWSQFDATVSRFEIHAAFDIIKSYVDFPNYETEQAFEITR------------------- 41
PinSV               IDWSGFDASVSAEGIDFAFSLIKRMLTFNSELDELCFEVSR------------------- 41
AoV                 LDFSSFDSKVPSWLIRVAFNILRQNINFETWNGQPVSKRDRQ--------KWRNVWDAMV 52
STV                 LDWSKFDRERPSEDLDFMIKVICSCFRPKDEVEAKLLRGYG--------------VCMR 45
                     *:* **       :   :       .

                                                5
VCV                 ENLFEAPIILPDGRMYRRHFAGIPSGLFITQLLDSWYNYTMLATILHALGFNPSNCIIKV 120
WCCV-1              ENLFEAPIILPDGRMYRRHFAGIPSGLFITQLLDSWYNYTMLATILSALHFDPLHCIIKV 120
HaV                 EAFFDSPIVLPNGHVYKRRFAGIPSGLFITQLMDSWYNYVMLCSILHYMDIDPKTCIIKV 116
ZbV-Z               ENFLNSKVDLSDGRLLQK-FGGVPSGSGFTSIIGSICNYIMLDTCFMKMGLDKNRWSAIV 100
BCV-3               ILFKHKKLAAPDGNIYMI-HKGIPSGSYYTSIVGSVVNRLRIEYIWRVLFSRSPHRCYTQ 100
PCV                 QLFIHKKVAAPDGYIYES-HKGIPSGSYYTSLVGSIINYLRINYLWRLLTGHPPQQCHTL 100
PinSV               LNFIHRKLIDPEGRILLK-HRGIPSGSYYTMLVDTIINARRILYMFHHLTGELP-TVHCQ 99
AoV                 YYFINTPILMPDGRMFRK-YRGVPSGSWWTQMVDSVVNDILVQYIC-LCQEIEPRDLRVL 110
STV                 RALVERRLITDDGGVIHI-DGMVPSGSLWTGWLDTALNILYIKSVLRSIDILEEEAVPKC 104
                      : .  :   :* :       :***   *  :.:  *   :

                    6
VCV                 QGDDSI 126
WCCV-1              QGDDSI 126
HaV                 QGDDSI 122
ZbV-Z               YGDDCL 106
BCV-3               -GDDSL 105
PCV                 -GDDSL 105
PinSV               -GDDSL 104
AoV                 -GDDSA 115
STV                 AGDDNL 110
                     ***
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(B)

