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Characterization of *Tomato curly stunt virus*: a new tomato-infecting begomovirus from South Africa

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The biological and molecular characterization of a virus recognized as a distinct begomovirus species, *Tomato curly stunt virus* (ToCSV), first observed in South Africa in 1997, is reported here. Whitefly-transmission and host-range studies were carried out using a *Bemisia tabaci* colony identified as the B-biotype. The experimental host range of ToCSV spanned primarily species in the Solanaceae and Fabaceae. The complete ToCSV genome (2·766 kb) was amplified by PCR, cloned, and the DNA sequence determined. Phylogenetic analysis revealed that ToCSV was most closely related to *Tobacco leaf curl Zimbabwe virus* (TbLCZV), at 84% nucleotide identity, indicating that ToCSV is a new species in the genus *Begomovirus* that is probably endemic to southern Africa. The ToCSV genome sequence contained all of the hallmark coding and non-coding features characteristic of other previously recognized monopartite begomoviruses. ToCSV is only the second begomovirus described from southern Africa that infects solanaceous species. Neither a begomoviral DNA-B component nor a satellite-like DNA molecule was detected by PCR in extracts of ToCSV-infected plants.

Keywords: *Bemisia tabaci*, emergent virus, *Geminiviridae*, *Lycopersicon esculentum*, *Solanum lycopersicum*, whitefly-transmitted virus

Introduction

A number of whitefly-transmitted geminiviruses (genus *Begomovirus*, family *Geminiviridae*) are known to infect tomato (*Solanum lycopersicum* formerly *Lycopersicon esculentum*) in Africa (Czosnek & Laterrot, 1997; Idris & Brown, 2005). Begomoviruses contain circular, single-stranded (ss) DNA and have either a monopartite or bipartite genome. Bipartite tomato-infecting begomoviruses are distributed in the Eastern and Western Hemisphere, but all tomato-infecting begomoviruses reported thus far from Africa have a monopartite genome arrangement (Fauquet *et al*., 2005).

During 1997 a new disease of tomato plants emerged in tomato production areas in the Onderberg region of South Africa. The affected tomato plants showed foliar symptoms similar to those induced by *Tomato yellow leaf curl virus* (TYLCV), including foliar chlorosis, leaf curling, stunting and reduced fruit set. Since first reported in 1997 (Pietersen *et al*., 2000), the disease has spread to additional tomato-growing localities throughout South Africa and was also recently identified in southern Mozambique (authors, personal observation). Between 1997 and 2003 the disease distribution radiated northward and southward from the epicentre in Onderberg where it was first observed (Fig. 1).

The name tomato curly stunt disease (ToCSD) was based on observed field symptoms in tomato plants. A preliminary investigation for the presence of a suspect begomovirus in affected tomato plants revealed the association of a provisional, new begomoviral species, referred to as *Tomato curly stunt virus* (ToCSV) (Pietersen *et al*., 2000). This conclusion was based on analysis of the nucleotide (nt) sequence of the core coat protein (CP) sequence (GenBank AF261885), which shared less than 86% nt identity with the most closely related begomoviruses. ToCSV was shown to be experimentally transmissible by the B-biotype whitefly *Bemisia tabaci*, a recent introduction to the tomato-producing region of South Africa (Brown, 2000). The specific biotype haplotype was determined using the mitochondria cytochrome oxidase I (COI) marker (Brown, 2000; Pietersen *et al*., 2000).

Field screening of TYLCV-resistant tomato lines for resistance to ToCSV (Pietersen & Smith, 2002) revealed that TYLCV-tolerant tomato accessions developed mild symptoms of the disease, compared to susceptible reactions

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Figure 1 Map showing the progression of *Tomato curly stunt* disease in South Africa.

for TYLCV-susceptible accessions. This suggested possible cross-resistance to ToCSV and TYLCV, and a possible genetic source of disease resistance.

This paper reports the molecular and phylogenetic characterization of ToCSV, and results of experimental host range, vector transmission and epitope profile studies.

Materials and methods

Natural spread of ToCSV

The emergence and spread of ToCSV in South Africa during 1998–2003 was examined during surveys of the Onderberg region where ToCSD was first observed. Samples showing typical field symptoms of the disease, i.e. yellow leaf curling and stunting, were obtained from local fields and tested for ToCSV presence by grafting. Total nucleic acids were extracted from the leaf samples for PCR-based assays using the core CP (575–577 bp) primers (Wyatt & Brown, 1996). Subsequently, leaf samples from tomato plants in newly affected areas and fields bordering those locations were assessed for virus presence by PCR and distribution of the virus was determined. The disease incidence in newly affected areas was estimated by counting the number of plants with symptoms in groups of 20 plants at 10 randomly selected sites within each field.

Virus source plants

Isolate 99/0631 of ToCSV reported herein was collected from an infected tomato plant in the Onderberg region of South Africa in 1999. The virus was established in culture by two serial passages using a single whitefly allowed feeding access (24 h) to field tomato plants. After two serial passages to tomato cv. Red Khaki, the virus was maintained by side grafting and/or serial transmission using 30–40 adult *B. tabaci* at approximately 6- to 8-week intervals (Pietersen *et al.*, 2000). The identity of ToCSV in culture was confirmed by PCR amplification, cloning and DNA sequencing of the core CP gene using the protocol and PCR primers described by Wyatt & Brown (1996). ToCSV-infected source plants were maintained in an insect-proof growth room at 22–24°C under fluorescent lights (Osram L36W/77) with a 12-h photoperiod. Transmission and host-range experiments were conducted under the same environmental conditions in separate growth rooms.

