A Distinct Strain of *Tomato leaf curl Sudan virus* Causes Tomato Leaf Curl Disease in Oman

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Abstract

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Tomato leaf curl disease (ToLCD) is a significant constraint for tomato production in the Sultanate of Oman. The disease in the north of the country has previously been shown to be caused by the monopartite begomoviruses (family *Geminiviridae*) *Tomato yellow leaf curl virus* and *Tomato leaf curl Oman virus*. Many tomato plants infected with these two viruses were also found to harbor a symptom enhancing betasatellite. Here an analysis of a virus isolated from tomato exhibiting ToLCD symptoms originating from south and central Oman is reported. Three clones of a monopartite begomovirus were obtained. One of the clones was shown to be infectious to tomato and *Nicotiana benthamiana* and to induce symptoms typical of ToLCD. Analysis of the cloned sequences show them to correspond to isolates of *Tomato leaf curl Sudan virus* (ToLCSDV), a virus that occurs in Sudan and

Yemen. However, the sequences showed less than 93% nucleotide sequence identity to previously characterized ToLCSDV isolates, indicating that the viruses represent a distinct strain of the species, for which we propose the name “Oman” strain (ToLCSDV-OM). Closer analysis of the sequences showed them to differ from their closest relative, the “Tobacco” strain of ToLCSDV originating from Yemen, in three regions of the genome. This suggests that the divergence of the “Oman” and “Tobacco” strains has occurred due to recombination. Surprisingly, ToLCSDV-OM was not found to be associated with a betasatellite, even though the isolates of the other ToLCSDV strains have been shown to be. The significance of these findings and the possible reasons for the distinct geographic distributions of the tomato-infecting begomoviruses within Oman are discussed.

The family *Geminiviridae* encompasses phytopathogenic viruses that characteristically have genomes of circular, single stranded (ss)DNA encapsidated in geminate particles. The family consists of four genera, with viruses assigned on the basis of insect vector, host specificity, and genome organization (7,9). The genus *Begomovirus* constitutes the largest genus of the family *Geminiviridae* that includes viruses which are transmitted by the silverleaf whitefly (*Bemisia tabaci* Genad.) and infect a wide range of economically important dicotyledonous crops. The majority of begomoviruses native to the New World (NW) are bipartite, having genomes consisting of two genomic components (known as DNA A and DNA B). Recently, the first monopartite begomovirus, with a genome consisting of only a homolog of the DNA A of bipartite viruses, which is native to the NW, has been identified (20,23). In the Old World (OW), although a small number of bipartite begomoviruses are known, the majority are monopartite (7).

Numerous begomoviruses, of both the monopartite and bipartite types, cause tomato leaf curl disease (ToLCD) in the warmer parts of the world (1). Probably the most important, and geographically widespread, is *Tomato yellow leaf curl virus* (TYLCV). This virus has its origins in the Middle East/Mediterranean and has spread to

most tomato producing regions of the world (16). Monopartite begomoviruses, such as TYLCV, encode six genes. Two genes in the virion-sense encode the coat protein (CP), the only structural protein encoded by geminiviruses that is involved in insect transmission and movement of the virus in plants, and the V2 protein, that is involved in virus movement in plants and overcoming RNA-based host defenses (18,22). The four complementary sense genes encode the replication-associated protein (Rep; the only virus encoded protein required for viral DNA replication; 11), the transcriptional activator protein (TrAP; involved in up-regulating late virus genes and overcoming RNA-based host defenses), the replication enhancer protein (REN; which interacts with Rep and host factors to enhance virus replication; 25), and the C4 protein (which is a symptom determinant and overcomes RNA-based host defenses; 18).

Although described as monopartite, many such begomoviruses are often associated with an additional class of molecules known as betasatellites. The sequences of these ssDNA satellites are unrelated to their helper begomoviruses, and require the helper begomovirus for replication, movement in plants, and transmission between plants (6). In some cases, the helper begomovirus requires the betasatellite to infect the natural host, whereas in other cases the interaction is more relaxed. Although the precise requirement, by the helper virus, for the presence of the betasatellite remains unclear, the betasatellite acts to suppress RNA-based host defenses leading to enhanced viral DNA levels in hosts (8,24).