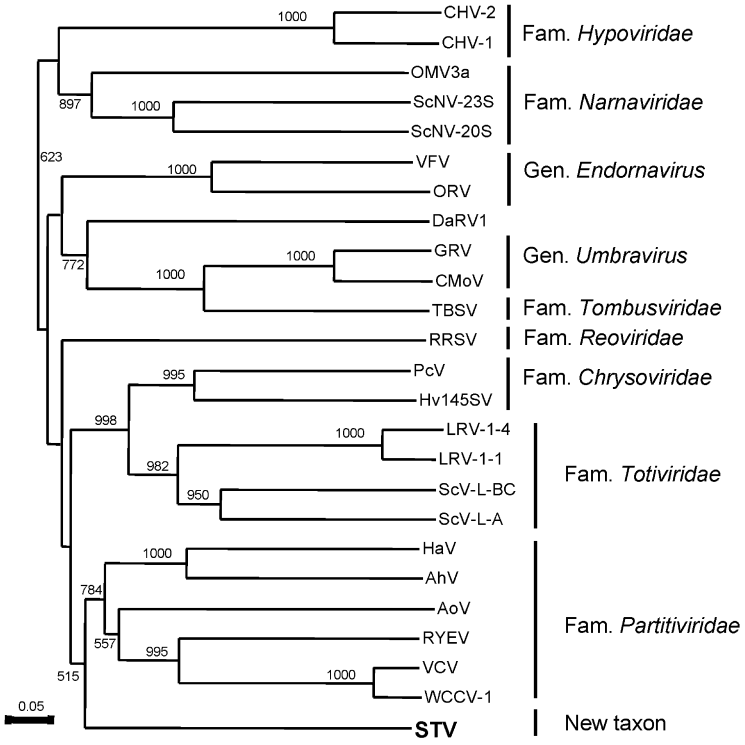

Fig. 4. (A) Comparison of motifs 4–6 of the viral RdRps of STV and some partitiviruses. Asterisks denote identical residues in all proteins used in analysis. Viruses used for comparison are: *Vicia cryptic virus* (VCV), *White clover cryptic virus 1* (WCCV-1), *Heterobasidion annosum virus* (HaV), Zygosaccharomyces bailii virus Z (ZbV-Z), *Beet cryptic virus 3* (BCV-3), Pepper cryptic virus-1 (PCV-1), *Aspergillus ochraceous virus* (AoV) and Pinus sylvestris partitivirus NL-2005 (PinSV). (B) Unrooted phylogenetic tree generated from the alignment of the amino acid sequences of motifs 4–6 (Bruenn, 1993) of the putative RdRp of STV and viral species of several families infecting plants/fungi/protozoa. Viruses used in analyses and their GenBank accession numbers are: *Atkinsonella hypoxylon virus* (AhV, NP_604475), *Aspergillus ochraceous virus* (AoV, EU118277), *Heterobasidion annosum virus* (HaV, AAK52739), *Radish yellow edge virus* (RYEV, AY748911), *Vicia cryptic virus* (VCV, EF173396), *White clover cryptic virus 1* (WCCV-1, NC_006275),

2.5. Northern blots

DsRNA of isolates MS-7 and UC-82 were run on a 1% TAE agarose gel (TAE: 40 mM Tris–acetate, 1 mM EDTA, pH 8.0) and denatured by soaking the gel in 50 mM NaOH for 30 min, neutralized for 15 min with 1.5 M Tris–HCl (pH 7.5) containing 0.5 M NaCl and double rinsed with bidistilled RNase-free water. DsRNA was then transferred to a Hybond N+ nylon membrane by capillary blotting using 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0). After transfer, dsRNAs were cross-linked to the membrane by UV radiation for 2 min. Membranes were hybridized with a digoxigenin (DIG)-labeled probe complementary to the genomic segment between nt 2731 and 3038 according to the manufacturer's instruction (Roche Applied Science, IN, USA). DIG-labeled DNA Molecular weight marker III (Roche Applied Science, IN, USA) was used as a reference. The same probe was used in hybridization tests on total RNAs extracted from STV-infected tissue and healthy controls.

2.6. Detection

Total RNAs were extracted from symptomatic and asymptomatic tomato leaves using the Plant RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was heat denatured at 95 °C for 5 min and reverse transcribed by Superscript Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) in a total volume of 30 μl. Five microlitres of cDNA was submitted to PCR with a virus-specific primer pair, STV-F (5′-CGTTATCTTAGGCGTCAGCT-3′) and STV-R (5′-GGAGTTTGATTGCATCAGCG-3′), designed to amplify a 440 bp portion of the viral genome. After initial denaturation at 94 °C for 2 min, the cDNAs were amplified by 35–40 cycles as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 53 °C, extension for 45 s at 72 °C and a final extension for 10 min at 72 °C. PCR products were analyzed by electrophoresis through ethidium bromide-stained 1.5% agarose gels in TAE buffer, pH 8.0.

DsRNA-positive tomatoes were tested for the presence of endophytic fungi by molecular methods, and by light and electron microscopy. For molecular tests, DNA was extracted from 0.1 g of leaf tissue from cvs. 'Loica', 'Celebrity' and 'Better Boy' using the DNeasy Plant Minikit (Qiagen) and then subjected to PCR using the broad-spectrum fungal primers, ITS-1/ITS-4 (White et al., 1990). Light and transmission electron microscopic observations were performed on thin-sections of diseased tomato leaf tissue as described by Martelli and Russo (1984).

2.7. Transmission studies

Attempts to transmit the putative virus by mechanical transmission were conducted using tomato tissue homogenized in 0.1 M phosphate buffer, pH 7.2, and rubbing it onto celite-dusted leaves of herbaceous hosts including: *Nicotiana benthamiana*, *N. tabacum*, *Chenopodium amaranticolor*, and tomato seedlings cv. Rutgers.