Mechanical transmission

Virus inoculum was prepared by grinding ToCSV-infected, tomato leaves with symptoms in a chilled mortar and pestle in a ratio of 1:5 (w/v) with 0·1 M potassium phosphate buffer, pH 7·2, containing diatomaceous earth. Ten tomato cv. Red Khaki seedlings approximately 6 weeks old were inoculated with virus extracts in two replicated experiments. Four additional plants in each replicated experiment were mock-inoculated with buffer only. Plants were maintained as described above and monitored periodically for symptom development for 6 weeks. Plants were tested for ToCSV presence by PCR using core CP primers (Wyatt & Brown, 1996) at 6 weeks post-inoculation.

Whitefly vector colony

A virus-free colony of *B. tabaci* B-biotype (Pietersen *et al.*, 2000) was used for whitefly-transmission experiments. Based on morphological characteristics, the whitefly colony was also identified as *B. tabaci* by I. Millar of the Biosystematics Division of the ARC-Plant Protection Research Institute. The B-biotype identity was confirmed periodically throughout the study by PCR amplification and DNA sequencing of COI (Brown, 2000). The colony was established from adult whiteflies collected in the Onderberg tomato fields and transferred to laboratory-sown cotton seedlings. The colony was confirmed to be begomovirus-free based on the negative results of PCR amplification for 10 groups of whiteflies (10 adults per sample) using core CP primers (Wyatt & Brown, 1996). The virus-free whitefly colony was maintained thereafter on cotton (*Gossypium hirsutum* cv. Sabie), a non-host of the virus (see Table 1), in whitefly-proof cages at 27°C. The cages containing colony whiteflies were maintained in a quarantine-approved insectary for the entire study.

Table 1 Experimental host-range study for *Tomato curly stunt virus* determined by whitefly-mediated transmission employing a 24-h acquisition access period followed by a 24-h inoculation access period

Test plant[a]	Plants with symptoms/ inoculated plants	PCR virus detection/ inoculated plants
Brassica pekinensis	0/10	0/10
Capsicum annuum cv. Anaheim	0/10	0/10
C. annuum cv. Capistrano	0/10	0/10
C. annuum cv. Golden Wonder	0/10	0/10
Cucurbita maxima cv. Big Max	0/10	0/10
Datura stramonium	2/10	10/10
Glycine max cv. Rampage	0/10	0/10
***G. max* cv. Buffalo**	2/10	2/10
Gossypium hirsutum cv. Delta Pine 90	0/10	0/10
Malva parviflora	0/10	0/10
Manihot esculentum	0/10	0/10
Nicotiana benthamiana	0/10	5/10
N. clevelandii	0/10	4/10
N. glutinosa	2/10	1/10[b]
N. tabacum cv. TL33	0/10	4/10
Phaseolus vulgaris cv. Black Turtle Soup	0/10	0/10
***P. vulgaris* cv. Bonus**	2/10	2/10
***P. vulgaris* cv. Bountiful**	5/10	7/10
Pisum sativum cv. Green Feast	0/10	0/10
***Solanum lycopersicum* cv. Star 9006**	10/10	10/10
S. melongena cv. Burpee's Black Beauty	0/10	0/10
Vicia faba cv. Aquadulce	0/10	0/10
Vigna unguiculata cv. California Blackeye	0/10	0/10
V. unguiculata cv. Tvu 1582	0/10	0/10

[a]Plants in bold are susceptible to ToCSV.

[b]Only one of the two *N. glutinosa* plants with symptoms was PCR-positive.

Virus-vector transmission

Six-week-old tomato cv. Red Khaki seedlings were used as the virus source and as diagnostic indicator species. Source plants were inoculated with ToCSV 4 weeks prior to initiating the transmission experiments. The source plants were observed periodically for symptom development during 4 weeks and tested for virus presence by PCR using the core CP primers.

The acquisition access period (AAP) was determined by allowing virus-free, adult whiteflies access feeding for 5 min, 15 min, 30 min, 1 h, 2 h, 6 h or 24 h on ToCSV-infected tomato plants or a cutting from the virus source plant, and then transferred to healthy 6- to 8-week-old tomato seedlings for 24 h. The inoculation access period (IAP) was determined by allowing whiteflies a 24-h AAP on a virus-source plant or cutting from a source plant, followed by an IAP of 5 min, 15 min, 30 min, 1 h, 2 h, 6 h or 24 h on virus-free tomato seedlings. AAPs and IAPs of 24 h were used when testing the IAP and AAP, respectively, based on previous studies showing that 24 h is sufficient to transmit the related begomovirus, TYLCV (Picó *et al*., 1998; Czosnek *et al*., 2001).

Whiteflies were collected and used for transmission bioassays, irrespective of sex or age. Experiments were conducted using 15 whiteflies per plant and six seedlings for each AAP or IAP determination. The negative experimental control comprised exposure of whiteflies to virus-free tomato plants using the respective AAP and IAP combination.

Whitefly feeding was terminated by the application of a systemic insecticide (Imidacloprid SC350, Bayer; 100 mL of a 0·5-mL L^{-1} suspension applied as a soil drench) and by spraying with a contact insecticide (Chlorpirifos, Efekto). Plants were maintained in the isolation facility under artificial light for 3 days then moved to an insect-free greenhouse or growth room and observed weekly for symptom development over a 6-week period in two experimental replicates.

