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Research Article
Biochemical, Serological, Molecular and Natural Host Studies on Tomato Chlorosis Virus in Egypt

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Abstract

Background and Objective: Tomato Chlorosis Virus (ToCV) is a white fly-transmitted and phloem-limited crinivirus reported in this study for the first time in Egypt. ToCV caused drastic reduction in tomato yield since 2013. The aim of this study is to characterize the virus incidence using biological, serological and molecular tools. **Materials and Methods:** The *B. tabaci* MEAM1 white fly was used for virus isolation and propagation. Identity of ToCV , its natural hosts were confirmed with RT-PCR using a specific primer pair for ToCV-heat shock protein 70 homologue (HSP70h) gene, sequencing and phylogenetic studies. ToCV was purified using the innovative electro-elution technique. The induced antiserum for the Egyptian isolate of the virus (ToCV-Giza) was used for DAS-ELISA and dot blotting immuno-assays to evaluate the virus presence in tomato and other natural hosts. **Results:** The ToCV-Giza isolate was donated an accession number "MH667315.1" from the GenBank. Blastx sequence analysis of the HSP70h gene indicated 97-99% of amino acid similarities with many tested ToCV isolates. Phylogenetic studies showed the clustering of all ToCV isolates including ToCV-Giza in a separate group from the other tested criniviruses. The virus had a UV spectrum of a nucleoprotein with A_{max} and A_{min} at 260 and 240 nm, respectively and $A_{260/280}$ ratio of 1.33. Out of 52 different tested plant species within 22 families, 44 were positive hosts for ToCV. Thirty seven out of these 44 plant species were considered as new hosts for ToCV in the present study. These included *Ammi majus* and *Coriandrum sativum* (*Apiaceae*), cabbage (*Brassicaceae*), sweet potato (*Convolvulaceae*), melon, cucumber, luffa (*Cucurbitaceae*), soybean, cowpea, faba bean (*Fabaceae*), Egyptian and American Cotton (*Malvaceae*). Several ornamentals either herbal type or woody trees belonging to *Acanthaceae*, *Amaranthaceae*, *Euophorbiaceae*, *Moraceae* and *Rubiaceae* were also recognized for the first time as hosts for ToCV. **Conclusion:** The obtained results confirmed the wide distribution of ToCV in its natural hosts in Egypt. Hygienic measures including control of the virus vector and removing of natural hosts should be strictly implicated.

Key words: Crinivirus, natural hosts, *Tomato chlorosis virus*, tomato, whiteflies

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tomato Chlorosis Virus (ToCV) is one of the most devastating viruses in tomato (*Solanum lycopersicum* L.) grown in the fields and greenhouse facilities worldwide[1-3]. ToCV was reported for the first time in Florida, USA[4] and many European, American, African and Asian countries[5-25].

ToCV, *Crinivirus,* Closteroviridae has non-enveloped flexuous filamentous particles of approximately 800-850 nm in length[4,26] with two segments of linear, positive-sense and single-stranded RNAs, encapsulated separately[4,18]. The RNA1 (8595nt) contains four open reading frames (ORFs) which encode replication proteins. RNA2 (8247nt) encodes nine ORFs necessary for virus encapsulation, movement and vector transmission[27].

ToCV is mostly a phloem-limited virus[4,28] and transmitted in a semi-persistent manner by species and biotypes of Aleyrodidae including: The more polyphagous vector *Bemisia tabaci* New World(NW), Middle East-Asia Minor 1(MEAM1) and Mediterranean (MED), *Trialeurodes abutiloneus* and *T. vaporariorum* [2,6,29]. ToCV was not transmitted by seed or mechanical inoculation[3,29,30]. Recently Lee *et al*.[3] have achieved graft transmission of ToCV onto healthy tomato.

Generally, members of *Crinivirus* induce symptoms readily mistaken for mineral deficiencies or pesticidal phytotoxicity. Infected plants show discoloration due to low-photosynthesis efficacy, loss of plant vigor and early senescence[2, 31].

ToCV-infected tomato exhibits interveinal chlorosis on lower leaves followed by leaf thickening, bronzing accompanied by brown necrotic flecks. Sometimes lower leaves exhibit inward leaf curling. Symptoms developed on fruits are incoherent, with significant yield reduction, due to sterile flowers and photosynthesis deterioration[2, 4].

Diagnosis of ToCV on tomato based solely on symptoms is difficult due to its co-infection with other criniviruses or begomoviruses transmitted by white flies which alters and aggravates symptoms[2,3].

The induced symptoms by ToCV can easily be confused with those caused by the white fly-transmitted *Tomato Infectious Chlorosis Virus* (TICV)[4] present as a single or mixed infection with ToCV in tomato as recorded in many Mediterranean countries[5,8,13,32]. The two viruses can be identified in mixed infection by specific antisera, specific primers, differential hosts and nucleic acid hybridization where the two viruses do not cross hybridize[5]. In addition TICV is transmitted only by *Trialeurodes vaporariorum*, while ToCV is transmitted by *T. vaporariorum*, *T. abutilonea* and *Bemisia tabaci* biotypes A, B and Q[2,4].

ToCV has rather a wide host range. Approximately 60 species of cultivated and weed plants belonging to 18 families have been reported naturally or experimentally susceptible to ToCV[20,22,29,33-35].

Unfortunately, there are no known available resistant varieties of tomato to ToCV[36,37] and the chemical control of the insect vector is not fully successful[2,38].

In the autumn of 2013, an unusual yellow leaf disorder of tomato similar to ToCV infection was observed both in the greenhouses and fields of the experimental station of the Faculty of Agriculture, Cairo University at Giza governorate, Egypt. The present investigation elucidates the nature of the virus etiology based on biological, chemical, serological and molecular analysis tools. To the best of our knowledge, this is the first report of ToCV in Egypt.