To attempt graft-transmission of the putative dsRNA virus associated with diseased plants, scions from disease-free and dsRNA negative tomatoes (cv. 'Rutgers') were grafted onto dsRNA-positive 'Celebrity' rootstocks. The inoculated plants were observed periodically for symptoms. In addition, scion leaves were collected monthly for 3 months, and tested for the presence of STV by RT-PCR and PAGE.

In order to study the incidence of this virus in different tomato genotypes, 5–10 seedlings grown from commercially available seeds or obtained directly from retail stores were tested by dsRNA and/or virus-specific RT-PCR as described. To investigate the possible vertical transmission of the dsRNA virus, seeds were collected from ripe tomato fruits of self-pollinated, dsRNA-positive plants of cv. Celebrity. Seeds were planted and grown under greenhouse conditions. Seedlings were collected randomly and tested for the presence of dsRNA and/or for STV using virus-specific RT-PCR.

3. Results

When tomato leaf samples tested negative by ELISA and/or RT-PCR for viruses that commonly infect tomato, dsRNA was extracted to determine if the causal agent of the disease could be a previously undescribed RNA virus. Using this approach a prominent 3.5 kb dsRNA band (Fig. 1) was obtained from diseased plants of 'UC-82', 'Celebrity', MT-1, and MS-7. No additional bands that may indicate the presence of satellite and/or defective RNAs were observed in purposely overloaded polyacrylamide or agarose gels (not shown). The concentration of the dsRNA was estimated at ca. 1.4 μg/g of tomato tissue (mean of three independent tests). Moreover, all four samples from Mexico and five of seven plants from Mississippi contained this dsRNA species. The dsRNA, however, was not detected in the symptomatic 'Peto95' plants collected from a field in California. All attempts to transmit the putative virus to selected herbaceous hosts, including tomato, either by mechanical inoculation or by grafting, failed. The inoculated plants did not yield any dsRNA and tested negative for the 3.5 kb dsRNA by RT-PCR, confirming that the molecule was not transmitted by any of the methods used. Purification attempts from dsRNA-positive tomatoes failed to yield virions.

The complete genome sequences of two STV isolates, from Mexico (MT-1) and Mississippi (MS-7), and partial sequences from 'UC-82' were deposited in GenBank and were given the accession numbers EF442780 (MT-1), EU413670 (MS-7) and DQ361006 and DQ361007 ('UC-82'). The genome length of MT-1 and MS-7 isolates was identical, each consisting of 3437 bp. Pairwise comparisons showed that they differed with respect to nine nucleotides (nt) at 0.26% divergence, with A-to-G substitutions encountered five times. Similar results (sequence identity >99%) were obtained when the partial sequences of 'UC-82' and two additional isolates from Mexico were compared (data not shown), revealing extremely high sequence conservation among isolates obtained from various tomato cultivars grown in different geographic locations.

STV contains 51.6% A+U and analysis of the genome showed the presence of two partially overlapping open reading frames (ORFs) on the positive-sense RNA strand, a genome organization resembling members of the *Totiviridae* (Fig. 2). The 5′ and 3′ untranslated regions (UTRs) were 137 and 110 nt long, respectively. Both UTRs were richer in A + U than the coding regions of the genome (ca. 61% and 68% in the 5′ and 3′ UTR regions, respectively). The 5′ end of the positive-strand RNA was uncapped and highly structured, while the 3′ end did not contain a poly(A) tract.