Host-range studies

The experimental host range of ToCSV was determined by allowing whiteflies (>50 per plant) access to ToCSV source plants then transferring them to test plant seedlings (Table 1) using the method of Picó *et al.* (1998). Groups of *B. tabaci* adults were collected from the virus-free colony and allowed a 24-h AAP on virus source plants followed by a 24-h IAP on healthy test plants (10 seedlings per replicate) as described by Idris & Brown (1998). Negative controls included a 24-h exposure of adult B-biotype whiteflies from cotton plants to tomato seedlings that had been reared in a virus-free growth room. Whitefly feeding was terminated by killing adults with an insecticide, as described above. Test plants were monitored for symptom development at weekly intervals and symptoms were recorded. Plants were assayed for ToCSV presence by PCR 30 days post-inoculation (DPI).

Epitope profiles

Epitope profiles were determined using triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) (Swanson *et al*., 1992) with a panel of monoclonal antibodies (MAbs) raised against purified *African cassava mosaic virus* (ACMV) (numbered SCR 17, 18, 20, 23), *Indian cassava mosaic virus* (ICMV) (SCR 52, 53, 55, 56, 60, 62) or *Okra leaf curl virus* (OkLCuV) (SCR 106) (Thomas *et al*., 1986; Swanson, 1992; Swanson *et al*., 1992; Swanson & Harrison, 1993). TAS-ELISA tests were carried out at the Scottish Crop Research Institute (SCRI). Young tomato leaves with symptoms were collected 4 weeks after whitefly-mediated inoculation (parameters as described above) and provided to the SCRI under the Scottish Executive Environment and Rural Affairs Department license no. PH/73/2000. TAS-ELISA controls were sap extracts from uninfected tomato leaves and extraction buffer alone.

Virus DNA isolation from plants

For the studies carried out in South Africa, viral DNA was isolated from tomato leaves as described by Pietersen & Smith (2002). For PCR, cloning and sequencing in the Arizona laboratory total nucleic acids were extracted from four tomato leaf samples with symptoms (20 DPI) by the method of Doyle & Doyle (1987).

PCR amplification, cloning and DNA sequencing

Total DNA isolated from the four leaf samples was used as template for virus amplification by PCR. To obtain the full-length sequence of ToCSV, two overlapping fragments of ~1·3 and ~2·3 kbp were amplified by PCR using Taq DNA polymerase (Eppendorf) and cloned into the T-Easy vector as described by Idris & Brown (2005). The 1·3-kbp fragment was amplified using the degenerate PCR primers prV2644 and prC1154, whereas the 2·3-kbp fragment was amplified using the degenerate primers prV878 and prC344 (Idris & Brown, 1998). Three full-length viral clones obtained by PCR amplification were completely sequenced. The two fragments overlapped by greater than 500 bases and if the overlapping sequences shared 100% identity that would be consistent with amplification from the same viral genome. The full-length viral genome was assembled using the algorithm FAKtory 1·41, an on-line program (http:bcf.arl.arizona.edu/biodesk) provided by the Biotechnology Computing Facility, University of Arizona.

To detect a possible DNA-B component in ToCSV-infected plant extracts, PCR was carried out using the degenerate primers BV1855 and BC2571 (Idris & Brown, 2004) that amplify a begomoviral fragment of 800 bp.

Total DNA extracts from ToCSV-infected tomato plants were also subjected to PCR amplification to detect the presence of an associated satellite DNA molecule (satDNA) (Dry *et al*., 1997; Saunders *et al*., 2000; Briddon *et al*., 2001; Idris & Brown, 2005) using degenerate primers based on a region conserved to date for all begomovirus-associated satDNAs (Briddon *et al*., 2001; Idris *et al*., 2005).

The apparent full-length genome sequence was determined and assembled into a genome of 2766 nucleotides. The full-length viral genome sequence was analysed using the EDITSEQ program available in the LASERGENE software package (DNASTAR Inc.) to identify predicted ORFs of 10 kDa or larger in size. The ToCSV genome sequence was compared against sequences available in the NCBI GenBank database using the on-line BLAST algorithm. The DNA sequences for the closest ToCSV relatives were downloaded from the database and used for phylogenetic and recombination analyses, together with selected tomato-infecting begomoviruses for which sequences are available in GenBank.

Phylogenetic analysis

The CLUSTAL V in MEGALIGN program (DNASTAR Inc.) was used to align full-length begomovirus DNA-A components or complete monopartite genome sequences for selected begomoviruses (Table 2). Percentage nucleotide identity was calculated using CLUSTAL V. The CLUSTAL W alignment program, also available in MEGALIGN, was used to align viral sequences for phylogenetic analysis. The alignment was subjected to maximum parsimony (MP) analysis using PAUP* version 4·01b10 (Swofford, 2005) to reconstruct the phylogenetic tree. The most parsimonious tree was sought using a heuristic search method, stepwise addition, and the tree-bisection-reconnection random branch-swapping options for 1000 iterations. Bootstrap values estimated at a >70% confidence limit were placed at the major tree nodes (Fig. 2). The tree was rooted using the monopartite *Cotton leaf curl Gezira virus* (CLCuGV) from the Nile Basin in sub-Saharan Africa.

Recombination analysis

The aligned nucleotide sequences were subjected to recombination analysis using the BOOTSCAN, CHIMAERA, GENCONV, MAXCHI, RDP and SISCAN options available in the Recombination Detection Program (RDP version 2 beta 08) (Martin & Rybicki, 2000). Parameters were established using a window size of 40 nt with a maximum acceptable probability of 0·01. To corroborate the putative recombinant predictions that were identified in the search, phylogenetic analysis using the maximum likelihood (ML) option, available in the PAUP software package, was carried out using the viral fragments identified as 'recombinatorial' (data not shown).