MATERIALS AND METHODS

Virus isolation and propagations: The ToCV was isolated from infected tomato plants grown at the experimental station of the Faculty of Agriculture, Cairo University in Giza Governorate.

Non-viruliferous *B. tabaci* MEAM1, maintained on cucumber (*Cucumis sativus* L.) plants, in insect-proof cages, were fed onto infected tomato plants using 20 insects per plant. Subsequently, viruliferous insects were transferred onto healthy tomato seedlings (15 insects/plant) using 24 h and 48 h acquisition and inoculation access feeding periods, respectively. Insects were then killed with an insecticide. Plants were grown for further 6 weeks in an insect proof green house for virus propagation.

ToCV host analysis: Three-leaf samples from each plant grown in the vicinity of tomato plantations were collected and subjected to serologic and molecular analysis.

Virus purification: ToCV virus was purified from100 g frozen Tissues. Tissues were ground (1:4 w/v) in cold extraction buffer (10 mM K_2HPO_4, 20 mM Na_2SO_3, 1 mM EDTA, 0.01% Thioglycolic acid, 0.01% 2-mercaptoethanol), pH 8.7. The extract was passed through 4 layers of cheesecloth. The filtrate was clarified with ½ volume of cold chloroform: butanol, (1:1,v/v) mixture and centrifuged 8000 rpm for 10 min at 4°C. The virus was precipitated with 10% polyethylene glycol (6000 mw) plus 1% NaCl by stirring over night at 4°C then centrifugation (8000 rpm/10 min/4°C). The supernatant was removed and pellet was suspended in 1 mM K_2HPO_4 buffer containing 1 mM EDTA, pH 8.7 (suspension buffer, SB)

using 1/10 volume of the original filtrate recovered after the cheesecloth-filtration step. Suspended preparation was dialyzed twice overnight at 4°C in two changes of SB then received another cycle of low-speed centrifugation. Virus pellet was suspended in SB (1/30 volume of the original filtrate) and electro-eluted in ISCO Blue Tank, ISCO INC, Lincoln, USA, by applying 4 mA per cell and with tank buffer concentration equals to 20 folds of the SB. Virus concentrations was estimated using an extinction coefficient (A0.1%, 1cm, 260 nm) value of 3.0 described for the crinivirus *Beet Pseudo-Yellows Virus* [39]. Physical properties of purified virus were also examined spectrophotometrically.

Serological studies

Production of polyclonal antisera for ToCV: The protocol described by Abdel-Salam[40] was followed for the production of ToCV antiserum. The prepared antiserum and its purified IgG were cross absorbed with healthy non-diluted tomato sap (20%, v/v) to remove non-specific antibodies according to Abdel-Salam[40].

Serological tests

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA): The DAS-ELISA procedure described by Clark and Adams[41] was followed with some modification including an additional blocking step with 5% non-fat dry milk and 1% bovine serum albumin after the IgG coating and washing steps. Extraction buffer used for sap extraction from tested tissues composed of 0.1 M sodium citrate, pH 6.0, containing 2.5 mM EDTA and 2% Triton X-100 (TX100). Samples were extracted at a dilution 1/20 (w/v). IgG-alkaline phosphatase conjugation was prepared according to Converse and Martin[42]. IgG concentrations used for plate coating was tested at a range between 1-4 μg mL^{-1}, while IgG-enzyme conjugate was tested at range of dilutions between 1/500 up to 1/2000. Absorbance values were read at optical density (OD) at 405 nm using MR-96 Microplate Reader (Bio-Medical Electronics Co. LTD). Samples were considered positive if their OD_{405nm} value, after subtracting the buffer absorbance value, were $\geq$2.9 times the healthy control value[43]. Samples with OD_{405nm} close to the threshold of 2.9 OD were rechecked with dot blotting immunobinding assay.

Dot blotting immunobinding assay (DBIA): Symptomatic leaves were collected from several plant species from several locations at the experimental fields of the Faculty of Agriculture, Cairo University in Giza governorate. Leaves were extracted (1:20 w/v) with either TBST buffer (20 mMTris-HCl containing 150 m M NaCl, 0.5% Tween-20, pH 8.0) or in sodium citrate buffer as described in DAS-ELISA above. The procedure of DBIA described by Abdel-Salam *et al.*[44] was followed with some modifications. These included blocking the nitrocellulose membranes (NCM), with 5% (w/v) non-fat dry milk (NFDM) and 1% (w/v) bovine serum albumin, incubation in goat anti-rabbit alkaline phosphatase conjugate diluted at 10^{-4} in PBST containing 5% NFDM and 2% (w/v) polyvinyl pyrrolidone and staining with Naphthol/Fast red complex as described before[44]. Samples stained red are considered virus positive, while samples remained green are considered negative to virus presence.

Molecular studies

Oligonucleotide primers for ToCV and TICV: The specific oligonucleotide primers ToCV-172(+) (5'GCT TCC GAA ACT CCG TCT TG 3') and ToCV-610 (-) (5' TGT CGA AAG TAC CGC CAC C 3') for ToCV[5] and TICV-32(+) (5' TCA GTG CGT ACG TTA ATG GG3') and TICV-532(-) (5'CAC AGT ATA CAG CAG CGG CA 3') for TICV[32] were designed to amplify 439 and 501 bp for ToCV and TICV, respectively of the corresponding coding sequence of the heat shock protein 70 homologue (HSP70h)gene.

Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR): Total RNA was extracted from 100 mg of infected leaves using RN easy plant mini kit, Cat No. 74903, Qiagen Sciences, Maryland, USA. Duplex RT-PCR was employed to check the presence of ToCV in tested host range or in tomato plants propagated for virus purification, respectively. GoTaq Flexi DNA polymerase was used in PCR analysis. The total RNAs were first heat-denatured at 65°C/5 min and then chilled immediately in ice, the reaction mixture was added to the PCR tubes. The first reverse transcription step was done at 50°C/30 min using M-MLV Reverse Transcriptase. After a brief denaturation step (94°C/4 min), 35 cycles, each (94°C/30, 55°C/30, 72°C/30 sec), were performed and ending with a final cycle at 72°C for 10 min using the Techne™ TC-312Thermal Cycler. The PCR products were examined in 1.5% agarose gel electrophoresis, stained with 0.5 μg mL^{-1} ethidium bromide and examined with UV illuminator.

Cloning, sequencing and phylogenetic studies: This part is carried out at the Department of Biotechnology, College of Agriculture and Food Sciences, King Faisal University. The DNA bands of interest were cut from the agarose gel, purified, cloned into pGEM T-Easy vector (Promega). The ligation

mixtures were used to transform *Eschericia coli*, strain DH5α, using the procedure of Sambrook *et al*.[45]. Three plasmids from selected colonies were purified by miniprep then sequenced in both directions using automated, capillary DNA sequencing and dye terminator sequencing. The DNA sequence for the ToCV-Giza isolate was submitted to the Gen Bank to obtain an accession number. DNA sequences and expected translation amino acids were compared with some available ToCV reference sequences using NCBI/Blastx, www.ncbi.nlm.nih.gov. Phylogenetic relationships were measured using MEGA6 programs.

RESULTS

Biological studies

Symptomatology: ToCV causes yellowing disease on tomato. Early symptoms consist of interveinal chlorosis on lower leaves, resembling nutrient deficiency. With disease progress, the interveinal yellowing become obvious and leaves show slight inward leaf curling, bronzing and necrotic flecking often occur within the yellowing areas (Fig. 1). Leaves become thickened and easily broken. Symptoms mostly developed on lower and middle leaves, where upper leaves appear normal. Infected plants exhibit drastic reduction in vigor and fruit yield due to flower sterility. Infected fruit appear normal.

On other natural hosts for ToCV symptoms of infected plants are very similar to ToCV-infected tomato including interveinal chlorosis, yellowing and bronzing on old leaves. Some hosts developed necrotic flecking on leaves which later develop to shoot holes (Fig. 2, Table 1).

Chemical studies: Purified virus preparations of ToCV with the electro-elution technique had a UV spectrum of a nucleoprotein with A_{max} at 260 nm, A_{min} at 240 nm and $A_{260/280}$ ratio of 1.33. Purified virus yield was 0.33 mg g^{-1} fresh tissue.

Serological studies

DAS-ELISA: Results showed that the optimum concentration for IgG used for coating the micro-plates was 4 μg mL^{-1}. The optimum dilution for IgG-enzyme conjugate was at 1/1000 dilution. DAS-ELISA detected ToCV-infected tomato plants in the field (Fig. 3). However some infected tomato plants gave $OD_{405\ nm}$ values close to the threshold of 2.9 O.D. The test detected the virus in several economic plant families as well as in many ornamentals (Fig. 3, Table 1).

DBIA: Detection of ToCV in infected tissues using TBST as an extraction buffer gave very poor or negative results. On the other hand, extraction with sodium citrate buffer containing the neutral detergent Triton X-100 enables the detection of the virus presence on NCM (Fig. 4, 5). The ToCV-antiserum detected ToCV in several plant families (Table 1) and was able to differentiate between infections with ToCV and a begomovirus from pepper (Fig. 4).

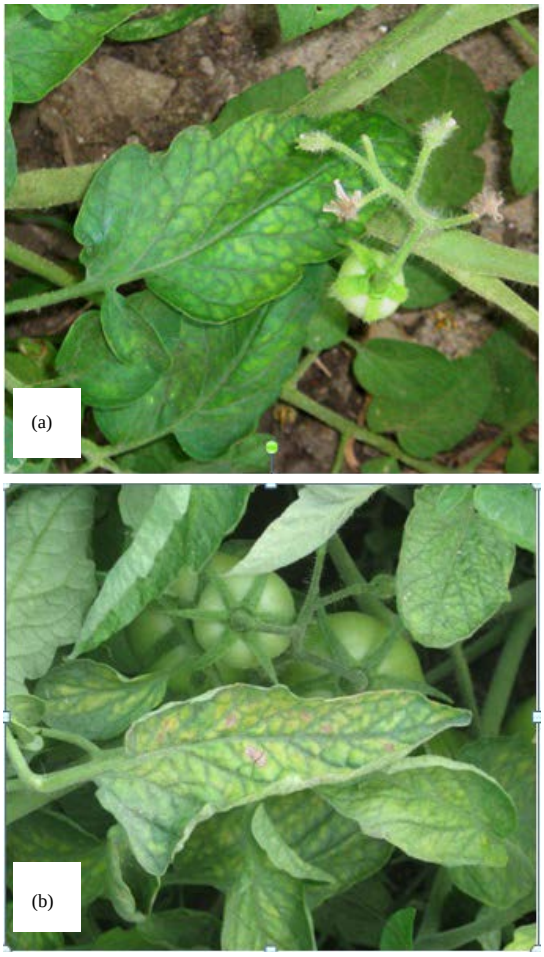

Fig. 1(a-b): Symptoms developed on tomato upon ToCV infection (a) Interveinal chlorosis and flower withering and (b) Interveinal yellowing and necrotic flecking

Molecular studies

RT-PCR: ToCV-infected tomato, propagated in the greenhouse, after whitefly transmission was tested with duplex RT-PCR and the primer pairs ToCV-172(+)/610(-) for ToCV and TICV-32(+)/532(-) for TICV. Only amplicons for ToCV with 439 bp was detected. No amplicons for TICV at 501 bp was observed. In addition, no amplicons was detected in healthy tomato (Fig. 6). Such results indicated the sole presence of ToCV in the propagated plants.

