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Tomato Leaf Curl Sudan Virus (TLCSDV) causing leaf curl disease on a new host *Amaranthus cruentus* L.

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Abstract

Amaranthus leaf curl disease symptom was observed in the farmer's field based at Jeddah, Saudi Arabia. This crop used as leafy vegetables in many countries. In this study, a field survey was conducted in April 2014 and naturally infected Amaranthus leaf samples were collected to identify the associated virus with leaf curl disease. The causative agent was transmitted through whiteflies (*Bemisia tabaci*) from naturally infected leaves to healthy Amaranthus seedlings. The begomovirus infection was identified by PCR by using specific primers. The full viral genome was amplified by rolling circle amplification. The presence of betasatellites was also confirmed by using betasatellites specific primers. The full viral genomes as well as betasatellites were amplified, cloned and sequenced. The full-length viral genome sequence analysis showed the highest (99.9%) homology with *Tomato leaf curl Sudan Virus* infecting tomato reported from the Arabian Peninsula. The betasatellites sequence analysis showed the highest identity (99.3%) with Tomato leaf curl betasatellites-Yemen. The phylogenetic analysis was performed by using both full as well as betasatellites genome and full genome formed the closest cluster with *Tomato leaf curl Sudan virus* while betasatellites genome formed closed cluster with tomato yellow leaf curl Yemen betasatellites. The recombination analysis was performed and results showed that the associated virus could be a variant of Tomato leaf curl Sudan virus, a virus that occurs in Sudan, Yemen and Arabian Peninsula. This is the first report that about the Tomato leaf curl Sudan virus causing leaf curl disease on a new host Amaranthus in Jeddah, Saudi Arabia.

Key words: Amaranthus, Leaf curl disease, Begomovirus, Phylogenetic and Recombination relationships, Saudi Arabia.
Abbreviations: AgEV_Ageratum enation virus, CaCV_Capsicum chlorosis virus, CMV_Cucumber mosaic virus, ChiLCV_Chili leaf curl virus, PALCV_Papaya leaf curl virus, ToLCSDV_Tomato leaf curl Sudan virus, TYLCV_ Tomato yellow leaf curl virus.

Introduction

Amaranthus belongs to the family, *Amaranthaceae* with approximately 60 species and mostly are known as cosmopolitan weeds (*A. retroflexus* L., *A. hybridus* L., *A. powellii* L. and *A. spinosus* L.). Amaranthus is broad leaf and warm-seasoned plant grown in Asia, South-East Asia, Manchuria and North America for various purposes like ornamental, edible greens, leafy vegetable, forage crops, herbs and grain (Hauptli and Jain 1977, Bhatia 2005). Additionally, Amaranthus is a very rich source of iron, calcium, zinc, magnesium, Vitamins (A, B, C) and cholesterol-lowering soluble fiber (http://archive.indianexpress.com/news/thirdmillennium-grain-highprotein-amaranth-is-rich-in-ironcalcium/1111172/).The Amaranthus cultivation is being affected by many viral diseases (Horvath 1991; Costea and Tardif, 2003) and among them, the most common viruses are known as *Amaranthus leaf mottle virus* (Segundo et al., 2007), *Amaranthus mosaic 'potyvirus'* (Brunt, 1996), *Cucumber mosaic virus* (CMV) (Srivastava and Raj, 2004) and begomoviruses like ; *Tomato yellow leaf curl virus* (TYLCV) (Abou-Jawdah et al., 1999), *Chili leaf curl virus* (ChiLCV) (George et al., 2014), *Ageratum enation virus* (AgEV) (Raj et al., 2008; Srivastava et al., 2013), *Papaya leaf curl virus* (PALCV) (Srivastava et al., 2015) and *Capsicum chlorosis virus* (CaCV) (Sharma and Kulshrestha 2014). Begomoviruses belongs to family *Geminiviridae* are serious problems for many crops globally. The family *Geminiviridae* has now been reported to have seven genera known as *Mastrevirus, Curtovirus, Begomovirus, Topocuvirus, Eragrovirus, Turncurtovirus, Becurtovirus* (Muhire et al., 2014; Brown et al., 2015; Varsani et al., 2014). Members of the genus begomovirus have circular single-stranded DNA (ssDNA) with either a mono-or bipartite genome.The bipartite begomovirus genome has two ssDNA molecules (~2.7 kb), known as DNA-A and DNA-B while mono-partite begomovirus has only DNA-A with satellite molecule known as betasatellites (Briddon et al., 2003). Betasatellites are small (~1.4kb), highly diverse with circular ssDNA. The betasatellites replication, movement, and transmission between plants are mediated by their helper virus. Betasatellites have an ORF; beta C1, an adenine-rich region, and a satellite conserved region with 45-93% sequence identity (Sivalingam et al., 2010). The dicotyledonous plants are infected by begomovirus transmitted by whitefly vector (*Bemisia tabaci)* in a persistent manner. Globally, the whiteflies have become serious pathogens for many dicotyledonous crops in tropical, sub-tropical and warmer temperate regions and mostly Solanaceous crops are being affected by begomovirus in East and Southeast Asia (Kenyon et al., 2014; Idris and Brown 2005). The whitefly posing a serious threat to global food security and there is an urgent need to develop effective strategies to control crop loss and