ORF1 encodes a putative 377 amino acids (aa) protein with a molecular mass of 42.4 kDa (p42), initiated at nt 138 and termi-

Leishmania RNA virus 1-4 (LRV-1-4, NC_003601), *Leishmania RNA virus 1-1* (LRV-1-1, NC_002063), *Saccharomyces cerevisiae virus L-A* (ScV-L-A, NC_003745), *Saccharomyces cerevisiae virus L-BC* (ScV-L-BC, NC_001641), *Helminthosporium victoriae 145S virus* (Hv145SV, NC_005978), *Penicillium chrysogenum virus* (PcV, NC_007539), *Rice ragged stunt virus* (RRSV, NC_003771), *Tomato bushy stunt virus* (TBSV, NC_001554), *Carrot mottle virus* (CMoV, NC_011515), *Groundnut rosette virus* (GRV, NC_003603), Diaporthe ambigua RNA virus 1 (DaRV-1, NC_001278), *Ophiostoma mitovirus 3a* (OMV3a, NC_004049), *Saccharomyces 20S RNA narnavirus* (ScNV-20S, NC_004051), *Saccharomyces 23S RNA narnavirus* (ScNV-23S, NC_004050), *Cryphonectria hypovirus 1* (CHV-1, NC_001492), *Cryphonectria hypovirus 2* (CHV-2, NC_003534), *Vicia faba endornavirus* (VFV, NC_007648), *Oryza rufipogon endornavirus* (ORV, NC_007649).

nated with a UGA termination codon at nt 1269. BLASTp searches revealed no statistically significant matches with sequences available in GenBank. The same result was obtained following a search for conserved domains in CDD. Because the ORF1 in totiviruses encodes the viral coat protein, a pairwise comparison was performed with coat proteins of different dsRNA viruses belonging to the *Totiviridae* and *Partitiviridae*. The analyses revealed less than 10% amino acid sequence similarity between STV and members of these two viral families (data not shown).

In addition, folding predictions using PSIPRED software (McGuffin et al., 2000) revealed that secondary structure of this protein consisted almost exclusively of helices and coiled folding patterns (Fig. 3). Coat proteins encoded by totiviruses and partitiviruses normally fold to form β-strand-rich secondary structures that are almost completely absent in STV p42. The overlapping region between ORFs 1 and 2 was 293 nt long and ORF2 was in the +1 frame relative to ORF1.

The second cistron spanned the region from nt 1039 to 3325, predicted to encode a 762 aa protein with a molecular mass of 87.4 kDa (p87). This protein is likely expressed via the +1 frameshift as a fusion protein with ORF1 to produce a "gag/pol-like" protein with molecular mass of 121.5 kDa (p122). The heptanucleotide sequence, GGGAAGA, located between nts 984 and 990 was identified as a putative slippery sequence that could facilitate the ribosomal frameshift. Computer-assisted secondary structure prediction using the KnotSeeker program (Sperschneider and Datta, 2008) detected pseudoknot structures that could be involved in this phenomenon immediately downstream of the putative frameshift site (nt position 1005–1087; not shown).

A search for conserved domains revealed several viral RNA-dependent RNA-polymerases (RdRp) conserved motifs, located between aa 596 and 791 of p122. The segment between aa 616 and 722 of the p122 contained RdRp motifs 4–6 (Bruenn, 1993) and represented the most conserved region of the polypeptide (Fig. 4A). This portion of the polyprotein shared minimal aa sequence similarity (<20%) with several myco- and plant cryptic viruses including (GenBank accession numbers are shown parenthetically): Zygosaccharomyces bailii virus Z (ZbV-Z, a putative totivirus; NP_624325), Pepper cryptic virus-1 (PCV-1, a putative partitivirus; ABC96789) and *Beet cryptic virus 3* (BCV-3, gen. *Alphacryptovirus*, fam. *Partitiviridae*; S63913).

In phylogenetic trees, either using the whole product encoded by ORF2 or conserved RdRp motifs 4–6, STV grouped weakly with members of the family *Partitiviridae* (Fig. 4B) indicating that it indeed belongs to a partiti-like lineage rather than to the *Totiviridae*.

Northern hybridization assays did not reveal the presence of any 3′end-proximal sub-genomic RNAs in two tested isolates. The hybridization signal was obtained only with a full-genomic size molecule (Fig. 5A) in dsRNA and total nucleic acid blots.