Table 1: Detection of ToCV in plants adjacent to tomato plantations in Giza governorate

		Detection Methods*		
Family/host plant	Symptoms	RT-PCR	DBIA	DAS-ELISA
Acanthaceae				
Adahtoda vasica (Malabar nut)	ICh	- (3/3)	- (3/3)	ND
Daedalacanthus nervosus	ICh, NF	+ (3/3)	+ (3/3)	ND
Amaranthaceae				
Amaranthus sylvestris (pigweed amaranth)	ICh	+ (3/3)	+ (3/3)	0.534
Apiaceae				
Ammi majus (Bishop's weed)	ICh, Y	+ (3/3)	+ (3/3)	ND
Coriandrum sativum (Cilantro)	ICh, Y	+ (3/3)	+ (3/3)	ND
Apocynaceae				
Metaplexis japonica	ICh, Y	+ (3/3)	+ (3/3)	0.649
Araceae				
Anadendrum affine	ICh, Y	+ (3/3)	+ (3/3)	ND
Philodendron bipinnatifidum (Horsehead philodendron)	ICh	- (0/3)	- (0/3)	ND
Araliaceae				
Schefflera arboricola	ICl	+ (3/3)	+ (3/3)	0.520
Asteraceae				
Bidens bipinnata (Spanish needles)	NS	+ (3/3)	+ (3/3)	ND
Brassicaceae				
Brassica oleracea var. *Capitata* (Cabbage)	ICh	+ (3/3)	+ (3/3)	ND
Sismbrium irio (Wild Mustard)	ICh, Y	+ (3/3)	+ (3/3)	ND
Chenopodiaceae				
Chenopodium album (Goosefoot)	ICh	+ (3/3)	+ (3/3)	ND
Convolvulaceae				
Ipomea batatas (Sweet potato)	ICh	+ (3/3)	+ (3/3)	1.545
Cucurbitaceae				
Cucumis melo (Melon)	ICh	+ (2/3)	+ (3/3)	0.425
Cucumis sativus (Cucumber)	ICh, Y	+ (2/3)	+ (2/3)	0.338
Luffa aegyptiaca (Luffa)	ICh	+ (3/3)	+ (3/3)	0.298
Euophorbiaceae				
Codiaeum variegatum (Croton)	MY, MW	+ (3/3)	+ (3/3)	0.826
Codiaeum variegatum cv. Gold finger	Y	+ (2/3)	+ (3/3)	0.546
Euphorbia geniculata	ICh	+ (2/3)	+ (3/3)	0.712
Jatropha integerrima (Peregrina)	ICh, Y	+ (3/3)	+ (3/3)	0.565
Ricinus communis (Castor bean)	ICh	+ (3/3)	+ (3/3)	ND
Fabaceae				
Arachis hypogaea (Peanut)	ICh	- (0/3)	- (0/3)	ND
Bauhinia variegate (Orchid tree)	ICh	+ (3/3)	+ (3/3)	0.378
Leucaena leucocephala (White leadtree)	ICh	+ (3/3)	+ (3/3)	ND
Phaseolus vulgaris (French bean)	ICh	+ (3/3)	+ (3/3)	ND
Glycine max (Soybean)	B, NF, ICH, SH, Y	+ (3/3)	+ (3/3)	0.420
Vigna unguiculata (Cowpea)	ICh	+ (3/3)	+ (3/3)	ND
Vicia faba (Faba bean)	ICh	+ (3/3)	+ (3/3)	ND
Geraniaceae				
Pelargonium hirsutum (Geranium)	ICh	+ (2/3)	+ (3/3)	ND
lamiaceae				
Ocimum basilicum (Basil)	ICh	- (0/3)	- (0/3)	ND
Malvaceae				
Althaea rosa (Hollyhock)	MCh, Y	+ (3/3)	+ (3/3)	0.547
Corchorus olitorius (Jew's mallow)	B, R, ICh,	+ (3/3)	+ (3/3)	0.419
Gossypium barbadense (Egyptian cotton)	MCh, VR,Y	+ (3/3)	+ (3/3)	0.690
G. hirsutum (American cotton)	MCH, VR, Y	+ (3/3)	+ (3/3)	0.289
Hibiscus cannabinus (Mesta)	ICh	+ (3/3)	+ (3/3)	0.594
H. piment (Wax mallow)	ICh	-- (0/3)	-- (0/3)	ND
H. rosa-sinensis (China rose)	ICh	+ (2/3)	+ (3/3)	0.438
Malva parviflora (Cheese weed) mallow	MCh, R,Y	+ (3/3)	+ (3/3)	0.606
Meliaceae				
Cedrela odorata (Spanish cedar)	ICh	- (0/3)	- (0/3)	ND
Moraceae				
Ficus carica (Fig)	ICh	+ (3/3)	+ (3/3)	0.477
F. nitida	ICh	+ (2/3)	+ (3/3)	ND
Morus alba (white mulberry)	ICh	+ (3/3)	+ (3/3)	0.402