manage the begomoviral disease. Currently, the natural occurrence of mono-partite begomoviruses and their associated alpha and betasatellites are reported to be endemic in Eastern Hemisphere (Briddon et al., 2001; Idris et al., 2014).

In this study, the associations of begomovirus with leaf curl disease of Amaranthus from; Jeddah, Saudi Arabia has been discussed. In April 2014, during field survey, leaf curl disease was observed on Amaranthus plant growing in and around the tomato growing farmer's field at Jeddah, Saudi Arabia. Severe leaf curl infection was also observed in tomato crops. Naturally infected Amaranthus leaf samples exhibiting typical leaf curl symptoms were collected from natural open plots of Jeddah, Saudi Arabia. The causative agent was detected by begomovirus specific PCR, efficiently transmitted by whitefly vector and characterized at molecular level by cloning and sequencing of full genome and betasatellites and analyzing the sequence identity, phylogenetic relationship with selected begomovirus isolates from different locations. The sequence identity and phylogenetic analysis results strongly support the causative agent is a variant of *Tomato leaf curl Sudan virus* (ToLCSDV), a virus that has been reported earlier to cause tomato leaf curl disease in Sudan, Oman and Yemen. The association of begomovirus with leaf curl disease on various crops in Arabian Peninsula has been published in many reports (Ajlan et al., 2007; Khan et al., 2008; Idris et al., 2012; Akhtar et al., 2014; Idris et al., 2011; Khan et al., 2013a; (Ajlan et al., 2007; Khan et al., 2008; Idris et al., 2012; Akhtar et al., 2014; Idris et al., 2011; Khan et al., 2013b Al-Saleh et al., 2014; Hosseinzadeh et al., 2014; Idris et al., 2014).

Results

Field survey, collection of sample and transmission of virus by whiteflies

Amaranthus plants were growing in and around infected tomato fields and approximately 90 % plants were observed to be infected with leaf curl disease as compared to healthy ones (Figure. 1 a & 1 b).The whiteflies (*B. tabaci*) were also observed in the infected Amaranthus and tomato leaves. The causative agent was successfully transmitted to healthy Amaranthus seedling. Total 11 samples were collected from symptomatic and non-symptomatic plants and processed for virus detection. The causative agent was successfully transmitted to 14/21 healthy tomato seedling by whiteflies inoculation which further developed similar leaf curl symptoms after 18-20 days post inoculation (dpi) as observed in the field.

Molecular detection of virus, cloning and sequencing of viral genome

The begomoviral infection was confirmed by PCR and ~856 bp amplicon was observed in 9 samples by using begomovirus-specific primers. Two clones were obtained from betasatellites genome but only one sequence was used for further analysis. Restriction of RCA products with E*co*RI yielded fragments of ~2.8 kb from infected samples and cloned into pGEM7Zf + (Promega, Madison, WI) from the RCA-amplified products. The confirmed clones were sequenced bi-directionally at our special infectious agents unit (SIAU), King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Assembled sequences from both full length and betasatellites genomes were submitted to GenBank under the accession numbers (KT033710 & KT 199104).

