

## MOLECULAR CHARACTERIZATION AND CONSTRUCTION OF AN INFECTIOUS CLONE OF A NEW STRAIN OF TOMATO YELLOW LEAF CURL VIRUS IN SOUTHERN IRAN\*

A. PAKNIAT<sup>1</sup>, S.A.A. BEHJATNIA<sup>1\*\*</sup>, S. KHARAZMI<sup>2</sup>, M. SHAHBAZI<sup>1</sup>  
AND K. IZADPANA<sup>1</sup>

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### Abstract

Complete nucleotide sequence of an infectious cloned DNA of Tomato yellow leaf curl virus from Abadeh (TYLCV-[Ab]) was determined and found to be comprised of 2782 nucleotides. This isolate shares 95% sequence identity with an Israeli isolate of TYLCV and is considered as a new strain of TYLCV from Iran. In addition to Abadeh, the same virus was detected in naturally infected tomato field samples in Firouzabad, Boushehr and Yasouj. It seems that the TYLCV-[Ab] is a major component of tomato leaf curl disease in southern Iran. A subgenomic DNA, about one third the size of the viral genome, was also detected in naturally infected tomato plants. A partial dimeric (1.5 mer) TYLCV-[Ab] DNA clone was constructed in a binary vector and used to agroinoculate tomato (*Solanum lycopersicum* cv. Grosse Lisse) and tobacco (*Nicotiana tabacum* cv. Turkish) plants. Typical yellowing and leaf curl symptoms were observed on tomato, while tobacco appeared to be a symptomless host of the virus. The viral DNA was detected in newly emerging leaves of infected tomato and tobacco plants, showing that the single DNA component of TYLCV-[Ab] is sufficient for infectivity. Both molecular analysis and symptomatology indicate that this isolate is a severe strain of TYLCV.

**Keywords:** Geminivirus, Tomato leaf curl virus, Subgenomic DNA, Tobacco, Nucleotide sequence.

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\*: Part of PhD. Thesis of The First Author Submitted to College of Agriculture, Shiraz University, Shiraz, Iran.

\*\* : Corresponding Author, Email: behjatni@shirazu.ac.ir

1. PhD Student, Assoc. Prof., MSc. Student and Prof. of Plant Pathology, Respectively, Plant Virology Research Center, Shiraz University, Shiraz, Iran.

2. MSc. of Plant Biotechnology Research Center, Shiraz University, Shiraz, Iran.

## Introduction

Tomato leaf curl is one of the most serious and economically damaging diseases caused by a number of begomoviruses, all of which share the name Tomato (yellow) leaf curl virus [T(Y)LCV]. The level of damage may reach severe to complete crop loss (Makkouk *et al.* 1979; Nakhla *et al.* 1994; Polston *et al.* 1994). Recent spread of these viruses has paralleled the worldwide expansion of the B biotype of their whitefly vector, *Bemisia tabaci* Gen., which has a wider host range, greater fecundity and more aggressive feeding habit than other biotypes (Bedford *et al.* 1994; Czosnek & Laterrot 1997; De Barro 1995). T(Y)LCVs belong to the genus *Begomovirus*, family *Geminiviridae*, and are characterized by small (about 18 X 30 nm) twinned isometric particles with genomes consisting of either one or two circular single-stranded DNA species of 2.5-3.0 kb. Although most begomoviruses have bipartite genome, that of TYLCVs are monopartite. These viruses infect dicotyledonous plants including tomato, pepper, bean and cucurbits worldwide.

TYLCV infection was regarded as an economic problem in the Mediterranean region (Al-Musa 1982; Cherif & Russo 1983; Cohen & Nitzany 1966; Czosnek *et al.* 1990; Makkouk 1978; Makkouk & Laterrot 1983; Makkouk *et al.* 1979; Mazyad *et al.* 1979). Since these early reports, TYLCVs have spread rapidly throughout the Middle East (Hajimorad *et al.* 1996; Mansour & Al-Musa 1992) and have also been reported in Southeast Asia (Rochester *et al.* 1990), North and Central Africa (Harrison *et al.* 1991), the America (Brown & Bird 1992; Brown & Nelson 1988; Harrison *et al.* 1991; McGlashan *et al.* 1994; Nakhla *et al.* 1994; Polston *et al.* 1994), Europe (Kheyr-Pour *et al.* 1991; Macintosh *et al.* 1992; Noris *et al.* 1994) and Australia (Dry *et al.* 1993). Biological, molecular and epidemiological characteristics and management of the TYLCV complex have been reviewed in Picó *et al.* (1996), Moriones and Navas-Castillo (2000) and, more recently, in Czosnek (2007) indicating that the leaf curl disease has been spread all around the world in recent years.

