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Full Length Article

Molecular Characterization of a Leaf Curl Disease Infecting Zucchini Squash in Iraq

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Abstract

Leaf curl diseases symptoms were observed in a zucchini squash field west Baghdad. Total DNA was extracted from leaves collected from three symptomatic plants using a commercial plant DNA extraction kit. Polymerase chain reaction (PCR) using genus specific primers were used to amplify the viral DNA fragments. PCR products were visualized by gel electrophoresis and directly sequenced. Sequence analysis using MEGA6 software confirmed the detection of *Squash leaf curl virus* (SLCV) when shared 99% maximum nucleotide sequence identity with equivalent GenBank sequence from Israel (KT099131) and (KM595115), Jordan (JX444577) and (KM595211), Lebanon (KM595136) and Palestine (KM595230). The high nucleotide sequence identity suggests the virus could be introduced to Iraq from neighboring countries through infected plant materials. Restriction site similarity was tested to show sequence variability. *In silico* restriction digestion was applied to SLCV sequences to compare restriction sites similarities in partial CP region amplified. Both obtained and equivalent GenBank sequences were digested virtually using NEBcutter V2 software and compared on the basis of restriction enzyme and restriction site incidence. When compared to equivalent sequences, SLCV from Iraq shared 96% maximum restriction site (RS) similarity with SLCV from Israel (KM595115). While it scored 56% maximum RS similarity to TYLCV sequences from Iraq (JQ025990), (JQ025991) and (JQ025993). *In silico* restriction digestion showed to be a useful approach to differentiate among SLCV sequences.

Keywords: Phylogenetic analysis; Begomoviruses; Deng primers; ssDNA; Cucurbits; Baghdad

Introduction

Zucchini squash (*Cucurbita pepo*) is one of cucurbit crops grown in Iraq due to its economic importance (Al-Kuwaiti *et al*., 2016). Based on FAO statistics, the estimated Iraqi production of cucurbits (including zucchini) was 1,182,535 tons (FAO, 2013). Over 59 viruses have been reported to impact squash worldwide (Lecoq and Desbiez, 2012; Al-Kuwaiti *et al*., 2013), causing serious losses up to 100% (Babadoost, 2012). *Geminiviridae* is the largest among other plant virus families. It consists of at least 362 species belong to 7 genera; namely, *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus* (ICTV, 2017). Members within this family are considered emergent viruses (Brown, 2010; Fauquet and Nawaz-Ul-Rehman, 2010). Geminiviruses have been reported to impact several crops worldwide causing significant losses (Fauquet and Nawaz-Ul-Rehman, 2010). According to the recent taxonomy of ICTV (2017), *Begomovirus* is the largest known genus as it includes 322 species. It is vectored by the sweet potato whitefly *Bemisia tabaci*, the only vector for all members of the genus in a circulative persistent manner (Duffus and Stenger, 1998; Rosen *et al*., 2015). *Squash leaf curl virus* (SLCV) is one of the genus *Begomovirus* members (ICTV, 2017) threat to many cucurbits including squash (Duffus and Stenger, 1998). SLCV has bi-geminate virions with a circular ssDNA consisting of two segments DNA A and B (Duffus and Stenger, 1998). Typical symptoms on SLCV infected squash vary from interveinal mottle and green vein-banding of leaf veins, leaf yellowing to severe stunting and leaf curling on new growth. Enations usually form on the lower surface of symptomatic leaves. Occasionally, flowers fail to develop or set fruits. If formed, fruits may be small and malformed (Duffus and Stenger, 1998; Al-Musa *et al*., 2008; El-Dougdoug *et al*., 2009). At least 4 definite begomoviruses, namely, *Squash leaf curl virus*, *Squash leaf curl China virus* SLCCV (Hong *et al*., 1995), *Squash leaf curl Philippines virus* (SLCuPV) (Kon *et al*., 2003) and *Squash leaf curl Yunnan virus* (SLCuYnV) (Xie and Zhou, 2003) have been identified to cause leaf curl diseases on cucurbits worldwide. Squash leaf curl disease (SLCD) has been observed in The USA in 1977. Since then, SLCD incidence was restricted to USA (Lapidot *et al*., 2014). In 2003, this disease was reported in Israel, then many SLCV outbreaks have been reported on cucurbits in the Middle East region and Egypt as well (Lapidot *et al*., 2014). Besides Cucurbitaceae, SLCV has been found to infect hosts belong