Table 1: Continue

Family/host plant	Symptoms	Detection Methods* RT-PCR	DBIA	DAS-ELISA
Nyctaginaceae				
Bougainvillea spectabilis (Great bougainvillea)	ICh	- (0/3)	- (0/3)	ND
Poaceae				
Zea mays (Maize)	ICh	- (0/3)	- (0/3)	ND
Rubiaceae				
Pentas lanceolata (Egyptian star cluster)	ICh	+ (3/3)	+ (3/3)	ND
Solanaceae				
Capsicum annuum (Sweet pepper)	NF, ICh, LC, FW	+ (3/3)	+ (2/3)	0.412
Solanum lycopersicum (Tomato)	NF, ICh, IY, FW, LC, LT, S, SH	+ (3/3)	+ (3/3)	0.989
S. melongena (Eggplant)	NF, ICh, Y	+ (3/3)	+ (3/3)	0.506
S. pimpinellifolium (Currant tomato)	ICh	+ (3/3)	+ (3/3)	ND
S. tuberosum (Potato)	ICh	+ (3/3)	+ (3/3)	ND
Withania somnifera (Indian ginseng)	ICh, Y	+ (3/3)	+ (3/3)	0.479

B: Bronzing, FW: Flower withering, Ich: Interveinal chlorosis, IY: Interveinal yellowing, LC: Leaf curl, LT: Leaf thickening, MCh: Marginal chlorosis, MW: Marginal waving, MY: Marginal yellowing, ND: Not determined, NF: Necrotic flecks, NS: No symptoms, R: Reddening, Y: Yellowing, VR: Vein reddening, S: Stunting, SH: Shoot holes
*Each examined plant was tested with RT-PCR, DBIA and/or DAS-ELISA for three times in three separate occasions. Numbers between brackets represent numbers of infected plants/the number of collected plants, **Sample was considered positive if its $OD_{405\ nm}$ value $\geq$2.9 times the healthy tomato control value ($O.D_{405\ nm}$= 0.101). For each sample, results represent the average $O.D_{405\ nm}$ of three replicates minus the $O.D_{405\ nm}$ of buffer

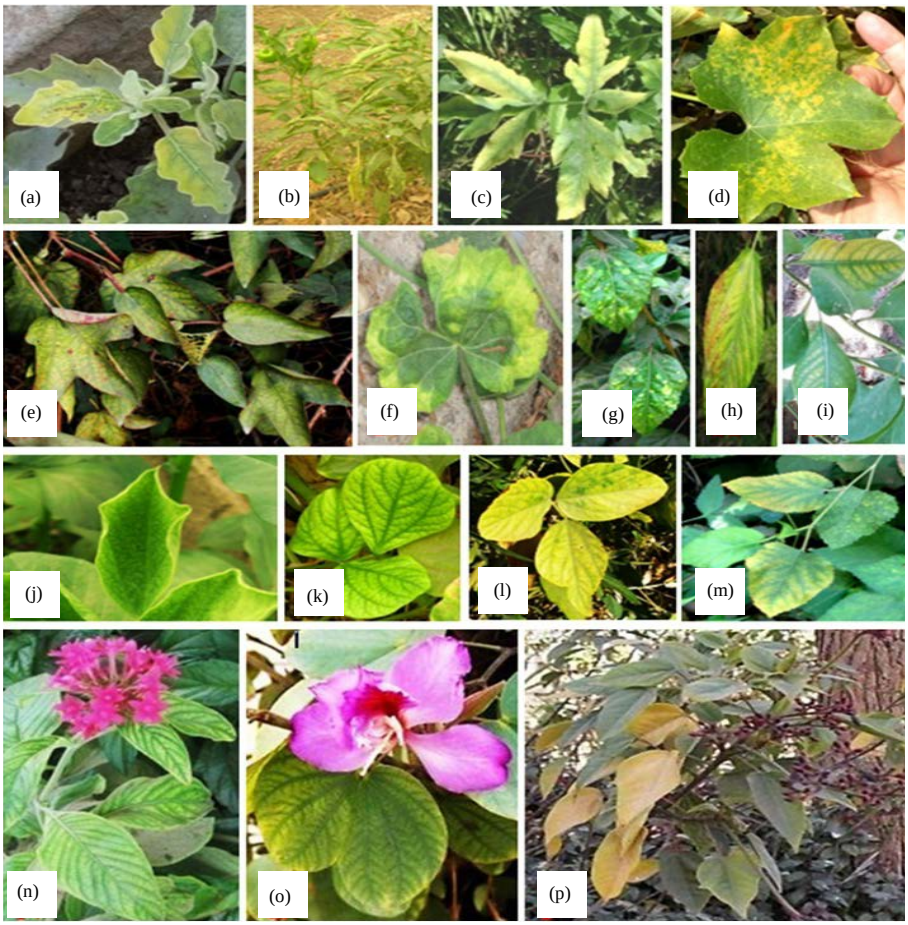

Fig. 2(a-p): Natural host plants for ToCV (a) Eggplant, (b) Sweet pepper, (c) Wild mustard, (d) Luffa, (e) Egyptian cotton, (f) Hollyhock, (g) China rose, (h) Jew's mallow), (i) Indian ginseng, (j) Faba bean, (k) Cowpea, (l) Soybean, (m) White mulberry, (n) Egyptian starcluster, (o) Orchid tree and (p) Peregrina. Table 1 refers to scientific names and symptom description