Previous studies in Oman have shown the presence of TYLCV and *Tomato leaf curl Oman virus* (ToLCOMV; a recombinant virus so far only identified in Oman) in northern regions (14,15). Both viruses were found to be associated with Tomato leaf curl betasatellite (ToLCB), a betasatellite previously only identified in Pakistan and India (15). Here a distinct virus causing ToLCD is shown to be present in south and central Oman. Sequence analyses

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show it to be a distinct strain of *Tomato leaf curl Sudan virus* (ToLCSDV) and to have a recombination pattern that differs from its closest relative occurring in Yemen. The significance of these findings is discussed.

Materials and Methods

DNA extraction and detection of virus. Total genomic DNA was extracted from leaves using a modified CTAB method (21), resuspended in sterile distilled water and maintained at -20°C until use. Samples were screened for the presence of begomoviruses by polymerase chain reaction (PCR)-amplification of a conserved sequence of begomoviruses using primers FDCP-382 (5'-CTCCTCTTCGACAGCCCTA-3') and RDCP-1038 (5'-TGMGTACAXGCCATATACAA-3') that amplify a product of ~650 bp of the precoat protein gene (2). Betasatellites were amplified using primers beta01/beta02 that amplify the full-length satellite (~1,350 bp; 5). Only PCR positive samples were used for further analyses.

The levels of viral DNA in plants were assessed by Southern blot hybridization. DNA samples were electrophoresed on 1% agarose gels in TAE buffer and blotted to nylon membranes (Roche GmbH, Germany). Blots were probed with an ~870 bp fragment of ToLCSDV-Oman clone ToS-93 produced by restriction with *Kpn*I and *Eco*RI. The restriction fragment was eluted and labeled with digoxigenin using a DIG high Prime DNA Labeling and Detection Starter Kit I (Roche GmbH, Germany) as described by the manufacturer.

Virus amplification, cloning, and sequencing. Circular DNA molecules were amplified by rolling-circle amplification (RCA; 10) using an Illustra TempliPhi 100 Amplification kit (GE Healthcare, Upsala, Sweden) following the manufacturer's instructions. RCA products were digested with selected restriction endonucleases to identify enzymes giving full-length (~2.8 kb) fragments. Putative full-length clones were purified using a GeneJET Plasmid Miniprep Kit (Fermentas, Lithuania) and sequenced commercially (Macrogen Inc., South Korea) in both orientations.

Sequence analysis. Sequences were assembled using SeqMan, part of the Lasergene package of sequence analysis software (DNASTar Inc., Madison, WI, USA). Assembled sequences were used to query the NCBI nucleotide sequence database using the nblast algorithm (3). Genes were predicted using ORF Finder (NCBI). Multiple sequence alignments were performed using the Clustal V algorithm in MegAlign (Lasergene) to determine sequence similarity values. The phylogenetic relationship of ToLCSDV-OM with other related begomoviruses was analyzed using maximum parsimony (MP) algorithms available in Phylogenetic Analysis Using Parsimony (PAUP), version 4.0a126 (26). A single most parsimonious tree was obtained by full heuristic search, stepwise addition, and tree-bisection-reconnection random branch-swapping options. Bootstrap values (1,000 iterations) were calculated based on the 70% majority rule and confidence limits were placed at the major nodes. Recombination analyses were performed using SimPlot (version 2.5; 17) and the Recombination Detection Program (RDP version 3; 19).

Agrobacterium-mediated inoculation. A construct for virus inoculation to plants was prepared by cloning an approximately 2-

kb *Pst*I and *Xba*I fragment of the full-length clone Mir1 in the binary vector pGreen0029 (12). The full-length insert of Mir1, excised using *Pst*I and *Bgl*II, was then ligated into pGreen0029 containing the 2-kb fragment at a unique *Pst*I site. The resulting partial direct repeat construct was electroporated into *Agrobacterium tumefaciens* strain AGL1.