to the families Fabaceae, Solanaceae, Euphorbiaceae, Chenopodiaceae and Malvaceae (Duffus and Stenger, 1998; Al-Musa *et al*., 2008; El-Dougdoug *et al*., 2009). *In silico* restriction digestion approach was applied to differentiate some animal viruses (e.g. Dalal *et al*., 2009), bacteria (Babu *et al*., 2014) and phytoplasmas (Nejat *et al*., 2010). This approach was combined with conventional restriction digestion method to distinguish TYLCV isolates amplified by PCR (Park *et al*., 2014).

In 2016 growing season, leaf curl disease symptoms were observed on squash plants, grown in a field located in Al-Radhwanya district west Baghdad, Iraq. This study, therefore, was initiated to investigate the virus causing leaf curl disease in squash in Iraq based on molecular and *in silico* restriction digestion comparisons.

Materials and Methods

Three leaf samples were collected from squash plants exhibited leaf curl symptoms. Total DNA was extracted from fresh leaves using AccuPrep® Plant DNA Extraction kit from (Bioneer, South Korea) following the manufacturer instructions. Polymerase chain reaction (PCR) was performed using AccuPower PCR PreMix kit from (Bioneer, South Korea) and Deng primer set (Deng *et al*., 1994). PCR products were visualized by ethidium bromide agarose gel electrophoresis, then PCR products were sent to (Bioneer, South Korea) for sequencing. Sequence data obtained were analyzed using MEGA6 software package (Tamura *et al*., 2013). Phylogenetic tree was constructed using Neighbour-Joining statistical method. *In silico* restriction digestion map was generated for each sequence using NEBcutter v 2.00 software from BioLabs (Vincze *et al*., 2003). *In silico* comparison similarities were calculated based on enzymes restriction sites (RS) and their frequencies within each sequence using the following equation:

$$RS\ similarity\% = \frac{the\ sum\ of\ similar\ restriction\ sites}{total\ number\ of\ restriction\ enzymes\ used} \times 100$$

Results

PCR results indicated zucchini squash plants, exhibiting leaf curl symptoms (Fig. 1A, B) were begomovirus infected, when Deng primers could amplify the ~530 bp DNA fragment from all three samples tested (Fig. 1). Sequence analysis showed all six sequences isolated, referred to as (SqLCV1-SqLCV6), belonged to Squash leaf curl virus (SCLV: genus *Begomovirus*; family: Geminiviridae) coat protein (CP) gene when compared to equivalent GenBank sequences (Table 1). The accession codes assigned by the GenBank were (KU724307), (KU724308) and (KX017581-KX017584) for SqLCV1, SqLCV2 and SqLCV3-SqLCV6 sequences, respectively. Iraqi SCLV sequences obtained scored 100% maximum nucleotide sequence identity

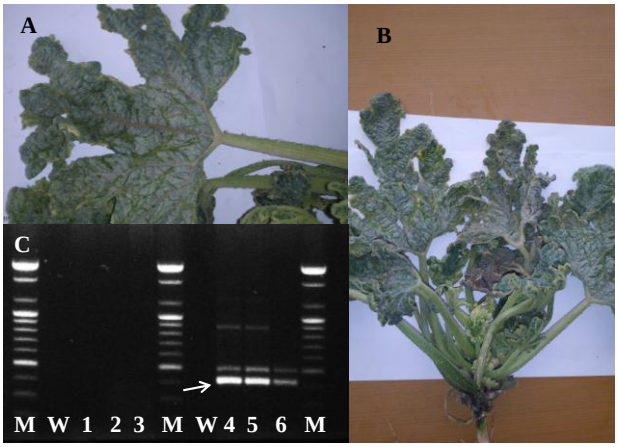

Fig. 1: Symptomatic zucchini plants exhibit leaf curl symptoms (A and B), ethidium bromide stained gel pattern shows ~530 bp DNA fragments (arrow tagged) amplified by Deng primers. Lanes 1-3 healthy zucchini plants, Lanes 4-6 symptomatic zucchini plants, W: water control, M: 1kb marker (Bioneer, South Korea)