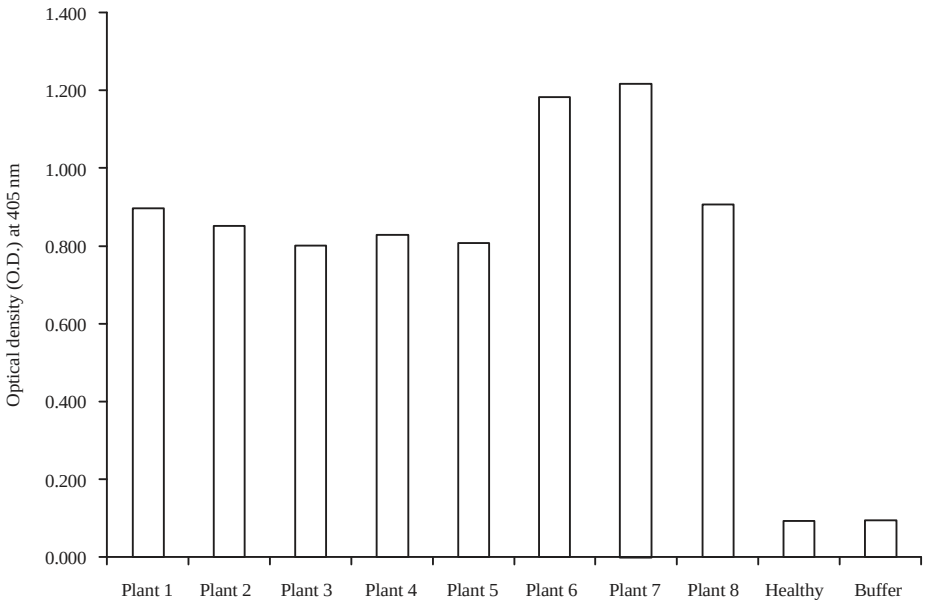

Fig. 3: DAS-ELISA for detection of ToCV-infected tomato plants in the field (plants # 1-7). Plant # 8 represents a positive control of ToCV-infected tomato plant within tomato plants, grown under greenhouse conditions and used for virus propagation
Data for each plant are the average of three ELISA readings

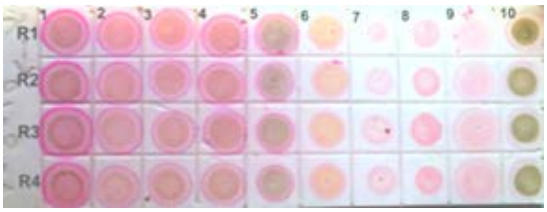

Fig. 4: DBIA test showing the reaction of ToCV-IgG (1/500 dilution) with tested hosts on nitrocellulose membrane (NCM).1, +ve control (ToCV/tomato); 2-4, tomato; 5, pepper; 6, 7, Egyptian cotton; 8, American cotton; 9, hollyhock; 10, -ve control (healthy tomato)
Each row was repeated four times (R1–R4)

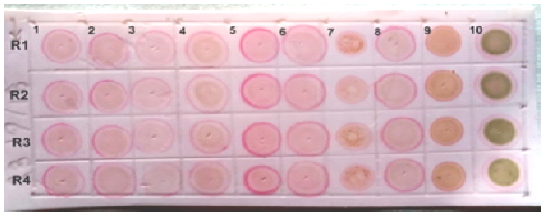

Fig. 5: DBIA test showing the reaction of ToCV antiserum (1/500 dilution) with tested hosts on nitrocellulose membrane (NCM). 1, +ve control (ToCV/tomato); 2,3, tomato; 4, luffa; 5, cucumber; 6, soybean; 7, melon, 8, Jew's mallow; 9, Egyptian cotton; cotton; 10, -ve control (healthy tomato)
Each row was repeated four times (R1–R4)

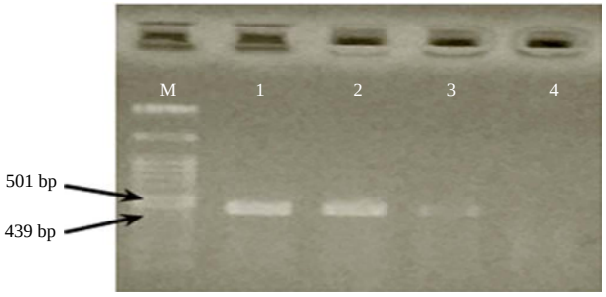

Fig. 6: Agarose gel electrophoresis of duplex RT-PCR using specific primers for ToCV and TICV. Lanes 1-3: Infected tomato; lane 4: Healthy negative control
M, 100 bp DNA ladder

ToCV was detected with RT-PCR in several plant families (Table 1). Out of 52 different tested plant species within 22 families, 44 were positive for ToCV. Thirty seven out of these 44 plant species were considered as new hosts for ToCV in the present study.

Cloning, sequencing and phylogenetic studies: Eleven *E. Coli* colonies, charged with the plasmid insert were tested using ToCV specific primers. One out of the tested colonies was negative and the rest were positive for the presence of ToCV-charged plasmid. The DNA sequence for the ToCV-Giza isolate submitted to the GenBank was donated the accession number "MH667315.1". Blastx

Table 2: ToCV and other criniviruses used in comparative Blastx* sequence analysis of the HSP70h gene