Sequence, phylogenetic and recombination analysis

The complete genome contained 2789 nucleotides (nt) encoding six potential open reading frames (ORFs): V1 (coat protein), V2 (pre-coat protein) in virion sense strand while C1 (replication associated protein), C2 (transcription activator protein), C3 (replication enhancer protein) and C4 (C4 protein) in complementary sense, which were typical of begomovirus genome. The betasatellites genome was found to have 1359 nucleotides (nt). Based on full genome sequences; BLAST analysis of the identified begomovirus isolate in this study (KT033710) showed highest 99.9% sequence identity with ToLCSDV-(HG530539) reported from Saudi Arabia and lowest identity was 69.0% with PALCV-(JN135233) infecting Amaranthus reported from India followed by 91.5%-92-4% with other ToLCSDV reported from Oman. The nucleotide sequence identity was varied from 91-92% with TYLCV reported from Saudi Arabia. Interestingly, three other begomoviruses reported infecting Amaranthus in India, showed only 69% identity (PALCV-JN135233), 71.7% (AgEV-EU867513) and 71.8% (ChiLCV-KF471061) with our isolate. The highest amino acid sequence identity was observed with ToLCSDV-HG530539 in all the 6 proteins (99.6%-V2, 99.8%-V1, 99.7%-C3, 99.8%-C2, 99.9-C1, and 99.8-C4) respectively, with respective sequences of selected begomovirus isolates (Table 1).

Since the association of a betasatellite molecule with ToLCSDV has been reported earlier (Idris et al., 2014), a separate PCR was performed using betasatellite specific primers (Briddon et al., 2002). An amplicon of ~1.4 kb was obtained from infected samples by PCR, indicating the presence of betasatellite. The highest nucleotide identity (99.3%) was found with Tomato leaf curl Yemen betasatellites (ToLCYEB) (JF919719) and the lowest (45.7%) was observed with Ageratum leaf curl betasatellites-India (JQ710745) (Table 2).

To analyze the phylogenetic relationships of identified begomovirus isolate used under this study (KT033710) with other selected begomovirus isolates, a neighbor-joining phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis tool (MEGA v 6.1) (Tamura et al., 2013). The phylogenetic analysis results based on complete genome sequences of selected begomovirus isolates indicates that the identified virus is a variant of either ToLCSDV, TYLCV as the closest cluster was observed with two begomovirus groups ToLCSDV, TYLCV and other three Indian begomovirus isolates reported from Amaranthus. The isolate formed closed cluster with ToLCSDV from other crops and showed the closest relationships with ToLCSDV-Tomato (HG530539, JN591386), Tobacco-(JF919733) reported from Saudi Arabia, Yemen and Oman (Figure. 2). Based on nucleotide sequence identity and close phylogenetic relationships with ToLCSDV and as per new criteria proposed for begomovirus classification in the ICTV report (Fauquet et al., 2008), the begomovirus isolate associated with leaf curl disease of Amaranthus from Saudi Arabia was identified as a variant of ToLCSDV. In a phylogenetic study based on betasatellites, the closest relationship was observed with ToLCYEB isolates infecting tobacco and tomato reported from Yemen (Figure. 3).

Table 1. Pairwise (%) sequence identities of DNA genome of begomovirus under study (KT 033710) with selected begomoviruses at nucleotide (nt) and amino acid (aa) levels.