Screening of 27 different tomato genotypes for this virus revealed that the dsRNA was present in apparently healthy seedlings of eight cultivars/hybrids ('UC-82', 'Celebrity', 'Sunny', 'Loica', 'Fresh Market', 'Sweet Heart', 'Trust' and 'Better Boy') (Table 1). Seed transmission was confirmed in a separate experiment showing that 15 out of 23 symptomless F1 'Celebrity' plants contained the 3.5 kb dsRNA band (ca. 65%). Additional experiments, using other seed sources of the same cultivar, confirmed the high rates of vertical transmission (70–90%).

Light and transmission electron microscopy trials failed to detect the presence of any fungi, a possible host for this virus, in the tomato tissues examined. PCR using the universal ITS primers yielded an amplicon of about 690 bp from three tomato cultivars that tested positive for virus presence. However, sequences of all 45 randomly selected clones (15 clones/cultivar) proved to be of plant origin, sharing 99–100% nucleotide identity with tomato ribosomal RNA and ITS-1 and -2 sequences.

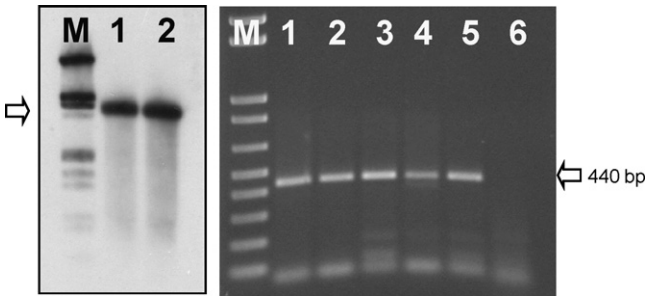

Fig. 5. (A) Northern blots. A single hybridization signal corresponding to the full size genomic RNA (arrow) was detected in tested isolates MS-7 and UC-82 (lanes 1 and 2, respectively). Lane M contains DIG-labeled DNA Molecular weight marker III (Roche Applied Science, IN, USA). (B) Detection of STV by RT-PCR. Amplicons of predicted size (440 bp) are present in infected samples/cultivars 'MS-4', 'MS-7', 'Loica', 'Celebrity' and 'Better Boy' (lanes 1–5, respectively), but not in the experimental control (lane 6). Lane M contains the reference 1 kb Plus DNA ladder (Invitrogen, USA).

In the RT-PCR assays with total RNA extracts, the primers designed to amplify the overlapping region between ORFs 1 and 2 produced the expected 440 bp DNA fragment from all dsRNA-positive samples tested. No amplicons were obtained from the negative experimental controls (dsRNA-free tomato plants) (Fig. 5B and Table 1).

4. Discussion

In this paper we present the detection and characterization of a dsRNA of 3.5 kb originally found in diseased greenhouse- and field-grown tomatoes from three different geographical locations that exhibited symptoms of yellowing, stunting and reduction in fruit quality and quantity. The dsRNA was detected in most tested samples from Mexico, California and Mississippi, with the exception of cultivar Peto95. Data presented here suggest that this dsRNA represents the genome of an unusual dsRNA plant virus that shares traits in common with viruses belonging to the families *Totiviridae* and *Partitiviridae*.

The family *Partitiviridae* includes dsRNA viruses infecting fungi (genus *Partitivirus*) and plants (genera *Alphacryptovirus* and *Betacryptovirus*) (Ghabrial et al., 2005). The dsRNA genome is composed of two segments, each monocistronic, encoding for RNA-dependent RNA-polymerase (dsRNA1) and viral coat protein (dsRNA2). In contrast, viruses belonging to the family *Totiviridae*, which infect fungal or protozoal hosts have undivided dsRNA genomes that contain two partially overlapping ORFs, encoding the viral coat protein and RdRp expressed through a ribosomal frameshift that produces a fusion protein (Wickner et al., 2005). *Helminothosporium victoriae virus 190S* is the only exception and presumably employs internal initiation mechanism to express RdRp (Huang and Ghabrial, 1996; Soldevila and Ghabrial, 2000).