Criniviruses	Country	Virus acronym in relation to its isolate/country	GenBank accession numbers	Host	Amino acids identity (%) with ToCV-Giza
Tomato chlorosis virus (Giza isolate)	Egypt	ToCV-Giza	MH667315.1	Tomato	100
Tomato chlorosis virus	Brazil	ToCV-Br	AMJ27375.1	Eggplant	98
Tomato chlorosis virus	China	ToCV-Ch	ATL24428.1	Tomato	99
Tomato chlorosis virus	Greek	ToCV-Gr	ABX89240.1	Tomato	98
Tomato chlorosis virus	Hungary	ToCV-Hun	ADQ27446.1	Tomato	98
Tomato chlorosis virus	Italy	ToCV-It	ABF69859.1	Tomato	97
Tomato chlorosis virus	Japan	ToCV_Jap	BAJ07974.1	Tomato	99
Tomato chlorosis virus	Jordan	ToCV-Jor	AMJ27375.1	Tomato	98
Tomato chlorosis virus	Lebanon	ToCV-Leb	ABB22756.1	Tomato	99
Tomato chlorosis virus	Mexico	ToCV-Mex	ABD24425.1	Tomato	98
Tomato chlorosis virus	Portugal	ToCV-Por	AAF37301.1	Tomato	99
Tomato chlorosis virus	Saudi Arabia	ToCV-SAr	AHX25815.1	Tomato	98
Tomato chlorosis virus	South Africa	ToCV-SAf	AUF72344.1	Tomato	99
Tomato chlorosis Virus	South Korea	ToCV-SK	AJS10659.1	Tomato	99
Tomato chlorosis virus	Spain	ToCV-Sp	AHH44921.1	Pepper	99
Tomato chlorosis virus	Taiwan	ToCV-Tai	ASE04576.1	Tomato	97
Tomato chlorosis virus	Tunisia	ToCV-Tun	AGH13385.1	Potato	98
Tomato chlorosis virus	Turkey	ToCV-Tur	ABS89223.1	Tomato	98
Tomato chlorosis virus	USA	ToCV-USA	AEK21306.1	Tomato	98
Beet pseudo-yellows virus	USA	BPYV-USA	AAB40655.1	*Nicotianaclevelandii*	66
Blackberry yellow vein associated virus	USA	BYVAV-USA	AAW67738.1	Blackberries	68
Cucurbit yellow stunting disorder virus	USA	CYSDV-USA	AAB40656.1	Cucumber	70
Diodia vein chlorosis virus	USA	DVCV-USA	ACU80556.2	*Diodia* sp.	65
Potato yellow vein virus	Peru	PYVV-Pe	AAD56773.1	Potato	67
Strawberry pallidosis associated virus	China	SPAV-Ch	AUT14080.1	Strawberry	67

* Search protein data base using a translated nucleotide query

sequence analysis, based on amino acid substitution of the HSP70 H gene of ToCV with similar worldwide ToCV isolates indicated range of similarities between 97-99% (Table 2). With other criniviruses, Blastx analysis, on the HSP70 H gene, indicated 65-70% similarities between these viruses and ToCV-Giza isolate (Table 2). Phylogenetic study (Fig. 7) based on amino acid substitution showed two major clusters of criniviruses. One cluster included all tested ToCV isolates, while the second cluster included the other tested criniviruses. Sixteen ToCV isolates including ToCV-Giza were grouped together on one sub-branch. ToCV-Mex and ToCV-USA were grouped on a second sub-branch. ToCV-Tai grouped alone on a separate sub-branch apart from all tested ToCV isolates.

DISCUSSION

Tomato chlorosis disease is one of the most devastating diseases in tomato in both greenhouses and fields worldwide[2,3,31]. ToCV acquired its potency as a pathogen from the fact that its spread is furnished by several species and biotypes of Aleyrodidae including several biotypes of the more polyphagous vector *B. tabaci*, *T. abutiloneus* and *T. vaporariorum*[2,6,29]. These vectors favor tropical and subtropical zones that allow the spread of ToCV in many countries in the Mediterranean basin, Africa and Asia as well as in north and south America[2,48]. In addition ToCV, thought first to be limited in its host range, turned out to have more than 60 plant species within 18 families[20,22,29,33-35,49,50]. Such natural hosts for ToCV are key factors in ToCV epidemiology, since they can serve as sources of inoculum for acquisition by whiteflies and drastically complicate virus control strategy[2,50]. In 2013, symptoms of leaf chlorosis, flower withering and drastic reduction in tomato yield were observed on tomato plantations in the experimental fields of the Faculty of Agriculture in Giza governorate. The ToCV-induced symptoms were successfully transmitted to healthy tomato seedlings in the greenhouse by the *B. tabaci* insects as described by Wintermantel and Wisler[29]. The induced symptoms were very similar to those described by several investigators[2-4,51]. The success of symptom transmission by *B. tabaci* to tomato excluded any contamination with TICV. The latter is transmitted only by *T. vaporariorum*[2,4,5]. Further, infected-tomato propagated plants in the greenhouse were tested positive for the specific primers of ToCV and negative to TICV specific primers, again confirming the sole presence of ToCV in tomato propagated stock plants.

ToCV was purified using the electro-elution (EE) technique which overcame problems of virus instability and virus particle aggregation as mentioned by Jacquemond *et al.*[52].

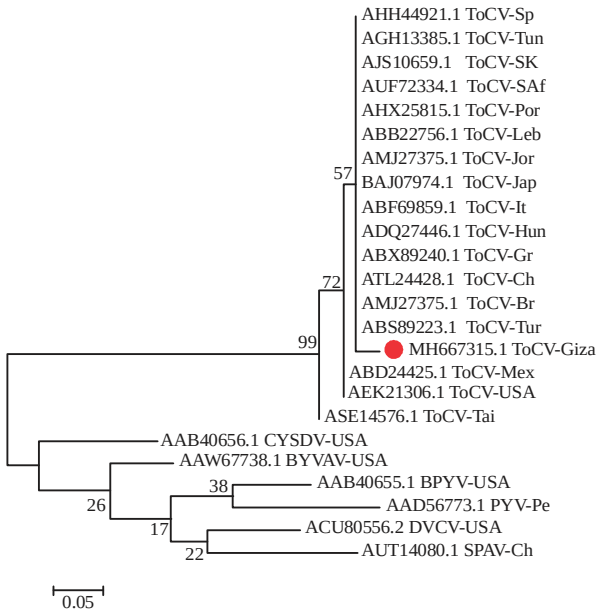

Fig. 7: Molecular phylogenetic analysis by Maximum Likelihood method showing the evolutionary history of the tested sequences based on the JTT matrix-based model Jones *et al.*[46]. The tree with the highest log likelihood (-700.2867) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search was obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. The tree is drawn to scale, with branch lengths measured in the number of amino acid substitutions per site. The analysis involved 25 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 79 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 Tamura *et al.*[47]. Full names of viruses in Fig. 7are mentioned in Table 2.