Begomoviruses causing tomato yellow leaf curl disease (TYLCD) belong to at least 42 species and 178 strains (Fauquet *et al.* 2008). So far, the full-length nucleotide sequences and other molecular and biological characteristics of five Iranian

T(Y)LCV isolates have been reported. Of these, Tomato yellow leaf curl virus-Iran [Iran: Iranshahr:1998] (TYLCV-IR; Bananej *et al.* 2004) and Tomato leaf curl Karnataka virus-Iran [Iran:Iranshahr] (ToLCKV-IR[IR:Ira]; Behjatnia *et al.* 2004), both isolated from infected tomato fields in Iranshahr (Sistan-Balouchistan province), Tomato yellow leaf curl virus strain TYLCV-IR2 (Acc. No. EU085423) isolated from infected tomato in Bandar Abbas (Hormozgan province) and the Kahnouj (Kerman province) isolate of TYLCV (TYLCV-[Kahnouj]) (Fazeli *et al.* 2008) have a single genomic component. The fifth isolate is a variant of Tomato leaf curl Palampur virus (ToLCPMV) which has two genomic components. It has been recently reported that the latter virus is the casual agent of an ongoing tomato leaf curl epidemic in Iran and in addition to infecting tomatoes; it has devastated greenhouse cucumber and melon farms in Jiroft, Kerman province (Heydarnejad *et al.* 2009). In addition to Kerman and Sistan-Balouchistan provinces, the TYLCD has widely spread to tomato fields of Boushehr, Hormozgan, Fars, Khorasan, Khuzistan, Markazi and Yazd provinces (Bananej *et al.* 2004; Bananej *et al.* 2009; Behjatnia *et al.* 2004; Fazeli *et al.* 2008; Hajimorad *et al.* 1996; Malekzadeh *et al.* 2008; Vahdat *et al.* 2008). It seems that different tomato-infecting begomoviruses including various T(Y)LCV species or strains are responsible for the disease (Fazeli *et al.* 2008).

Tomato plants showing severe leaf curl and yellowing were observed in many tomato greenhouses in Abadeh region of Fars province in November 2007. Partial sequence information of the viral genome identified the casual virus as a new strain of Israeli group of TYLCV, designated Abadeh isolate of TYLCV (TYLCV-[Ab]), differing from other T(Y)LCVs reported from Iran. Here we describe the genome structure of this virus, construction of its infectious clone and its subsequent agroinoculation to certain hosts.

## Materials and Methods

### Virus source

During October 2007, tomato plants showing yellow leaf curl symptoms were collected from greenhouses in Abadeh, Fars province. The virus was isolated from a single infected tomato plant showing yellow leaf curl symptoms used in

polymerase chain reaction (PCR), cloning and subsequent molecular characterization, and designated Abadeh isolate of TYLCV (TYLCV-[Ab]). Subsequently, surveys were made in tomato fields and greenhouses in Firouzabad (Fars province), Borazjan (Boushehr province) and Yasouj (Kohgiluyeh and Boyer-Ahmad province) in 2008 and 2009 growing seasons. A few plants showing yellowing, leaf curling and stunting symptoms were collected and tested by PCR using degenerate primers for whitefly transmitted geminiviruses (WTGs) (see below).

### Cloning and sequencing of the full-length viral genome

Total leaf DNAs were extracted from naturally infected tomato plants as described by Behjatnia *et al.* (1996) and used for DNA amplification.