Results

Geographical distribution of tomato chlorotic stunt disease

The distribution of tomato curly stunt disease during 1998–2003 is shown in Fig. 1. The disease was first

Table 2 List of begomovirus reference sequences from the NCBI GenBank database used in the phylogenetic analysis of *Tomato curly stunt virus*

Virus	Acronym	Accession number
Cotton leaf curl Gezira virus-Cotton Sudan	CLCuGV-[Co:SD]	AF260241
Cotton leaf curl Gezira virus-Okra Egypt	CLCuGV-[Ok:EG]	AY036010
East African cassava mosaic Cameroon virus	EACMCV	AF112354
East African cassava mosaic Malawi virus	EACMMV	AJ006460
East African cassava mosaic virus-UG2	EACMV-[UG2]	Z83257
Hollyhock leaf crumple virus	HoLCrV	AY036009
Pepper yellow vein virus	PepYVV	AY502935
South African cassava mosaic virus	SACMV	AF155806
South African cassava mosaic virus-M12	SACMV-[M12]	AJ422132
South African cassava mosaic virus-Zimbabwe	SACMV-[ZW]	AJ575560
Tobacco leaf curl Zimbabwe virus	TbLCZV	AF350330
Tomato curly stunt virus	ToCSV	AF261885
Tomato leaf curl Mali virus	ToLCMLV	AY502936
Tomato leaf curl Madagascar virus-Morondova	ToLCMGV-[Mor]	AJ865338
Tomato leaf curl Madagascar virus-Toliary	ToLCMGV-[Tol]	AJ865339
Tomato leaf curl Mayotte virus-Dembeni	ToLCYTV-[Dem]	AJ865341
Tomato leaf curl Mayotte virus-Kahani	ToLCYTV-[Kah]	AJ865340
Tomato leaf curl Sudan virus-Gezira	ToLCSDV-[Giz]	AY044137
Tomato leaf curl Sudan virus-Shambat	ToLCSDV-[Sha]	AY044139
Tomato yellow leaf curl Malaga virus	TYLCMlV	AF271234
Tomato yellow leaf curl virus-Almeria	TYLCV-[Alm]	AJ489258
Tomato yellow leaf curl virus-Sudan	TYLCV-[SD]	AY044138
Tomato yellow leaf curl virus-Egypt	TYLCV-[EG]	L12219
Tomato yellow leaf curl virus-Mild	TYLCV-[Mld]	X76319
Tomato yellow leaf curl virus	TYLCV	X15656

detected in the Onderberg region of South Africa (north eastern Mpumalanga) in 1998. A 1998 survey of 140 ha of tomato fields throughout the Onderberg region indicated that initially the disease was confined to a relatively small area in the Onderberg region, which is bounded by the Strydomblock, Malelane and Tonetti regions. The estimated disease incidence in tomato fields was ~50%, based on replicated random field counts. During 2000, the disease spread to the northern part of KwaZulu-Natal in the Pongola region. Disease incidence was assessed in two fields in the Pongola region, revealing 100% and 30% infection in two 1·5-ha 6-week-old tomato plantings. In 2001, KwaZulu-Natal to the south experienced an outbreak near Nkwalini, where disease incidence in 11 fields ranged from zero to 100%. In 2003, tomato curly stunt disease occurred at 5% incidence in two fields located northwest of the original outbreak, in Trichardtsdal, Limpopo, indicating continued virus spread. To date, ToCSV infection has been confirmed in tomato plants with symptoms from the Onderberg, Pongola Nkwalini and Trichardsdal regions. Tomato samples collected prior to 2000 from a number of tomato-growing areas in South Africa were assessed for ToCSV presence by PCR using the core CP primers, which yield a 579-bp fragment for most begomoviruses (Wyatt & Brown, 1996). The PCR products were cloned and the sequence determined for each to confirm ToCSV identity. PCR assays for tomato samples collected after 2000 were carried out using ToCSV-specific PCR primers designed by Pietersen & Smith (2002).

Mechanical transmission

Symptoms were not observed in any of 20 tomato cv. Red Khaki seedlings mechanically inoculated with ToCSV, nor was viral DNA detected by PCR in the inoculated or negative control tomato plants.

Whitefly transmission

The whitefly vector *B. tabaci* B-biotype, given an AAP of 30 min or less, failed to transmit the virus. Whiteflies transmitted ToCSV when the AAP ranged from 1 to 24 h. The rates of transmission ranged from 8% to 100% and increased with longer AAPs. The minimum IAP was 5 min. Transmission frequencies ranged from 8% to 83% and the rate of transmission increased with longer IAPs (Table 3). Negative control plants established by allowing virus-free-colony whiteflies a 24-h AAP did not develop disease symptoms and were negative for ToCSV presence by PCR using core CP primers.

Host range and symptoms

The results of the experimental host-range study and associated symptom phenotypes are summarized in Table 1. Of the 24 inoculated species and cultivars, only seven, including tomato, developed symptoms.

In tomato cv. Star 9006, and in Red Khaki included as the positive control, foliar chlorosis developed at the base of the youngest leaves approximately 24 days