This technique proved successful in purifying several viruses belonging to several genera including the crinivirus CYSDV[53]. The $A_{260/280}$ ratio of the purified virus was 1.33 which was very close to a corresponding ratio of 1.39 of a ToCV isolate from Japan[16]and to the purified crinivirus *Beet Pseudo Yellows Virus* with $A_{260/280}$ ratio of 1.315[39].

The ToCV-induced antiserum detected ToCV incidence in the greenhouse and the field using DAS-ELIA and/or DBIA. A described ToCV-induced antiserum was reactive only in DBIA but not in DAS-ELISA[54]. Several extraction buffers such as PBST, TBST and sodium citrate buffer containing 2% TX100 were examined. Only the citrate buffer with TX100 was successful in elucidating the presence of ToCV in the tested materials. In addition to TX100 as being detergent destabilizing membrane permeability[55] and a blocking agent in immunoassay, showed also role in reducing the non-specific antibody binding on membranes[56]. In DAS-ELISA, some tomato plants, though infected, showed OD_{405nm} reading below the defined threshold of 2.9 depicted for judging the positivity of samples in DAS-ELISA[43]. These samples were proven positive when examined with DBIA. Such similar problems was described by Jacquemond *et al.*[52] working with detection of ToCV with DAS-ELISA and was attributed by the authors to the low titer of ToCV as a phloem-inhabiting virus and to the heat instability of the tested virions. RT-PCR and DBIA surpassed DAS-ELISA in sensitivity and differentiated between samples infected with an unknown begomovirus and those infected with ToCV. In the present study, tested hosts with immuno-assays were re-evaluated with RT-PCR, using specific primers for ToCV. Thirty seven plant species were recorded for the first time as natural hosts of ToCV as *Ammi majus* and *Coriandrum sativum* (Apiaceae), cabbage (Brassicaceae), sweet potato (Convolvulaceae), melon, cucumber, luffa (Cucurbitaceae), soybean, cowpea and faba bean (Fabaceae), Egyptian and American Cotton (Malvaceae). Several ornamentals either herbal type or woody trees belonging to Acanthaceae, Amaranthaceae, Euophorbiaceae, Moraceae and Rubiaceae were also recognized for the first time as hosts for ToCV. The present results also confirmed previously recorded hosts of ToCV as *Capsicum annuum*[57,58], *Chenopodium album*[22], *Malva parviflora*[59], *Phaseolus vulgaris*[59], *Solanum lycopersicum*[4], *S. melongena*[60], *S. pimpinellifolium*[50], *S. tuberosum*[51,58] and *Withania somnifera*[59].

Blastx sequence analysis, on HSP70 H gene of ToCV-Giza, with similar worldwide ToCV isolates indicated range of similarities between 97-99%. With other criniviruses, only 65-70% similarities between these viruses and ToCV-Giza isolate was observed (Table 2). Similarly phylogenetic analysis on the same HSP70H gene corresponding sequences of the criniviruses understudy indicated the clustering of ToCV isolates in one group apart from the other tested criniviruses. These results confirmed the identity of ToCV-Giza isolate and its relatedness to ToCV. All ToCV isolates are known to share high similarity in the HSP70h gene as this region of ToCV genome is highly conserved between all ToCV isolates worldwide[29].

CONCLUSION

An important measure for the disease control of ToCV is the strict management of the alternative hosts of the virus and control of whitefly transmission. So far, there are no known available resistant varieties of tomato to ToCV and the chemical control of the insect vector is not fully successful.

SIGNIFICANCE STATEMENT

The present study utilized the electro-elution technique for purifying ToCV for the first time. This facilitates, to a great extent, problems encountered researchers upon purifying criniviruses such as low yield, virus instability, poor immunogenicity and tendency of virus particle aggregation. This, in turns, overcame the problem of the induction of poor quality antisera with low titer, non-specific and improper for DAS-ELISA analysis. Most previous studies on natural hosts for ToCV depended on whitefly-transmission bioassay and molecular detection with RT-PCR. Yet the present study used bioassay, serology and molecular tools to consolidate obtained results. Modification of extraction buffer in immunoassay in this study by involving 2% Triton X-100 in buffer composition represents a corner stone in detection of ToCV. The TBST was not successful in ToCV extraction from tissues. However, in other criniviruses TBST was ample enough for virus extraction in immunoblotting analysis. In the present study, 37 plant species were considered as new hosts for ToCV, in different families, for the first time. Of these hosts, Egyptian and American cotton species are expected to represent a big challenge to plant breeders searching for varieties of cotton resistant to both begomo and criviruses in disease complex situation resulted from the co-transmission of members of the two virus genera with *B. tabaci* whitefly. Previous results recorded the infection of the Egyptian cotton with a begomovirus. Perhaps, the production of good quality antiserum for ToCV, in the present study would facilitate studying the incidence of each virus group separately not only in cotton but also in other ToCV-susceptible plant species recorded in this study.

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