Accession No	Abbreviation	Host	Location	Percent sequence identity matrix at nucleotides and amino acid level						
				DNA-A Full (nt)	V2 (aa)	V1 (aa)	C3 (aa)	C2 (aa)	C1 (aa)	C4 (aa)
HG530539	ToLCSDV	Tomato	S.Arabia	99.9	99.6	99.8	99.7	99.8	99.9	99.8
JF919733	ToLCSDV	Tobacco	Yemen	91.5	95.6	97.2	85.8	85.9	91.6	74.0
JF919734	ToLCSDV	Tobacco	Yemen	90.3	93.9	97.2	87.3	87.4	88.8	56.0
KC845301	TYLCV	Tomato	S.Arabia	91.6	62.3	98.1	85.8	88.1	91.6	69.0
KF561125	TYLCV	Tomato	S.Arbia	91.5	94.8	91.0	73.1	86.6	84.8	68.6
KF040453	TYLCV	Tomato	S.Arabia	91.2	98.2	91.0	74.6	89.6	82.8	67.6
JN591386	ToLCSDV	Tomato	Oman	92.2	96.5	98.0	88.0	88.1	86.2	77.0
FJ956700	ToLCV	Tomato	Oman	88.5	93.1	77.9	85.0	81.4	75.7	73.0
KF444467	ToLCSDV	green bean	S.Arabia	89.7	92.3	96.4	85.8	85.9	87.1	71.0
KF561125	TYLCV	Tomato	S.Arabia	91.5	98.2	91.0	73.1	86.6	84.8	68.6
HE819244	ToLCSDV	Tomato	Oman	91.5	98.2	91.8	74.6	88.8	85.7	70.0
JN591385	ToLCSDV	Tomato	Oman	91.2	98.2	92.2	75.3	87.4	85.9	77.0
JN591386	ToLCSDV	Tomato	Oman	92.1	98.2	98.0	88.0	88.1	86.2	77.0
AY044139	ToLCSDV	Tomato	Sudan	92.4	98.2	97.2	85.8	88.1	93.8	82.0
JX483708	ToLCSDV	Tomato	Sudan	91.9	95.6	97.2	85.8	81.4	93.3	75.0
JF919731	ToLCSDV	Tomato	Yemen	89.7	96.5	96.8	78.3	77.0	91.6	69.0
EF110891	ToLCSDV	Tomato	Yemen	89.7	96.5	96.8	78.3	77.0	91.6	69.0
KC763630	ToLCSDV	Tomato	Sudan	88.3	90.5	96.1	86.5	87.4	91.0	72.0
AY044137	ToLCSDV	Tomato	Sudan	88.5	93.9	97.2	85.8	81.4	89.9	72.0
AY044138	ToLCSDV	Tomato	Sudan	82.9	93.9	76.3	82.8	84.4	93.0	66.0
GU180085	ToLCSDV	Tomato	Sudan	88.5	92.2	96.8	85.8	82.2	90.2	72.0
DQ358913	TYLCMV	Tomato	Ethiopia	83.2	94.8	76.7	85.0	86.6	90.2	72.0
DQ644565	TYLCV	Tomato	Oman	78.9	93.9	77.9	83.5	82.9	82.4	71.0
KF471061	ChiLCV	Amaranthus	India	71.8	68.9	72.7	70.8	66.6	79.2	44.1
JN135233	PALCV	Amaranthus	India	69.0	72.2	73.9	65.6	62.2	68.6	37.0
EU867513	AgEV	Amaranthus	India	71.7	72.4	73.9	62.6	61.4	78.9	56.0
GU076448	TYLCV	Tomato	Iran	83.7	92.2	77.9	83.5	82.2	81.2	66.0
KC106648	TYLCV	Tomato	Iran	79.1	92.2	78.2	82.0	80.0	80.0	38.0

Abbreviations: AgEV: Ageratum enation virus, CaCV: Capsicum chlorosis virus, CMV: Cucumber mosaic virus, ChiLCV:Chilli leaf curl virus,PALCV: Papaya leaf curl virus,ToLCV: Tomato leaf curl virus,ToLCSDV: Tomato leaf curl Sudan virus , TYLCV: Tomato yellow leaf curl virus, Tomato yellow leaf curl Mali virus, S.Arabia (Saudi Arabia)

Figure. 1a. Healthy *Amaranthus cruentus* plant, **Figure. 1b.** Infected *Amaranthus cruentus* plant showing severe leaf curl stunting symptoms as compared with healthy plant.

Recombination analysis was carried out with full genome sequences of selected begomovirus isolates using the RDP4 program (Martin et al., 2015) Three of these algorithms, RDP (P 1.657×10^{-14}), GENCONV (P 1.274×10^{-15}), MaxChi (P $7.395\times^{10-14}$), Chimera (P $8.767\times^{10-13}$), Si Scan (P $3.509\times^{10-35}$) and 3 Seq (P $1.328\times^{10-12}$) predicted that the genome of the herein newly described ToLCSDV variant exhibit evidence of inert-specific recombination. The boot scan and RDP analysis of the ToLCSDV-Amaranthus genome together indicated definite evidence of recombination within the viral genome. Two recombinant fragments (co-ordinates 2025 to 2742 in the C1 gene and 1945 to 2678 toward the 3′ end of the Rep gene) were detected and these fragments shared high levels of sequence identity with ToLCSDV-HG-530539-S.Arabia (99.9% identity). ToLCSDV-Saudi Arabia was indicated as the major parent and ToLCSDV-Mir-Oman (JM591386) minor parent and ToLCSDV-Yemen-JF919733 was found as a recombinant isolate.The recombination analysis results suggest that the ToLCSDV–Amaranthus isolate evolved either from ToLCSDV-Oman or Yemen by recombination (Table 3).