Agrobacterium inocula were prepared by growing cultures with antibiotic selection at 28°C for 48 h with continuous shaking. Cultures were then centrifuged and cells resuspended in 10 mM MgCl_2 containing 100 μM acetosyringone to an OD_{600} of 0.6-1 and incubated at room temperature for 3 to 4 h. *Nicotiana benthamiana* and tomato var. Indam plants were infiltrated on the underside of the leaves using 5-ml syringes.

Results

Cloning and sequencing. Leaf samples were collected from tomato plants exhibiting typical ToLCD symptoms in the Mirbat (southern Oman) and Manah (central Oman) Governorates of Oman in 2011 (Table 1). Total genomic DNA was used to PCR amplify an expected product of ~650 bp using CP primers (2). The PCR positive DNA samples were used as template for RCA to amplify circular DNA molecules. Restriction of RCA products with *Pst*I yielded fragments of 2.8 kb, which were cloned into the plasmid vector pUC19. The complete sequences were determined for three begomovirus isolates (Mir1, Mir2, and Mnh77) from cloned fragments and shown to be 2,773, 2,774, and 2,772 nt in length. The sequences were deposited in nucleotide sequence databases under the accession numbers given in Table 1. None of the samples showed the presence of betasatellites by PCR using primers beta01/beta02 (5). Digestion of RCA products with *Xba*I (a restriction site commonly found in ToLCB; the only betasatellite so far identified in Oman) did not yield the ~1.4-kb fragment typical of betasatellites.

Sequence analysis. Sequence alignments showed the three sequences originating from Oman to share greater than 95% nucleotide sequence identity, indicating that they are isolates of a single begomovirus species (the species demarcation threshold for begomoviruses being 89% nucleotide sequence identity; 9).

Analyses of the three sequences using ORF Finder showed them to encode the six genes typical of OW monopartite begomoviruses (Table 1). The only unusual feature was that the sizes of the predicted products of the Rep genes of the three virus isolates were larger than is typical of begomoviruses (378 aa for the three Oman isolates). However, all three Rep genes included an in-frame ATG codon at a position conserved with the usual Rep ATG of begomoviruses. It is thus likely that the predicted large Rep is an artifact and that Rep translation initiates from the downstream ATG giving a size (359 aa) more typical of begomoviruses.

Comparisons to sequences available in the databases. An initial comparison of the sequences using NCBI database was performed by BLAST. The sequences from Oman showed the highest levels of nucleotide identity to isolates of ToLCSDV (11 of which are available at this time). Subsequent sequence comparison using MegAlign showed them to share between 86.5 and 92.2% nucleotide sequence identity with ToLCSDV sequences available in the

Table 1. The origins and features of *Tomato leaf curl Sudan virus* isolates from Oman

Isolate	Origin	Isolate descriptor	Accession number	Size (nt)	Position of genes (coordinates)/ number of amino acids (predicted coding capacity in kDa)					
					CP	V2	Rep	TrAP	REn	C4
Mir1	Mirbat	[OM:Mir1:11]	JN591385	2,773	296-1030/ 244 [27]	136-486/ 116 [13]	1527-2663/ 378 [42.9 ^a]	1211-1618/ 135 [15]	1126-1470/ 114 [13]	2159-2449/ 96 [11]
Mir2	Mirbat	[OM:Mir2:11]	JN591386	2,773	296-1069/ 257 [28]	136-486/ 116 [13]	1527-2663/ 378 [42.9 ^a]	1211-1618/ 135 [15]	1066-1470/ 134 [15]	2159-2449/ 96 [11]
Mnh77	Manah	[OM:Mnh:11]	HE819244	2,772	296-1030/ 244 [27]	136-486/ 116 [13]	1527-2663/ 378 [42.9 ^a]	1211-1618/ 135 [15]	1126-1470/ 114 [13]	2159-2449/ 96 [11]

^a For all three isolates, the open reading frame encompassing the Rep gene contains an extended leader sequence. However, the sequences include a downstream ATG codon, conserved in position with other begomoviruses, which, if used to initiate translation, would yield a predicted product of 359 amino acids (40.7 kDa) more typical of the Rep proteins of begomoviruses.