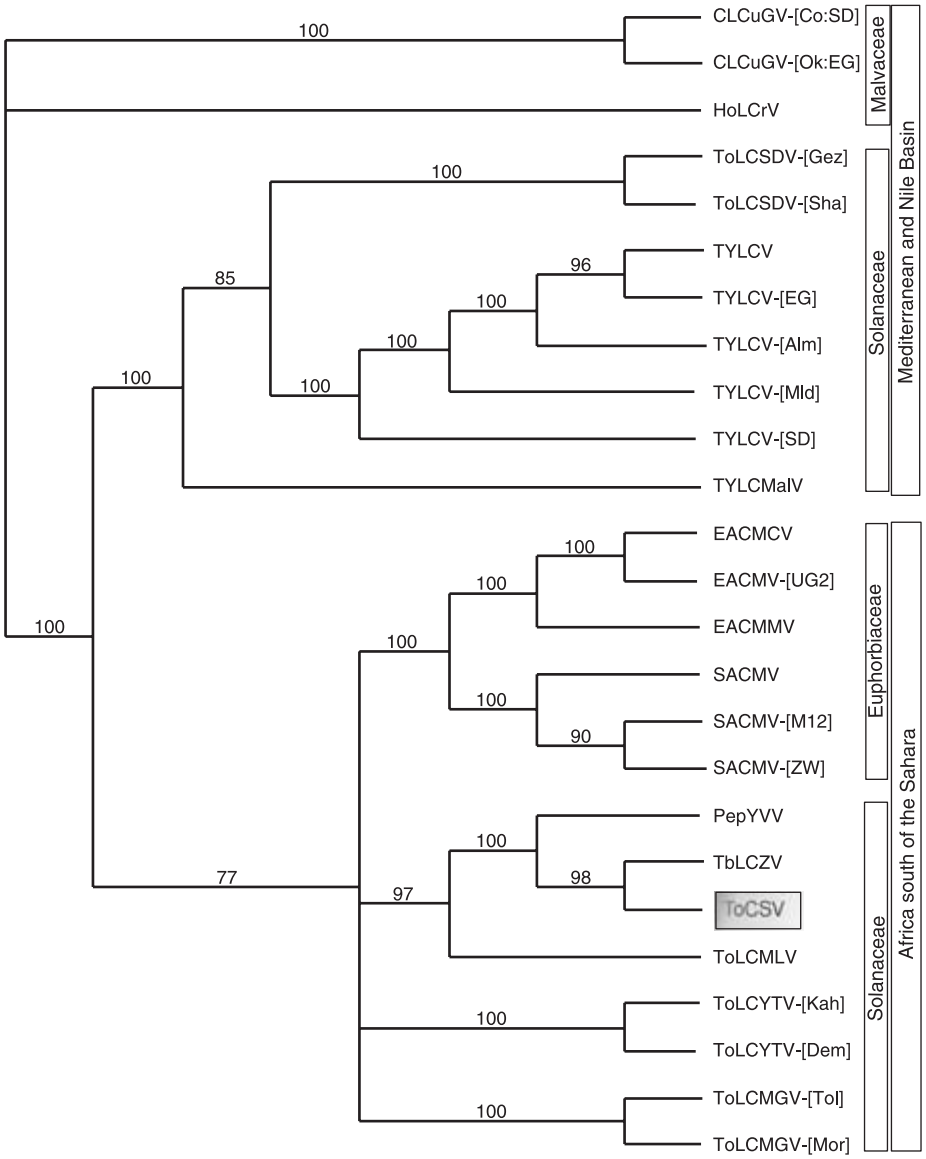

Figure 2 Phylogenetic tree indicating the relationships for *Tomato curly stunt virus* (ToCSV) and selected begomoviruses using maximum parsimony, available in PAUP 4·0bv10. The vertical and horizontal branch lengths are arbitrary. Bootstrap (1000 replicates) values are placed at the major nodes on the tree.

after exposure to viruliferous whiteflies. Subsequently, the older leaf margins developed upward curling, the leaves were stunted, overall plant growth was severely inhibited, and the stems and petioles did not elongate normally. During flowering, the majority of flowers aborted and few or no fruits developed on plants with symptoms.

The youngest leaves of *Datura stramonium* developed chlorosis from the leaf base and leaf curling within 12 DPI, with all subsequent systemically infected leaves developing symptoms. Symptoms became progressively more chlorotic and curled and plants were stunted.

The emerging leaves of *Glycine max* (soyabean) cv. Buffalo were curled and deformed by 4 weeks post-inoculation (PI). In *Nicotiana glutinosa*, the youngest leaves became slightly rugose one month PI, followed by mild, but increasingly severe curling on subsequently developing leaves. No further elongation of stems or internodes occurred and the plants were stunted.

Two cultivars of *Phaseolus vulgaris* developed systemic leaf chlorosis, downward leaf curling and deformation 4 weeks PI, followed by stunting of the plants. One bean cultivar, Black Turtle Soup, did not show symptoms and was negative for ToCSV by PCR.

Nicotiana benthamiana, *N. clevelandii* and *N. tabacum* cv. TL33 were identified as symptomless hosts.

Table 3 Transmission parameters for *Tomato curly stunt virus* and the introduced B-biotype of *Bemisia tabaci* for a range of acquisition access periods (AAP) and inoculation access periods (IAP) at 15 adult whiteflies per plant

AAP (24-h IAP)	Plants with symptoms/inoculated plants (% infected)	IAP (24-h AAP)	Plants with symptoms/inoculated plants (% infected)
5 min	0/12 (0)	5 min	1/12 (8)
15 min	0/12 (0)	15 min	2/12 (17)
30 min	0/12 (0)	30 min	2/12 (17)
1 h	1/12 (8)	1 h	4/11 (36)
2 h	5/12 (42)	2 h	4/12 (33)
6 h	11/12 (92)	6 h	10/12 (83)
24 h	12/12 (100)	24 h	8/12 (67)
Control	0/7 (0)	Control	0/7 (0)

Table 4 Epitope profile for *Tomato curly stunt virus* when tested with a panel of monoclonal antibodies raised against begomovirus species from different geographical regions worldwide

Monoclonal antibody[a]	ToCSV 99/0631 rating[b]
SCR17	4
SCR18	4
SCR20	4
SCR23	4
SCR52	0
SCR53	0
SCR55	0
SCR56	0
SCR58	0
SCR60	0
SCR62	0
SCR106	4

[a]All from the Scottish Crop Research (SCR) Institute, Invergowrie, UK.
[b]Overnight ELISA readings from duplicate wells, with 4 ≥1·80; 3 = 1·21–1·80; 2 = 0·61–1·20; 1 = 0·15–0·60.