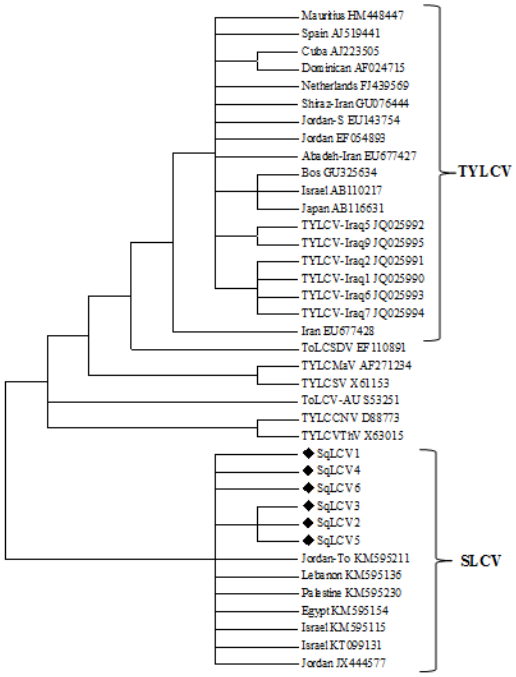

Fig. 2: Evolutionary relationships of *Squash leaf curl virus* (SLCV) isolated from zucchini in Iraq

Neighbor-joining phylogenetic tree constructed from partial CP sequences amplified (tagged with ♦) and equivalent begomoviruses sequences from the GenBank shows sequences relatedness. Virus/Isolates were referred to in table(1). The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed . Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 38 nucleotide sequences. Evolutionary analyses were conducted in MEGA6

Table 1: Begomovirus sequences from GenBank used for sequence comparison in this study

	GenBank acc. code	Isolate/virus names	Location
1.	KT099131	SLCV-Israeli	Israel
2.	JX444577	SLCV-Jordanian	Jordan
3.	KM595136	SLCV-Lebanon	Lebanon
4.	KM595211	SLCV-To	Jordan
5.	KM595115	SLCV-Israeli	Israel
6.	KM595230	SLCV-Palestinian	Palestine
7.	KM595154	SLCV-Egyptian	Egypt
8.	JQ025990	TYLCV-Iraq1	Iraq
9.	JQ025991	TYLCV-Iraq2	Iraq
10.	JQ025992	TYLCV-Iraq5	Iraq
11.	JQ025993	TYLCV-Iraq6	Iraq
12.	JQ025994	TYLCV-Iraq7	Iraq
13.	JQ025995	TYLCV-Iraq9	Iraq
14.	HM448447	TYLCV-Mauritius	Mauritius
15.	AJ519441	TYLCV-CB1/99	Spain
16.	GU076444	TYLCV-IL	Shiraz-Iran
17.	FJ439569	TYLCV-3181291	Netherlands
18.	AB110217	TYLCV-Ng	Israel
19.	GU325634	TYLCV-Bos	South Korea
20.	EU677427	TYLCV-Abadeh	Iran
21.	AJ223505	TYLCV-Cuban	Cuba
22.	AF024715	TYLCV-Dominican	Dominican
23.	EU143754	TYLCV-Jordan-S	Jordan
24.	EF054893	TYLCV-Jordan	Jordan
25.	AB116631	TYLCV-Japan:Misumi:Stellaria	Japan
26.	EU677428	TYLCV-Roodan-8	Kerman-Iran
27.	EF110891	*Tomato leaf curl virus Sudan virus* (TLCSDV)	Yemen
28.	S53251	*Tomato leaf curl virus-AU* (ToLCV-AU)	Australia
29.	AF271234	*Tomato yellow leaf curl Malaga virus* (TYLCMaV)	Spain
30.	D88773	*Tomato yellow leaf curl China virus* (TYLCCNV)	China
31.	X63015	*Tomato yellow leaf curl Thailand virus* (TYLCVThV)	Thailand
32.	X61153	*Tomato yellow leaf curl Sardinia virus* (TYLCSV)	Italy