Table 2. Sequence comparisons of *Tomato leaf curl Sudan virus* isolates from Oman with sequences of other tomato leaf curl disease-associated begomoviruses

Begomovirus ^a	Highest and lowest percent nucleotide sequence identities and predicted amino acid sequences for pairwise comparisons of gene sequences of ToLCSDV-OM isolates with selected other begomoviruses ^b								
	ToLCSDV-Oman isolate			CP			V2		
	Mir1	Mir2	Mnh77	Mir1	Mir2	Mnh77	Mir1	Mir2	Mnh77
ToLCSDV-Gez (2) ^c	86.8-87	87.1-87.3	88.4-88.9	95.9-96.5 (97.1-97.5)	96.8-97.4 (98.1-98.4)	96.2-96.7 (97.5-98)	92.6-93.4 (92.2-94)	92.6-93.4 (92.2-94)	92.6-93.4 (92.2-94)
ToLCSDV-Sha	89.3	91.5	89.6	96.7 (97.5)	97.5 (98.4)	97 (98)	97.7 (98.3)	97.7 (98.3)	97.7 (98.3)
ToLCSDV-YE (6) ^c	87.5-87.6	88.1-88.3	86.5-87	95.2 (98)	95.7 (98.8)	95.5 (98.4)	96.6 (98.3)	96.6 (98.3)	96.6 (98.3)
ToLCSDV-Tob (2) ^c	89.3-91.1	89.9-92.2	87.2-90	96.7-96.9 (97.5)	97.4-97.5 (98.4)	97-97.1 (98)	97.2-97.4 (95.7-96.6)	97.2-97.4 (95.7-96.6)	97.2-97.4 (95.7-96.6)
TYLCMLV (7) ^c	77.7-79.9	78.4-80.1	78.5-81	69.3-70.7 (74.6-76.6)	70.3-71.6 (75.9-77.8)	69.7-71.2 (75-77)	92.9-93.7 (92.2-94.8)	92.9-93.7 (92.2-94.8)	92.9-93.7 (92.2-94.8)
TYLCV ^d	81.1	81.1	80	70.3 (75.9)	71.4 (77.1)	70.7 (76.3)	93.2 (91.5)	93.2 (91.5)	93.2 (91.5)
ToLCOMV (2) ^c	73.4-75	73.9-75.5	73.2-74.7	69.9-70.2 (76.6)	71.1-71.3 (76.2)	70.3-70.6 (77.4)	93.2-97.7 (94-96.6)	93.2-97.7 (94-96.6)	93.2-97.7 (94-96.6)

^a *Tomato leaf curl Sudan virus* (ToLCSDV) strains are indicated as Gezira (Gez), Shambat (Sha), Tobacco (Tob), and Yemen (YE).

^b Values in parentheses are highest and lowest percent amino acid sequence identity values for the predicted product of each gene.

^c Number of sequences used in the analysis.

^d Only the *Tomato yellow leaf curl virus* (TYLCV) isolate showing the highest levels of nucleotide sequence identity (GU076448) was used for comparisons.

Fig. 1. Phylogenetic relationship of *Tomato leaf curl Sudan virus* (ToLCSDV) isolates from Oman and selected other begomoviruses available in the databases using maximum parsimony algorithm. Numbers at nodes are percent bootstrap confidence scores (1,000 iterations). Begomovirus species used are *Papaya leaf curl virus* (PaLCV), *Tomato leaf curl Bangalore virus* (ToLCBV), ToLCSDV, *Tomato yellow leaf curl Mali virus* (TYLCMLV), *Tomato yellow leaf curl virus* (TYLCV), *Tomato leaf curl Oman virus* (ToLCOMV) and *Tomato yellow leaf curl Sardinia virus* (TYLCV). The tree was arbitrarily rooted on the sequence of the DNA A component of *Squash mild leaf curl virus* (SMCuV), a distantly related begomovirus, as outgroup. In each case, the isolate descriptor and database accession number is given. ToLCSDV-OM isolates are highlighted in gray. Origins of strains of ToLCSDV isolates are shown on the right. ToLCSDV strains are indicated as Gezira (Gez), Shambat (Sha), Oman (OM), Tobacco (Tob), and Yemen (YE).