Discussion

Amaranthus is an important subsidiary food crops in the tropical and subtropical highlands of Asia and South America and utilized as food grains, leafy vegetables, and forage crops in America, China, Greece, Italy, Russia, Nepal, and India.

Table 2. Pairwise (%) sequence identities of betasatellites under study (ToLCSDB-Amaranthus-KT199104) with selected betasatellites at nucleotide (nt) levels.

Accession Nos.	Acronyms	Location	Host	% identity
JF919717	ToLCYEB	Yemen	Tobacco	99.1
JF919718	ToLCYEB	Yemen	Tobacco	99.2
JF919719	ToLCYEB	Yemen	Tobacco	99.3
JF919720	ToLCYEB	Yemen	Tobacco	99.1
JF919721	ToLCYEB	Yemen	Tobacco	99.1
JF919722	ToLCYEB	Yemen	Tobacco	99.0
NC_010126	TYLCβ-Om	Oman	Tomato	49.5
DQ644566	TYLCβ01-Om	Oman	Tomato	49.5
HG969297	TYLCβ-Om	Oman	Papaya	49.0
HG969296	TYLCβ-Om	Oman	Papaya	48.0
HE800552	TYLCβ-Om	Oman	Tomato	49.1
HE800551	TYLCβ-Om	Oman	Tomato	49.1
HE800550	TYLCβ-Om	Oman	Tomato	48.4
HE800549	TYLCβ-Om	Oman	Tomato	49.1
HE800548	TYLCβ-Om	Oman	Tomato	49.1
HE800547	TYLCβ-Om	Oman	Tomato	49.1
HE800546	TYLCβ-Om	Oman	Tomato	49.5
HE800545	TYLCβ-Om	Oman	Tomato	48.6
HE800544	TYLCβ-Om	Oman	Tomato	48.7
HE800543	TYLCβ-Om	Oman	Papaya	47.8
HE800542	TYLCβ-Om	Oman	Capsicum	49.3
HE800541	TYLCβ-Om	Oman	Capsicum	49.4
HE800540	TYLCβ-Om	Oman	Capsicum	49.3
KJ396939	OkLCV satDNA-10	Jordan	Tomato	46.0
KF471033	TYLCTHB	India	Amaranthus	47.8
JQ710745	AgeLCB	India	Amaranthus	45.7
NC_004903	TYLCTHVβ	Thailand	Tomato	47.2
DQ641714	TYLCVVβ	Vietnam	Tomato	48.5
NC_007485	TYLCMVβ	Mali	Tomato	47.6
KC677734	ToLCJaB	Java	Tomato	47.5

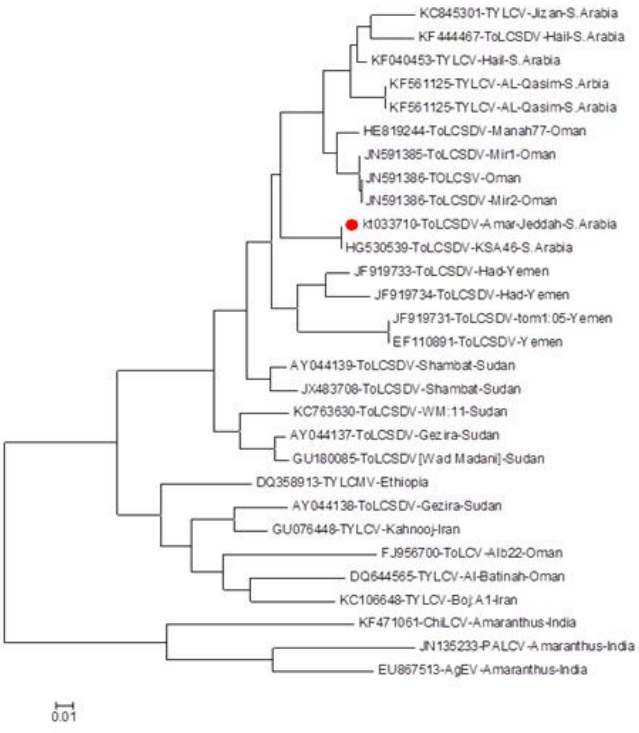

Figure 2. Phylogenetic relation ships of begomovirus genome under study with selected strains (selected based on full DNA -A BLASTn analysis) determined by NJ method within MEGA v6.1 with 1000 bootstrap replicates.