Epitope profiling

The monoclonal antiobody epitope profile for ToCSV is summarized in Table 4. Monoclonal antibodies raised against two viruses from Africa, ACMV and OkLCuV, reacted strongly (A_{405nm} > 1·8) to ToCSV antigen, but no reaction was observed to antibodies for ICMV, a virus infecting cassava in India.

PCR amplification, cloning and DNA sequencing

A full-length begomoviral genome for ToCSV of 2766 nt was assembled from the two overlapping PCR fragments. The complete genome sequence for ToCSV was deposited in the NCBI GenBank database as accession number AF261885.

Repeated attempts to detect a DNA-B component or a satDNA molecule in ToCSV-infected tomato plant extracts failed using the 'universal' B-component- and satDNA-specific PCR primers, respectively.

ToCSV has a genome organization similar to other monopartite begomoviruses in that it contains six ORFs: V1, V2, C1, C2, C3 and C4. A 259-nt intergenic region (IR) was identified between the C1 and V2 ORFs, which contained the nonanucleotide motif TAATATTAC, a conserved begomovirus sequence involved in binding to the replication associated protein (REP). The ToCSV REP-binding site was identical to that of *Tomato leaf curl Sudan virus*-Shambat (ToLCSDV-[Sha]) (Idris & Brown, 2005). The ToCSV IR contained two directly repeated sequences of GGTAC located at nucleotide coordinates 2624–2627 and 2663–2667, and two inverted repeats at coordinates 2624–2628 and 2664–2668. These iterated sequences are predicted to serve as REP-binding sites.

Phylogenetic analysis

Sequence comparisons showed that ToCSV shared the highest nucleotide identity with *Tobacco leaf curl Zimbabwe virus* from Zimbabwe (TbLCZV) [AF350330], *Pepper yellow vein virus* from Mali (PepYVV) [AY502935] and *Tomato leaf curl Mali virus* (ToLCMLV) [AY502936] at 84%, 82% and 78%, respectively. Phylogenetic analysis using MP indicated that ToCSV from South Africa was most closely related to two other African viruses of solanaceous hosts (PepYVV and ToLCMLV) and TbLCZV. Phylogenetic analysis using maximum parsimony (Fig. 2) showed that ToCSV grouped with begomoviruses from eastern and southern Africa and the Indian Oceanic Islands, forming a clade with TbLCZV, also from southern Africa, and PepYVV from Mali, western Africa. These results suggest that ToCSV is probably indigenous to southern Africa and that it is not an introduced virus.

Recombination analysis

Recombination analyses using six programs available in RDP (Martin & Rybicki, 2000) identified two recombinant fragments in the C1 and V1-V2 regions of ToCSV (Fig. 3). Three programs, BOOTSCAN, RDP and MAXCHI, recognized the source of the first recombinant fragment to be a close relative to *South African cassava mosaic virus* (SACMV) with probability ranging between 1·69 E-17 and 3·48 E-05. Four programs, BOOTSCAN, GENECONV, RDP and SISCAN, identified, with a probability ranging between 2·44 E-18 and 2·25 E-05, that the source of the second recombinant fragment was probably a close

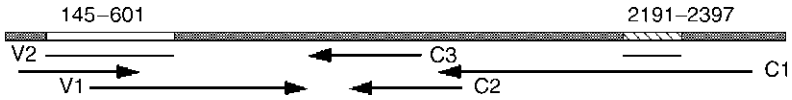

Figure 3 Genomic map of *Tomato curly stunt virus* showing opening reading frames and two recombination fragments (first fragment between nt coordinates 145 and 601 and second fragment between nt coordinates 2191 and 2397) identified by at least three recombination analysis methods.

relative of *East African cassava mosaic virus* (EACMMV) and SACMV. The recombinant fragment spanned the core of the C1 ORF at the nucleotide coordinates 2191–2397. Only two programs, RDP and SISCAN, identified a fragment having a significant match with nucleotide coordinates 2262–2735 to be close relative of ToLCSDV-[Sha], with probabilities of 3·41 E-07 and 5·17 E-08, respectively. Only SISCAN recognized a fragment between coordinates 2058–2477 to be close relative of ToLCMLV with 1·16 E-09 probability. Independent phylogenetic analysis of recombinant segments corroborated the validity of the predicted recombination events for ToCSV-EACMMV, ToCSV-SACMV, ToCSV-ToLCSDV-[Sha] and ToCSV-ToLCMLV (data not shown). Finally, a GenBank BLAST search identified a fragment at coordinates 1673–2766 which shared 96% nt identity with TbLCZV. However, the algorithm did not detect a recombinant fragment between ToCSV and TbLCZV.

Discussion

Transmission studies for ToCSV carried out using the B-biotype whitefly as the vector indicated that the virus was transmitted in a persistent manner, consistent with all other begomoviruses known to date (Briddon *et al*., 1990; Brown, 1994). The minimum AAP for ToCSV was 1 h and the transmission frequency increased with longer AAPs, in agreement with previous virus acquisition studies for the related TYLCV using the B-biotype of *B. tabaci* as the vector (Czosnek *et al*., 2001). Similarly, inoculation efficiency was found to be greater for whiteflies given increasingly longer IAPs. However, transmission was demonstrated in 5 min following an initial AAP of 24 h, indicating that the latent period had been satisfied in 24 h or less, also characteristic of begomoviruses. The IAP for ToCSV was somewhat shorter than that reported for TYLCV (Israel), which was 15–30 min (Czosnek *et al*., 2001).