Table 2. (continued from previous page)

Highest and lowest percent nucleotide sequence identities and predicted amino acid sequences for pairwise comparisons of gene sequences of ToLCSDV-OM isolates with selected other begomoviruses (continued) ^b											
Rep			TrAP			REn			C4		
Mir1	Mir2	Mnh77	Mir1	Mir2	Mnh77	Mir1	Mir2	Mnh77	Mir1	Mir2	Mnh77
87.3-87.4 (89.4)	87.5 (81.1)	88.6-88.7 (90.8)	85.3-86.5 (78.5-79.3)	86-87 (79.3-80)	86.8-87.7 (80-80.7)	88.1 (87.7-89.5)	89.4 (87.3)	88.7 (88.6-90.4)	83.2 (70.8)	83.2 (70.8)	84.2 (71.9)
90.1 (90.8)	90.2 (81.9)	88.5 (88.6)	87.5 (83.7)	88 (84.4)	88 (83)	88.7 (88.6)	91.1 (89.6)	88.7 (87.7)	85.9 (77.1)	85.9 (77.1)	83.2 (71.9)
86.3-86.6 (86.9-87.2)	86.4-86.7 (79.7-79.9)	85.9-86.2 (86.1-86.4)	81.6 (75.6)	82.1 (76.3)	82.6 (76.3)	84.6 (80.7)	87.4 (82.1)	85.2 (80.7)	85.9 (70.8)	85.9 (70.8)	83.5 (68.8)
85.7-90.7 (87.7-92.8)	85.8-90.8 (79.1-83.6)	83.4-89.7 (85.2-90)	95.1-95.3 (92.6-94.1)	95.6-95.8 (93.3-94.8)	92.4-93.1 (87.4-88.9)	93.6-95.4 (89.5-92.1)	95.6-97 (91-93.3)	93.6-95.4 (88.6-91.2)	84.1-92.4 (70.6-84.4)	84.1-92.4 (70.6-84.4)	81.4-92.4 (68.2-88.5)
84.3-87.1 (87.7-88.3)	84.4-84.9 (88-88.3)	85.5-88.3 (90-90.8)	87.5-88.5 (84.4-85.9)	88-89 (85.2-86.7)	89.2-90.2 (85.9-87.4)	86.7-88.1 (84.2-85.1)	87.4-88.9 (82.1-83.6)	87.2-88.7 (84.2-85.1)	81.4-84.2 (63.5-68.8)	81.4-84.2 (63.5-68.8)	83.8-84.5 (65.6-70.8)
88.2 (88.6)	88.3 (88.9)	87.5 (86.9)	85.5 (76.5)	86 (77.2)	86.5 (78.7)	85.2 (82.6)	87.9 (82.2)	85.8 (82.6)	86.3 (73.2)	86.3 (73.2)	83.8 (70.1)
74.2-75.9 (72-74)	73-74.6 (65.3-66.9)	74.8-76.2 (73.7-75.1)	85-85.8 (80.7-83)	85-85.8 (81.5-83.7)	84.3-84.6 (80-81.5)	85.8-87 (86.8)	87.9-88.6 (85.8)	85.8-87 (86)	65.1-65.5 (42.4)	65.1-65.5 (42.4)	65.5-65.9 (43.5)

Table 3. Recombination analysis of *Tomato leaf curl Sudan virus* isolate Mnh77 using the RDP 3.0 program^a