Table 3. Recombination analysis of Tomato leaf curl Sudan virus isolate-Amaranthus using the RDP 4.0.

Break point positions				Detection Methods					
Start	End	Minor Parent	Major Parent	RDP	GENCONV	MaxChi	Chimaera	SiScan	3 Seq
2025	2742	ToLCSDV-SA(530539)	ToLCSDV-Mir-Oman (JM591386)	1.352×10^{-11}	1.176×10^{-11}	$7.263\times^{10-11}$	7.762×10^{-11}	$2.421\times^{10-31}$	$1.134\times^{10-11}$
1945	2678	ToLCSDV-SA(530539)	ToLCSDV-Yemen-JF919733	1.231×10^{-10}	1.154×10^{-15}	$6.237\times^{10-11}$	7.348×10^{-11}	$2.191\times^{10-21}$	$1.326\times^{10-11}$

Abbreviations: ToLCYEB: Tomato leaf curl Yemen betasatellites, TYLCβ-Om: Tomato yellow leaf curl Oman betasatellites, OkLCV:Okra leaf curl betasatellites, TYLCTHVβ: Tomato yellow leaf curl Thailand betasatellites,

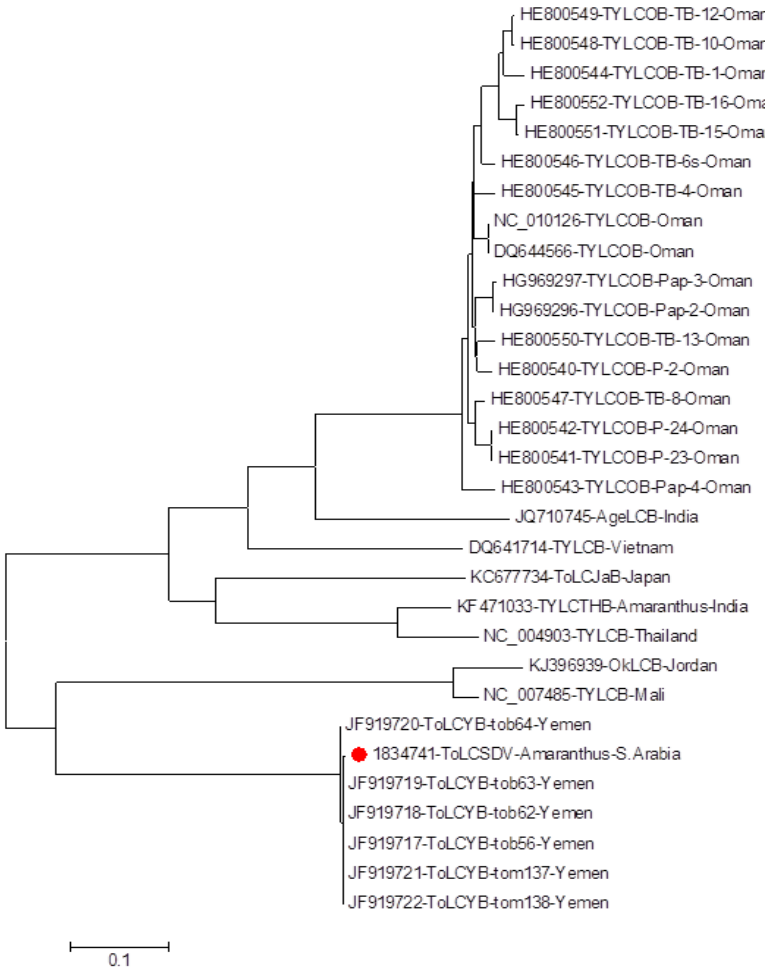

Fig 3. Phylogenetic relationships of betasatellites under study with selected strains (selected based on BLASTn analysis) determined by NJ method within MEGA v6.1 with 1000 bootstrap replicates.