(approximated) when compared against each other's, while they scored 99% maximum nucleotide sequence identity to SCLV GenBank sequences from Israel (KT099131) and (KM595115), Jordan (JX444577) and (KM595211), Lebanon (KM595136) and Palestine (KM595230) (Table). Neighbor-Joining phylogenetic tree grouped all sequences isolated together with SLCV GenBank isolates, separating them from other begomoviruses (Fig.). *In silico* restriction digestion analysis of partial CP gene showed Iraqi SLCV sequences amplified shared about 96% maximum restriction sites (RS) similarity with each other's and equivalent SLCV GenBank isolates from Israel (KM595115) (Table). While they showed 56% maximum RS similarity with the other begomoviruses from GenBank (Table 2).

Discussion

Many virus diseases have been detected in cucurbits (e.g. Al-Kuwaiti *et al*., 2016), however, no information regarding leaf curl disease caused by begomoviruses have been reported on cucurbits in Iraq. Disease caused by begomoviruses, namely *Tomato yellow leaf curl virus* (TYLCV) have been reported in Iraq since 1978 (Glick *et al*., 2009). However, the first molecular confirmation of a begomovirus incidence in Iraq was in 2013 (Al-Kuwaiti *et al*., 2013). The current study is the first molecular information regarding SLCV; another begomovirus isolated from Iraq. Deng primer set has been designed to amplify ~ 530 bp from the nucleotide position 1–530 within coat protein gene on DNA-A component of many begomoviruses (Deng *et al*., 1994). In this study shorter sequences of about 410 bp were amplified by Deng primers. However, Kumar and Singh (2015) showed that DNA fragments less than 500 bp were amplified by this primer set when used to detect begomoviruses. Besides, nucleotide sequence comparison confirmed that the 410 bp fragments were 99% identical to SLCV CP gene. To the best of the author`s knowledge, this is the first report of SLCV incidence on zucchini in Iraq. *In silico* restriction digestion approach (ISRDA), applied to SLCV sequences, could differentiate among closely related isolates of high identity percentages as well as diverged species. In this approach, each sequence was virtually digested, using NEBcutter program (Vincze *et al*., 2003) then compared to each other's. Criteria used for comparison were based on type of restriction enzymes and number of times that cleave the sequence. If it was identical for the two sequences compared, it was scored 1, otherwise it was scored 0. Then total scores were calculated for each sequences based on the formula mentioned previously. As much as the number of restriction sites is identical, the similarity percentage was higher for the compared sequences. Thus, SqLCV4 and

Table 2: Identity percentages (approximated) of partial CP nucleotide sequences (lower left) and restriction enzyme sites similarities (upper right) of SLCV sequences amplified (bold letters) and sequences from the GenBank