Breakpoint positions				Detection methods (<i>P</i> value)					
Begin	End	Minor parent	Major parent	RDP	Genconv	Bootscan	Maxchi	Chimera	Siscan
34	446	ToLCOMV (FJ956700)	ToLCSDV-Tob (JF919733)	3.55×10 ⁻⁰⁶	1.25×10 ⁻⁰³	1.43×10 ⁻⁰⁶	1.65×10 ⁻⁰⁵	3.99×10 ⁻⁰⁶	8.15×10 ⁻⁰⁹
1651	1976	ToLCOMV (FJ956700)	ToLCSDV-Tob (JF919733)	2.20×10 ⁻⁰⁷	6.86×10 ⁻⁰⁵	4.07×10 ⁻⁰⁷	6.17×10 ⁻⁰⁴	8.92×10 ⁻⁰⁴	3.57×10 ⁻⁰⁹
2340	2660	TYLCMLV (FM212663)	ToLCSDV-Tob (JF919733)	5.69×10 ⁻⁰¹	4.08×10 ⁻⁰⁹	2.10×10 ⁻⁰³	1.18×10 ⁻⁰³	5.11×10 ⁻⁰³	6.38×10 ⁻⁰³

^a ToLCOMV (*Tomato leaf curl Oman virus*); TYLCMLV (*Tomato yellow leaf curl Mali virus*); ToLCSDV-Tob (*Tomato leaf curl Sudan virus* – tobacco strain from Yemen).

database (GU180085, AY044139, EF919728-EF919734, and EF110891) followed by between 77.7 and 81% with isolates of *Tomato yellow leaf curl Mali virus* (TYLCMLV; DQ358913, EU847740) and less than 81% to *Tomato yellow leaf curl virus* (TYLCV; nine full-length sequences compared) isolates available in the database. This suggests that the begomovirus isolates characterized here are isolates of ToLCSDV.

Four distinct strains of ToLCSDV have been identified, based upon the guidelines set by Fauquet et al. (9) for distinguishing begomovirus strains from variants (a demarcation threshold of 93%). Two strains have been identified in Sudan, the “Gezira” and “Shambat” strains, and two in Yemen (13). Unfortunately, the report by Idris et al. (13) failed to provide distinct names for the two strains newly identified in Yemen; here these will be referred to as “Yemen” strain for the majority of isolates from Yemen (EF110891, JF919728-JF919732), and “Tobacco” strain for those isolates originating from Yemen and isolated from tobacco (JF919733, JF919734). The isolates from Oman showed the highest levels of sequence identity (87.2 to 92.2%; Table 2) to isolates of the Tobacco strain of ToLCSDV, indicating that these represent a distinct strain of ToLCSDV. The name “Oman” is proposed for this new strain.

A phylogenetic tree based upon an alignment of the sequences of all available ToLCSDV isolates with the sequences of selected begomoviruses from the databases is shown in Figure 1. This shows all ToLCSDV isolates to form a single clade and that the ToLCSDV isolates from Oman are distinct, with ToLCSDV isolates grouping in accordance with the above outlined strains. Two of the Oman nucleotide sequences (JN591385 and JN591386) presented in this report were al-

ready used for comparison by Idris et al. (13) and showed the same relationship.

Between the 5' ends of the V2 and Rep genes of all three sequences from Oman lies a noncoding, intergenic region (IR) which encompasses a predicted hairpin structure containing the conserved nonanucleotide (TAATATTAC) forming part of the loop, which contains the initiation point for rolling-circle replication of the virion-sense strand of the viral genome (11). Upstream of this, adjacent to the TATA box of the Rep promoter (coordinates 2665-2669) lie repeated sequences (known as iterons) that are sequence specific binding sites of Rep (4). For ToLCSDV-OM, these iterons have the sequence GGTAC, which is shared with ToLCSDV-YE, -Tob, and -Sha but not ToLCSDV-Gez (GGGGT).

The CP gene sequences of the ToLCSDV-OM isolates show high levels of identity (95.2 to 97.5% nucleotide sequence identity, 97.1 to 98.8% amino acid sequence identity) to those of the other ToLCSDV isolates available in the database, whereas for the V2 gene they show approximately equal levels of identity to the other ToLCSDV isolates (92.6 to 97.7%, 92.2 to 98.3%) and isolates of ToLCOMV (93.2 to 97.7%, 94.0 to 96.6%). For the complementary-sense genes (Rep, TrAP, Ren, and C4), the sequences of the ToLCSDV-OM isolates show the highest levels of identity to the other ToLCSDV isolates (85.8 to 90.8%, 81.6 to 95.8%, 84.6 to 97%, and 77.3 to 92.4% nucleotide sequence identity; 87.2 to 93.4%, 75.6 to 94.8%, 80.7 to 93.3%, and 68.2 to 88.5% amino acid sequence identity, respectively; Table 2). However, the complementary-sense gene sequences are more divergent from the other ToLCSDV isolates than are the virion-sense gene sequences (Table 2).