Amaranth grain is a highly nutritional pseudo cereal with a superior amount of proteins and possesses anti allergic and antioxidant activities (Huputli and Jain 1977). Only a few reports are available in the literature about viruses infecting Amaranthus. The present study reports about a disease complex comprising a monopartite begomovirus, betasatellite associated with Amaranthus leaf curl disease in Jeddah, Saudi Arabia. Although the extent of the spread of this disease is not known, the results emphasize the greater impact of Tomato leaf curl Sudan virus by expanding its host range to Amaranthus crops. This disease may emerge as a serious threat to other crops grown in Saudi Arabia and thus, more detail studies are required to determine the diversity of begomoviruses and associated satellites infecting Amaranthus crops as well as other crops being cultivated in this region and serving as an alternative host for begomovirus. A survey of the literature revealed that Amaranthus is also infected by begomoviruses like AgEV (Srivastava et al., 2013), ChiLCV (George et al., 2014), PALCV (Srivastava et al., 2015) and Ageratum leaf curl has been reported from India (Srivastava et al., 2015).

Globally, the emergence of begomoviruses over the last 20-30 years has become the most important groups of plant viruses affecting vegetables crop production due to increase of whitefly vector population in the tropics and subtropics and tomato leaf curl or tomato yellow leaf curl has become the most devastating viral disease worldwide which serves as source of inoculum to disease spread by whiteflies vector (Hanssen et al., 2010). The expansion and intensification of tomato cropping favored the increased populations of

whiteflies (*B. tabaci)* with a wider expansion of leaf curl disease incidence in tomato crops. For the past two decades, tomato production in Arabian Peninsula and Nile Basin has been affected by leaf curl disease caused by begomovirus (Ajlan et al., 2007; Idris et al., 2012; Duffy and Holmes 2008). The presence of begomovirus and association of ToLCSDV causing leaf curl disease of tomato in Gezira, Sudan (Nile Basin) has been cloned and sequenced as early as 1996 (Idris and Brown 2005).It is more than likely that ToLCSDV isolates circulating in Saudi Arabia has originated either from Yemen or Oman, as the studied virus being the closest relative to the Saudi Arabian, Oman and Yemen isolates. In Oman tomato leaf curl disease was first identified in 1993 but the etiology of the disease was confirmed recently and the natural occurrence of ToLCSDV has been reported in Oman (Khan et al., 2013a). In Yemen, tomato leaf curl virus is associated with a betasatellites that have only been identified and known as Tomato yellow leaf curl Yemen betasatellites (Idris et al., 2012). On the other side, Yemen is separated from Oman and Saudi Arabia by a vast harsh desert condition which plays potential barriers to virus and whitefly movement. However, the extant occurrence in both locations of two closely related viruses indicates that what may serve as deterrents to human movement, do not present impervious barriers to virus-vector dispersal, having fostered instead the apparently, unrestricted natural dispersal of TYLCV and ToLCSDV isolates in the kingdom (Idris et al., 2011).

Based on results obtained from field survey, virus detection, sequence identity, phylogenetic relationship and recombination analysis, it is clear that the virus circulating in Saudi Arabia is a variant of ToLCSDV. The complete genome sequence identity showed the highest sequences identity with ToLCSDV isolate identified earlier from Saudi Arabia and betasatellites sequence identity with Tomato leaf curl Yemen betasatellites. The phylogenetic relationship results based on full genome and betasatellites also provided strong relationships with ToLCSDV isolates from Saudi Arabia, Yemen, and Oman. In field survey, leaf curl disease and presence of whitefly vector were observed in tomato crops and same kind of leaf curl disease was observed in Amaranthus crops also. So based on above results, it is obvious that the virus has been transmitted by whiteflies and causing leaf curl disease in Amaranthus growing in and around the tomato growing field in Jeddah, Saudi Arabia.