The genome organization of STV is similar to totiviruses (Fig. 2), and consists of a single dsRNA molecule containing two partially overlapping ORFs. The presence of a putative slippery site followed by pseudoknot structures, both shown to be involved in ribosomal frameshift (Tzeng et al., 1992), suggests that the two proteins are likely expressed as a fused product. Pairwise comparisons and phylogenetic analyses of RdRps of various dsRNA and ssRNA viruses infecting plants/fungi/protozoas with that of STV suggest that it belongs to a partitivirus-like lineage rather than to the totivirus group.

Considering that the viral nucleic acids (dsRNAs) were abundant and easy detectable in host tissues and the failure to observe any associated virus particles, it may be concluded that this virus may poorly form virions or exist in the cell in the form of non-

encapsidated nucleic acids. The first hypothesis is in agreement with the properties of plant-infecting partitiviruses (cryptoviruses) virions of which are present at very low concentration in host tissue (Boccardo et al., 1987). However, the "standard" protocol applied for purification of plant cryptoviruses (fam. *Partitiviridae*) did not yield any virions.

According to the second scenario, STV may not form classical virions, but rather may exist and replicate in the form of "naked" nucleic acid molecules, comparable to life-cycles of endornaviruses (Gibbs et al., 2005), umbraviruses (Taliansky et al., 2005), or fungi-infecting hypoviruses (Nuss et al., 2005) and narnaviruses (Buck et al., 2005), that form peculiar structures ("virus-like particles") consisting of cytoplasmic vesicles with RdRp activities associated with genomic nucleic acids.

This hypothesis is supported by the computer-assisted analyses of p42. BLAST searches (including PSI- and PHI-BLAST) did not reveal statistically significant homology of p42 with other proteins, including coat proteins of totiviruses and partitiviruses. Analysis of the secondary structure of the protein revealed a unique folding pattern in that, unlike the coat proteins of other totiviruses and partitiviruses, the protein encoded by ORF1 virtually lacked β-sheet structures. Furthermore, the predicted coat protein of STV is much smaller in size (42 kDa) compared to the analogous totiviruses proteins at 73–88 kDa (Wickner et al., 2005). The differences between STV p42 and proteins encoded by the ORF-1 in totiviruses and/or partitiviruses indicate that p42 may not be the viral CP. Further electron microscopic, serological and molecular studies are needed to determine the function of p42 protein.

The biological properties of STV, including its seed transmission, are similar to previously described plant viruses with dsRNA genomes, partitiviruses and endornaviruses (Boccardo et al., 1985; Valverde and Gutierrez, 2007). Other means of transmission are unknown. Taking into account the apparently high incidence of this virus in affected tomato fields, we cannot exclude transmission by an aerial vector, possibly involving a helper-virus mechanism, similar to umbraviruses. The lack of proof for a helper-virus, after having sequenced hundreds of clones derived from dsRNA templates, could be due to a very low titer of the helper-virus or alternatively a minus strand RNA virus that produces or yields insufficient amounts of dsRNA (Tzanetakis, personal observation).

STV appears strongly implicated as a component of the disease, given a consistent association with similarly affected tomato plants from multiple geographical regions, with which no other plant viruses could be detected during sample analysis by five different laboratories. Failure to transmit STV by grafting and/or mechanical inoculations, and the inability to purify virions from infected tomato plants have impeded our ability to determine its exact role in the etiology of the tomato yellow stunt disease. However, its absence in certain symptomatic samples (i.e. cv. 'Peto95') together with the lack of symptoms in the seedlings of cv. 'UC82', 'Celebrity' and the other five cultivars/hybrids, pose doubts on its pathogenicity.

Although the STV genome organization is similar to totiviruses, it is evolutionarily closer to extant partitiviruses, collectively suggesting that it belongs to a new taxon yet to be established. It is possible that STV represents a missing link between two extant virus families, and could be the "totivirus-like" ancestor from which plant partitiviruses (cryptoviruses) may have arisen, following the separation of the genomes into two dsRNA molecules, as predicted by Ghabrial (1998). Also relevant to this discovery is the recent detection of similar dsRNA viruses in blueberry (Martin et al., 2006), redbud (Tzanetakis, et al., unpublished), and rhododendron (Sabanadzovic et al., unpublished). These discoveries suggest that this type of virus may be more common in nature than currently known.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2008.11.018.

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