The results of the ToCSV host-range study revealed that it infects a suite of species (Table 1) similar to those reported for TYLCV (Israel) (Dalmon & Marchoux, 2000; Ying & Davis, 2000), including the ability to infect bean. TYLCV was reported to cause economic loss in commercial bean (*P. vulgaris*) in Spain (Navas-Castillo *et al*., 1999), and disease symptoms in bean were reported to range from severe to symptomless, depending on cultivar (Lapidot, 2002). In this study, two of the three bean cultivars tested developed obvious disease symptoms. No *P. vulgaris* cv. Black Turtle Soup bean plants were susceptible to ToCSV (Table 1). It is of interest to learn whether the determinant(s) for TYLCV resistance in bean are also responsible for ToCSV resistance in this host, as was found for TYLCV-resistant tomato germplasm (Pietersen & Smith, 2002). Interestingly, ToCSV symptoms were not observed in commercial bean fields in close proximity to ToCSV-infected tomato fields (G. Pietersen, unpublished data), suggesting a barrier to natural spread, and in parallel to a report from Israel (Lapidot, 2002). Interestingly, pepper (*Capsicum* sp.) was not a host of ToCSV in the experimental host-range study, and indeed, pepper crops planted in the proximity of tomato fields with high incidence of ToCS disease did not show ToCSV- or TYLCV-like symptoms, even though certain pepper cultivars are known to be susceptible to TYLCV (Reina *et al*., 1999).

The epitope profile using a panel of monoclonal antibodies raised against several African begomoviruses revealed that ToCSV shares certain common epitopes with two African viruses represented: ACMV from cassava and OkLCuV from okra. It is not surprising that MAbs to ICMV did not cross react with ToCSV, given that African viruses have not previously been shown to share related epitopes with begomoviruses from India (Swanson *et al*., 1992; Swanson & Harrison, 1993), presumably because they are geographically and/or host-isolated.

Phylogenetic analysis indicated that ToCSV is most closely related to begomovirus lineages from southeastern and western Africa. ToCSV, together with three other tomato-infecting viruses in Africa: TbLCZV, PepYVV and ToLCMLV, formed a newly recognized sister clade of begomoviruses extant to southeastern and west Africa (Fig. 2), with 78–84% shared identity. Based on >89% shared nt identity (>11% divergence) with other known begomoviral species, the working cutoff for demarcating begomoviral species (Fauquet *et al*., 2003) ToCSV is a new species in the genus *Begomovirus*. The high shared nt identity between ToCSV and tomato-infecting begomoviruses from Mali and the southwest Indian Ocean islands (Delatte *et al*., 2005) also indicates that ToCSV is indigenous to Africa and that it is not likely to have been introduced from outside of the region or, specifically, South Africa.

That a satDNA molecule was not detected using degenerate primers that have been used to amplify satDNAs associated with other monopartite begomoviruses, was also not unexpected, because many monopartite begomoviruses cause full-blown disease symptoms in the absence of this type of accessory molecule. However, the possibility that ToCSV has a satDNA cannot be ruled out until direct proof of causality is provided.

Recombination analyses failed to detect recombinatorial fragments in the TbLCZV genome, the first begomovirus identified in tomato from southern Africa. In contrast, a BLAST search using the ToCSV genome sequence identified

a major fragment, located between coordinates 1673 and 2766, at 96% nucleotide identity, spanning in part the C1-IR region of TbLCZV. This predicted evolutionary relationship was not surprising given that TbLCZV and ToCSV occur in the same location, and furthermore, infect solanaceous species. It is not clear how frequently recombination algorithms fail to detect statistically significant events; however, in the case of ToCSV, it seems likely that the visual detection of two fragments of >200 bases (Fig. 3) could well represent a 'real event'.

Quite unexpectedly, similar fragments were identified in the IR for ToCSV and ToLCSDV-Sha, the latter being a tomato-adapted virus from the Nile Basin (Sudan). The two viruses share an identical REP-binding site, which is similar to that of TYLCV and its related species widespread in the Mediterranean and North African region (Fauquet *et al*., 2005; Idris & Brown, 2005). Two other members of the sister clade, PepYVV and ToLCMLV, have a REP-binding site most like that of the cassava-infecting EACMV; in contrast, the TbLCZV iteron is similar to the REP-binding site for ToCSV. Thus, despite the results of the algorithm-based recombination analysis, the BLAST analysis and visual scanning of sequences suggested recombination has occurred during the evolution of these otherwise divergent begomoviral genomes. Interestingly, the extant viruses are host-adapted to cassava and tomato, respectively, and also are presently geographically isolated. Finally, based on three different recombination algorithms, significant genome fragments of two cassava-infecting viruses, EACMMV and SACMV, both indigenous to southern Africa, were also identified in the ToCSV genome (Fig. 3). This possibly suggests that ToCSV and the extant cassava-restricted EACMMV or SACMV have shared (or presently share) a common host that facilitated the predicted recombination.

Because ToCSV is only the second begomovirus yet to have emerged in tomato in southern Africa, it will be of evolutionary interest to identify the endemic hosts of ToCSV and trace the path of host-adaptation to a cultivated solanaceous species.

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References

Briddon RW, Pinner MS, Stanley J, Markham PG, 1990. Geminivirus coat protein gene replacement alters insect specificity. *Virology* **177**, 85–94.

Briddon RW, Mansoor S, Bedford ID, *et al*. 2001. Identification of DNA components required for induction of cotton leaf curl disease. *Virology* **285**, 234–43.