	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
1	SqLCV1		87	87	96	88	90	92	91	93	90	94	91	85	56	56	55	56	45	54	50	52	52	50	52	51	51	52	52	50	50	52	49	49	47	51	49	52	43
2	SqLCV2	98		88	88	93	89	89	87	87	87	87	85	83	54	51	53	52	51	52	46	48	49	47	48	48	47	49	48	47	46	48	44	46	44	47	45	47	40
3	SqLCV3	98	98		87	86	82	85	84	84	83	84	83	80	55	54	54	54	54	55	50	52	52	49	52	53	51	53	53	51	51	52	48	48	45	50	48	50	43
4	SqLCV4	100	98	98		89	92	95	93	93	93	96	94	86	54	54	53	53	53	53	49	51	51	49	51	50	50	51	51	49	48	51	47	47	46	50	47	51	42
5	SqLCV5	98	99	98	98		90	88	86	86	86	89	87	81	52	51	53	52	50	51	46	47	48	47	48	48	47	48	48	47	46	47	44	46	43	47	45	47	41
6	SqLCV6	99	98	97	99	98		92	89	90	90	90	88	84	53	51	53	52	51	51	45	47	47	46	47	47	46	47	47	46	45	47	45	46	44	49	44	48	41
7	Israel KT099131	99	98	97	99	98	99		98	97	98	98	96	91	54	52	53	52	52	43	48	50	51	47	51	50	50	51	51	49	48	51	46	47	46	52	45	51	42
8	Jordan JX444577	99	98	97	99	98	99	100		97	96	97	95	89	53	51	51	51	51	52	48	50	50	46	50	50	49	51	50	48	48	50	46	48	45	51	45	51	41
9	Lebanon KM595136	99	98	97	99	98	99	100	100		96	96	94	90	54	52	52	51	52	52	48	50	51	47	51	50	50	51	51	49	48	50	46	48	46	51	45	51	42
10	Jordan-To KM595211	98	97	97	99	97	98	99	99	99		97	94	90	55	52	52	51	52	53	48	50	51	47	51	50	50	51	51	49	48	51	46	47	45	51	44	50	42
11	Israel KM595115	99	98	97	99	98	99	100	100	100	99		98	90	53	52	52	52	52	53	49	51	51	48	51	51	50	52	51	50	49	51	47	48	46	52	46	51	42
12	Palestine KM595230	98	97	97	99	97	98	99	99	100	99	99		88	52	51	52	51	51	52	48	50	50	47	50	50	49	51	50	48	48	50	46	47	45	51	45	50	41
13	Egypt KM595154	98	97	96	98	97	98	99	99	99	98	99	98		53	52	51	51	51	52	47	49	50	46	50	49	48	51	50	48	48	49	48	46	45	53	44	48	43
14	TYLCV-Iraq1 JQ025990	45	45	44	45	45	45	46	46	46	46	46	46	45		78	71	79	78	79	72	70	71	72	72	70	73	72	72	73	72	72	63	65	53	54	49	56	44
15	TYLCV-Iraq2 JQ025991	45	45	45	45	46	45	46	46	46	46	46	46	45	100		81	96	95	88	87	83	84	84	83	81	88	83	84	88	85	84	69	70	54	55	48	55	47
16	TYLCV-Iraq5 JQ025992	43	43	43	43	44	43	43	43	44	44	43	44	43	93	93		82	80	77	72	70	72	71	72	71	73	71	72	72	72	73	60	61	50	50	48	55	43
17	TYLCV-Iraq6 JQ025993	45	45	44	45	45	45	46	46	46	46	46	46	45	100	100	93		96	90	87	83	84	86	83	81	88	83	84	87	85	84	68	70	54	56	47	55	47
18	TYLCV-Iraq7 JQ025994	45	45	44	45	45	45	46	46	46	46	46	46	45	100	100	93	100		92	88	83	85	87	84	82	88	84	85	89	86	85	66	70	53	56	48	55	47
19	TYLCV-Iraq9 JQ025995	45	45	44	45	45	45	45	45	45	45	45	45	44	98	98	93	98	98		84	80	83	83	82	80	84	83	84	84	84	83	66	73	54	55	49	56	47
20	Mauritius HM448447	45	45	45	45	45	45	45	45	46	46	45	46	45	99	99	93	99	99	98		90	92	94	93	89	96	92	93	96	95	93	65	68	54	57	46	55	48
21	Spain AJ519441	45	45	45	45	45	45	45	45	46	46	45	46	45	99	99	93	99	99	98	100		84	86	84	82	87	83	84	87	86	84	65	69	55	60	50	55	49
22	Shiraz-Iran GU076444	45	45	45	46	46	45	46	46	46	46	46	46	45	99	99	93	99	99	98	100	99		89	89	86	92	88	90	91	92	90	63	66	54	55	44	59	46
23	Netherlands FJ439569	45	45	45	46	46	45	46	46	46	46	46	46	45	99	99	93	99	99	98	100	99	99		90	86	92	87	89	92	92	90	61	68	54	55	45	53	47