Recombination analysis. The three isolates of ToLCSDV-OM showed identical recombination patterns. For this reason only, the

Fig. 2. Analysis of *Tomato leaf curl Sudan virus-Oman* (ToLCSDV-OM) isolate Mnh77 (HE819244) for recombination. **A**, Boot scanning analysis of ToLCSDV-OM using the SimPlot program with a sliding window of 200 nucleotides moving in 20-nucleotide steps. The Bootscan graph was built by using the neighbor-joining tree algorithm, the Kimura 2-parameter distance model, and 100 pseudo-replicates. Dotted line shows an arbitrary 70% reliability threshold, and solid line shows the 89% demarcation threshold for begomovirus species. **B**, Schematic representation of the results of RDP3 analysis (Table 3) shown is a linear representation of the ToLCSDV-OM genome showing the origins of the fragments, recombination breakpoints, putative parental viruses, and *P* values determined by the RDP method.

results with one isolate, Mnh77 (HE819244), are shown here. The bootscan and RDP analysis of the ToLCSDV-OM (Mnh77; HE819244) genome together indicated definite evidence of recombination within the viral genome, which differed from an isolate of ToLCSDV-Tob (JF919733). Two recombinant fragments (coordinates 34 to 446 in the V2 gene and 1651 to 1976 toward the 3' end of the Rep gene) were detected for ToLCSDV-OM, and these fragments shared high levels of sequence identity with ToLCOMV (FJ956700; 96.8 and 93.9%, respectively). A further recombinant fragment donated by TYLCMLV (FM212663) was found at the 5' end of the Rep gene (coordinates 2340 to 2660) in ToLCSDV-OM. For all these recombination events, ToLCSDV-Tob (JF919733) is indicated as the major parent, and the nonrecombinant regions of ToLCSDV-OM, nucleotide coordinates 447 to 1650 and 1977 to 2339, show 96 and 98.6% identity, respectively, with the homologous regions of ToLCSDV-Tob (JF919733). This suggests that the

Oman strain of ToLCSDV evolved from the Tobacco strain by recombination (Fig. 2; Table 3).

Infectivity assay. A construct for the *Agrobacterium*-mediated inoculation of ToLCSDV-OM isolate Mir1 (JN591385) was produced. *N. benthamiana* and susceptible tomato var. Indam were infiltrated with the infectious clone. All inoculated *N. benthamiana* (out of 15 inoculated) and 6 tomato plants (out of 15 inoculated) showed the first symptoms of infection at 18 days postinoculation (dpi; the results of two independent experiments). In *N. benthamiana*, the symptoms consisted of vein swelling and severe upward leaf curling (Fig. 3). Once infected, plants ceased to grow. Infected tomato plants showed mild downward curling of the edges of leaves, crumpling of leaves, and interveinal yellowing. Although plants continued to grow, they remained stunted with reduced internodes and a reduced size of leaves, symptoms that were comparable to those seen in the field (Fig.

Fig. 3. Symptoms induced by the *Tomato leaf curl Sudan virus*-Oman (ToLCSDV-OM) isolate Mir1 (JN591385) in *Nicotiana benthamiana* and susceptible tomato plants. Mock inoculated *N. benthamiana* (A), and tomato (D) plants. An *N. benthamiana* plant (B) and a tomato plant inoculated with ToLCSDV-OM (E). Photographs were taken at 25 dpi. A field infected tomato showing typical leaf curl disease symptoms (C). Southern blot detection of ToLCSDV-OM in inoculated plants of *N. benthamiana* and tomato (F). The samples loaded on the gel were DNA extracted from non-inoculated *N. benthamiana* plant (lane 1), the undigested plasmid clone of ToLCSDV-OM (~200 ng; lane 2), DNA extracted from the symptomatic young leaves of a tomato plant (lane 3) and an *N. benthamiana* plant (lane 4) inoculated with ToLCSDV-OM. Approximately equal amounts of DNA (10 µg) extracted from plants was loaded in each case. The blot was probed with DIG-labeled ToLCSDV-OM fragment. The viral DNA forms detected are labeled as single-stranded (ss), super-coiled (sc) and open-circular (oc).