Brown JK, 1994. Current status of *Bemisia tabaci* as a plant pest and virus vector in agroecosystems worldwide. *FAO Plant Protection Bulletin* **42**, 3–32.

Brown JK, 2000. Molecular markers for the identification and global tracking of whitefly vector-begomovirus complexes. *Virus Research* **71**, 233–60.

Czosnek H, Laterrot H, 1997. A worldwide survey of tomato yellow leaf curl viruses. *Archives of Virology* **142**, 1391–406.

Czosnek H, Ghanim M, Morin S, Rubinstein G, Fridman V, Zeidan M, 2001. Whiteflies: vectors, and victims (?), of geminiviruses. *Advances in Virus Research* **57**, 291–322.

Dalmon A, Marchoux G, 2000. Quelles plantes hôtes pour le tomato yellow leaf curl virus? *Phytoma* **527**, 14–7.

Delatte H, Martin DP, Naze F *et al*., 2005. South West Indian islands tomato begomovirus populations represent a new major monopartite begomovirus group. *Journal of General Virology* **86**, 1533–42.

Doyle JJ, Doyle JL, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11–5.

Dry IB, Krake LR, Rigden JE, Rezaian MA, 1997. A novel subviral agent associated with a geminivirus: the first report of a DNA satellite. *Proceedings of the National Academy of Sciences, USA* **94**, 7088–93.

Fauquet C, Mayo M, Maniloff J, Desselberger U, Ball L, 2005. *Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses*. London, UK: Elsevier, Academic Press.

Fauquet CM, Bisaro DM, Briddon RW *et al*., 2003. Revision of taxonomic criteria for species demarcation in the family *Geminiviridae*, and an updated list of begomovirus species. *Archives of Virology* **148**, 405–21.

Idris AM, Brown JK, 1998. *Sinaloa tomato leaf curl geminivirus*: biological and molecular evidence for a new subgroup III virus. *Phytopathology* **88**, 648–57.

Idris AM, Brown JK, 2004. Cotton leaf crumple virus is a distinct western hemisphere begomovirus species with complex evolutionary relationships indicative of recombination and reassortment. *Phytopathology* **94**, 1068–74.

Idris AM, Brown JK, 2005. Evidence for interspecific-recombination for three monopartite begomoviral genomes associated with the tomato leaf curl disease from central Sudan. *Archives of Virology* **150**, 1003–12.

Idris AM, Briddon R, Bull SE, Brown JK, 2005.*Cotton leaf curl Gezira virus*-satDNAs represents a divergent, geographically-isolated Nile Basin lineage: predictive identification of a satDNA REP binding motif. *Virus Research* **109**, 19–32.

Lapidot M, 2002. Screening common bean *Phaseolus vulgaris* for resistance to tomato yellow leaf curl virus. *Plant Disease* **86**, 429–32.

Martin D, Rybicki E, 2000. RDP: detection of recombination amongst aligned sequences. *Bioinformatics* **16**, 562–3.

Navas-Castillo J, Sanchez-Campos S, Diaz JA, 1999. Tomato yellow leaf curl-Is causes a novel disease of common bean and severe epidemics in tomato in Spain. *Plant Disease* **83**, 29–32.

Picó B, Díez MJ, Nuez F, 1998. Evaluation of whitefly-mediated inoculation techniques to screen *Lycopersicon esculentum* and wild relatives for resistance to tomato yellow leaf curl virus. *Euphytica* **101**, 259–71.

Pietersen G, Smith MF, 2002. *Tomato yellow leaf curl virus* resistant tomato shows resistance to *Tomato curly stunt virus*. *Plant Disease* **86**, 528–34.

Pietersen G, Idris AM, Krüger K, Brown JK, 2000. *Tomato curly stunt virus*, a new begomovirus of tomato within the *Tomato*

yellow leaf curl virus-IS cluster in South Africa. *Plant Disease* **84**, 810.

Reina J, Morilla G, Bejarano ER, Rodriguez MD, Janssen D, 1999. First report of *Capsicum annuum* plants infected by *Tomato yellow leaf curl virus*. *Plant Disease* **83**, 1176

Saunders K, Bedford ID, Briddon RW, Markham PG, Wong SM, Stanley J, 2000. A novel virus complex causes Ageratum yellow vein disease. *Proceedings of the National Academy of Sciences, USA* **97**, 6890–5.

Swanson MM, 1992. *Antigenic Variation and Serological Relationships of Whitefly Transmitted Geminiviruses*. PhD thesis. Dundee, UK: University of Dundee.

Swanson MM, Harrison BD, 1993. Serological relationships and epitope profiles of isolates of okra leaf curl geminivirus from Africa and the Middle East. *Biochemie* **75**, 707–11.

Swanson MM, Varma A, Muniyappa V, Harrison BD, 1992. Comparative epitope profiles of the particle proteins of whitefly-transmitted geminiviruses from nine crop legumes in India. *Annals of Applied Biology* **120**, 425–33.

Swofford DL, 2005. *PAUP: Phylogenetic Analysis Using Parsimony (and Other Methods), Version 4·0b10*. Sunderland, MA, USA: Sinauer Associates.

Thomas JE, Massalski PR, Harrison BD, 1986. Production of monoclonal antibodies to African cassava mosaic virus and differences in their reactivities with other whitefly transmitted geminiviruses. *Journal of General Virology* **67**, 2739–48.

Wyatt SD, Brown JK, 1996. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathology* **86**, 1288–93.

Ying ZT, Davis MJ, 2000. Partial characterization and host range of tomato yellow leaf curl virus in South Florida. *Proceedings of the Florida State Horticultural Society* **113**, 185–90.