It is obvious that the expansion and intensification of cropping systems favor the emergence and increase the level of whitefly population and the emergence of begomovirus variants and acquisition of satellite DNA molecules with more aggressive or crop-adapted characteristics due to mutation, recombination, and pseudo-recombination. Apart from that, it is also suspected that there may the introduction and spread of begomovirus species from outside the region probably through the human movement, of infected plant materials, introduction of tolerant and susceptible tomato cultivars and climatic condition in the localized regions also promoted the favorable condition for the whiteflies vector and allowed the spread of the viruses in new areas (Kenyon et al., 2014). Here, it is ascertained the association of a begomovirus and the satellite molecules with leaf curl disease from Saudi Arabia. The presence of Alphasatellites and DNA-B could not be ascertained in the infected Amaranthus samples when degenerate primers were used (George et al., 2014). But we succeeded in the full genome amplification by RCA methods. However, the nature of the association of a monopartite begomovirus with betasatellite leading to leaf curl disease in Amaranthus is unclear which needs further experimentation. However, based on the literature and findings and the association of ToLCSDV with leaf curl disease of Amaranthus is the first report from Saudi Arabia. The present finding suggested that *A. cruentus* may serve as an alternative host for ToLCSDV and in near future, this virus may spread and cause disease to other crops growing in Saudi Arabia.

Materials and methods

Field survey, sample collection and whitefly transmission

In April 2014, during the field survey, leaf curl disease was observed on Amaranthus plants growing in and around farmer's field at Jeddah, Saudi Arabia. Symptomatic and non-symptomatic top emerging leaves were collected from field infected Amaranthus plant and immediately kept in ice with self-sealing plastic bags and brought to the lab for further processing. A fresh culture of non-viruliferous whitefly was raised from their un-hatched eggs and maintained on healthy eggplant under insect-proof condition. The adult whiteflies were allowed to feed on infected tomato leaves for 24 h for virus acquisition and then transferred onto healthy tomato seedlings (twenty whiteflies / seedling) to transmit the causal organism by giving 24h inoculation access period under insect-proof cages. Total 21 healthy seedlings were inoculated in three replicates and kept under insect-proof condition for symptoms development for 25 days.

Virus detection, cloning and sequencing

Total genomic DNA was isolated from 100 mg leaf tissue of infected as well as healthy Amaranthus plant using a DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany); and begomovirus infection was confirmed by polymerase chain reaction (PCR) using specific primers TYC1F (5'-GGGCCTAGAGACCTG GCCCAC-3') and TYC1R (5'-CCGGTAATATTATAC GGATGGC-3'), which amplify an 856-bp fragment of the 5' end of the C1 gene of TYLCV-IL (Hosseinzadeh et al., 2014). No amplicon was observed in healthy samples. The full-length genome was amplified by rolling circle amplification from symptomatic *A. cruentus leaf* sample using Phi-29 DNA polymerase (TempliPhi™ DNA amplification kit; GE Healthcare, Buckinghamshire, UK) as per manufacturer's instructions.

Total 3 µl RCA amplified product was digested with *Eco*R1 and 2.7kb fragments were separated and purified on 1% Agarose gel. The gel purified full-length genome (~2.7kb) was cloned into pGEM7Zf + (Promega, Madison, WI) and sequenced. The full-length betasatellites were amplified by using specific primers (Briddon et al., 2002) and cloned into a pGEMT-Easy vector (Promega, USA). The confirmed clones were bi-directionally sequenced in our lab by using primer walking methods and analyzed by using NCBI-BLAST.

Sequence, phylogenetic and recombination analysis

The full length sequences were aligned and the resulting nucleotide sequence was initially searched for similarity using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997).The sequences that showed higher score and similarity were selected for further analysis. Multiple sequence alignments were performed by ClustalW program (http://www.ebi.ac.uk/clustalw) using nucleotides sequences

of selected begomoviruses from the GenBank. A phylogenetic tree was constructed using MEGA v 6.1 program from the aligned nucleotide sequences with neighbor-joining and maximum parsimony methods using maximum composite likelihood for DNA substitution test (Tamura et al., 2013). The recombination detection program (RDP4) tool was used (darwin.uvigo.es/rdp/rdp.html) for detection of potential recombinant sequences, identification of likely parental sequences and localization of possible recombination breakpoints. The analysis was performed with default settings using a 0.05 *P-value* cutoff and standard Bonferroni corrections for multiple testing (Martin et al., 2015).

Conclusion

Based on results obtained from, field survey, virus detection, sequence identity, phylogenetic and recombination analysis using full genome and betasatellites genome, this study concludes that the identified virus could be a variant of ToLCSDV, a virus reported earlier from Sudan, Yemen, and Arabian Peninsula . This is the first report causing severe leaf curl disease of Amaranthus in Jeddah, Saudi Arabia.

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Competing interests:

The author declares that they have no conflict of interest.

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