24	Israel AB110217	45	45	44	45	45	45	45	45	45	45	45	46	45	99	98	92	99	99	97	99	99	99	99		94	95	91	93	93	95	98	67	68	53	56	46	54	48
25	Bos GU325634	45	45	44	45	45	45	45	45	45	45	45	46	45	98	98	92	98	98	97	99	99	99	99	100		91	87	89	90	91	94	66	68	52	54	46	53	45
26	Abadeh-Iran EU677427	45	45	44	45	45	45	45	45	45	45	45	46	45	99	98	92	99	99	97	99	99	99	99	99	99		92	93	98	95	95	65	69	53	56	45	55	47
27	Cuba AJ223505	45	45	45	45	45	45	45	45	46	46	45	46	45	99	99	93	99	99	98	100	99	99	99	99	99	99		98	93	93	92	65	67	53	58	48	57	48
28	Dominican AF024715	45	45	45	45	45	45	45	45	46	46	45	46	45	99	99	93	99	99	98	100	99	99	99	99	99	99	100		92	96	93	65	67	52	58	47	57	48
29	Jordan-S EU143754	45	45	45	45	45	45	45	45	46	46	45	46	45	99	99	93	99	99	98	100	99	99	99	99	99	99	99	99		96	94	65	69	55	57	45	55	47
30	Jordan EF054893	45	45	45	45	45	45	45	45	46	46	45	46	45	99	99	93	99	99	98	100	99	99	99	99	99	99	99	99	100		96	64	67	54	58	45	54	48
31	Japan AB116631	45	45	44	45	45	45	45	45	45	45	45	46	45	99	98	92	99	99	97	99	99	99	99	100	100	99	99	99	99	99		66	68	53	56	46	54	47
32	Iran EU677428	44	44	43	44	44	44	44	44	44	44	44	44	43	95	94	89	95	95	93	95	95	95	95	95	95	94	95	95	95	95	95		67	53	56	51	54	53
33	ToLCSDV EF110891	43	43	43	43	43	43	43	43	43	43	43	44	43	93	93	87	93	93	92	94	93	93	93	94	94	93	93	93	93	93	94	92		50	50	46	54	44
34	ToLCV-AU S53251	42	43	42	43	42	42	42	42	43	42	42	43	42	73	73	68	73	73	72	73	73	73	74	73	73	73	73	73	73	73	73	72	73		53	48	48	48
35	TYLCMaV AF271234	41	41	41	42	42	42	42	42	42	42	42	43	42	81	80	75	81	81	80	80	80	80	80	80	80	80	80	80	81	81	80	81	81	70		48	52	67
36	TYLCCNV D88773	41	42	41	42	41	41	42	42	42	41	42	42	41	74	74	71	74	74	74	75	74	74	74	75	75	75	74	74	74	74	75	75	74	72	73		50	49
37	TYLCVThV X63015	43	43	42	43	43	43	42	42	43	43	42	43	42	73	73	69	73	73	73	73	73	73	73	73	73	73	73	73	73	73	73	73	73	72	70	71	75		46
38	TYLCSV X61153	40	40	39	40	40	40	41	41	41	41	41	41	40	81	81	76	81	81	81	81	81	81	81	81	80	81	81	81	81	81	81	81	80	71	92	75	71	

SLCV from Israel (KM595115) shared 96% maximum RS similarity as they were identical in 277 out of 289 restriction sites. ISRDA, therefore, could be a useful tool, together with the nucleotide sequence comparisons to resolve species demarcation based on restriction sites similarity.

The high identity percent indicated that SLCV may have been introduced into Iraq from the bordering countries, however the way that SLCV was introduced to Iraq is still unknown. It most likely moved to Iraq through the movement of viruliferous whitefly across country boundaries (Lapidot *et al.*, 2014). SLCV could threat cucurbit production in Iraq, as SLCD outbreaks have been reported in Egypt (Idris *et al.*, 2006), Palestine (Ali-Shtayeh *et al*., 2014), Lebanon (Sobh *et al.*, 2012) and Jordan (Al-Musa *et al*., 2008). Further precaution procedures, therefore, should be considered to control SLCD in Iraq.

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