3). Southern blot analysis of DNA samples extracted from both symptomatic *N. benthamiana* and tomato showed the presence of viral single- and double-stranded DNA forms typical of gemini-virus replication (Fig. 3).

Discussion

Tomato is an important agricultural crop in the Sultanate of Oman for local consumption, with an annual production of ~81,000 tonnes (Ministry of Agriculture and Fisheries Wealth, Oman). ToLCD is a major constraint for tomato cultivation in many tropical and subtropical areas of the world. For the past two decades, tomato production in Oman has also been affected by ToLCD. The losses have been so severe that some small farmers have abandoned tomato cultivation. Earlier studies have shown two begomoviruses, a distinct strain of TYLCV and a species unique to Oman (ToLCOMV), as well as a betasatellite (Tomato leaf curl betasatellite) with origins on the Indian subcontinent, to be associated with leaf curl disease of tomato in Oman (14,15; Supplementary Table 1). The results presented here show a third begomovirus to cause disease in tomato in Oman.

In Oman, the major area for crop production is the Al Batinah region along the Gulf of Oman, which is bounded entirely by the Al Hajar Mountains to the south. Another area of crop production is in the southern Dhofar region, close to the border with Yemen. There is little crop production in the arid areas between these two. Despite extensive studies, ToLCSDV-OM has only been found south of Al Hajar mountain range (Table 1; Supplementary Table

1), which likely has posed a barrier to the spread of the virus into Al Batinah.

It is more than likely that ToLCSDV present in Oman has originated from Yemen—the Tobacco strain of this virus being the closest relative to the Oman strain. However, the fact that these two strains have diverged suggests that the movement of virus between Yemen and Oman is infrequent. In Yemen, most agricultural crops are grown in the far west. Between the major agricultural areas of Yemen and the southern agricultural area of Oman lies a desert, which would pose a significant barrier to the spread of the whitefly vector and consequently the virus.

Since TYLCV-Oman, ToLCOMV (14,15), and the two strains of ToLCSDV identified in Yemen (12) are associated with betasatellites, it is somewhat surprising to find that ToLCSDV-OM is not. In Yemen, ToLCSDV is associated with a betasatellite that has only been identified in Yemen (Tomato yellow leaf curl Yemen betasatellite; 13). The betasatellite (ToLCB) identified with TYLCV-OM and ToLCOMV (14,15) has thus far only been identified in tomato north of the Al Hajar Mountains, which have likely constrained the spread of these viruses to the rest of the country. This betasatellite also occurs in the south but in association with another begomovirus species (*unpublished*). This would seem to indicate that ToLCSDV-OM is not a betasatellite requiring virus, as the infectivity studies here suggest. Whether this apparent difference in the requirement for a betasatellite between ToLCSDV-OM and the other ToLCSDV strains has arisen due to the recombination that has led to ToLCSDV-OM will need to be further investigated.

The identification here of ToLCSDV in the south of Oman, and previous studies showing TYLCV and ToLCOMV present in the north (14,15), highlights differences between the north and south of the country regarding ToLCD etiology. There is a need for more detailed studies of the extent of spread of the tomato infecting begomoviruses in Oman. All these viruses have their origins outside of the country and, once introduced into Oman, are undergoing evolutionary changes leading to distinct strains and even speciation (in the case of ToLCOMV; 14). Oman is thus a microcosm for the study of what may happen when previously geographically separated viruses meet, something that is already happening on a macro scale with TYLCV, which now has an almost global distribution (16).

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