Five combinations of degenerate oligonucleotide primers: PAR1<sup>C</sup>496/PAL1<sup>V</sup>1978, PAR1<sup>C</sup>496/PCR<sup>V</sup>181, PCR<sup>C</sup>1/PAL1<sup>V</sup>1978, PrimerB<sup>C</sup>/PAL1<sup>V</sup>1978 and PrimerB<sup>C</sup>/PCR<sup>V</sup>181 (Table 1), shown to be specific for DNA-A of WTGs (Deng *et al.* 1994; Rojas *et al.* 1993), were used in PCR to amplify WTGs DNA fragments with discrete sizes from total leaf DNA of naturally infected tomato plants collected in Abadeh. DNA samples from agroinfected-ToLCKV-IR [IR:Ira] tomato plants growing in a glasshouse were used as positive control in PCR. The amplified DNA fragments of Abadeh samples were cloned into a pTZ57R/T plasmid using InsT/Aclone PCR Product Cloning Kit (Fermentas) and sequenced in both directions using M13 universal primers and an ABI PRISM system at Macrogen (South Korea). To amplify and clone the rest of the genome of TYLCV-[Ab], a pair of specific primers (TYLCV-[Ab] 349<sup>V</sup>/1997<sup>C</sup>, Table 1), designed from the partial sequence information, was used. The resulting clone was sequenced to the full-length genome. To amplify the full-length DNA genome of TYLCV-[Ab], two pairs of specific adjacent primers (TYLCV-[Ab] 2333<sup>V</sup>/2338<sup>C</sup> and TYLCV-[Ab] 1015<sup>V</sup>/1020<sup>C</sup>, Table 1) flanking the natural occurring *SacI* and *SphI* restriction sites, respectively, designed from the full-length sequence information, were used. The full-length PCR fragments were cloned. Two full-length monomer genome clones, designated pTZ-*SacI*1.0TYLCVAb and pTZ-*SphI*1.0TYLCVAb, respectively, were sequenced as mentioned above. A pair of degenerate oligonucleotide primers, PBL1<sup>V</sup>2040 and PCR<sup>C</sup>1 (Rojas *et al.*, 1993) (Table

1), shown to be specific for DNA-B of WTGs was used in PCR to amplify a fragment of approximately 600 bp in size.

The PCR<sup>V</sup>181/primer B<sup>C</sup> primer pair (Deng *et al.*, 1994; Rojas *et al.*, 1993) (Table 1) was used to detect the TYLCVs in infected samples collected from tomato fields and greenhouses in Firouzabad, Boushehr and Yasouj.

PCR was carried out in a volume of 20 or 50  $\mu$ l containing DNA template extracted from approximately 0.4 mg leaf tissue, oligonucleotide primers (each at 1 $\mu$ M), 200  $\mu$ M each of dCTP, dGTP, dTTP and dATP, 1.5 mM MgCl<sub>2</sub> and 0.5 or 1.25 units of *Taq* DNA polymerase (Cinnagen, Iran) in the reaction buffer provided by the same source. The mixture was subjected to a 30 cycle PCR program of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. The final cycle was followed by a 7-min incubation at 72°C. The amplification products were separated on 1.2 % agarose gel, and DNA fragments were eluted from the gels using a QIAquick gel extraction kit (QIAGEN, Germany) according to manufacturer's instructions.

### Comparison of T(Y)LCV sequences

The complete DNA nucleotide sequence of TYLCV-[Ab] was compared with those of TYLCV-IR [IR:Ira:98], ToLCKV-IR [IR:Ira], TYLCV-IR2, TYLCV-[Ka] and ToLCPMV-IR [IR:Jir] from Iran and closely related T(Y)LCV isolates from different parts of the world for which complete genome information was available (Table 3). The complete DNA nucleotide sequence of Abutilon mosaic virus-[United States of America:Hawaii] as an out-T(Y)LCV group was also used in comparisons. Sequence alignment and phylogenetic analysis were carried out using the programs Megalign and Seqman from DNASTAR group and ClustalX.

### Construction of an infectious clone of TYLCV-[Ab]

In this study an infectious head-to-tail 1.5-mer of TYLCV-[Ab] clone, pBin-1.5TYLCVAb, containing the full-length *SphI* monomeric DNA and a direct repeat of 1464 bp *SphI/SacI* DNA fragment, was constructed. The pTZ-*SacI*1.0TYLCVAb and pTZ-*SphI*1.0TYLCVAb constructs were used as DNA templates for synthesis of the infectious clone. A 1318 bp *SphI* fragment was released from the pTZ-*SacI*1.0TYLCVAb through digestion of

**Table1. Oligonucleotide primers used in this study**

Primers	Size (nt)	Nucleotide positions	Sequences from 5' to 3' <sup>a, b</sup>
PCR <sup>V</sup> 181	18	degenerate	TAATATTACCGWTGGCC
Primer B <sup>C</sup>	23	degenerate	TGGACYTTRCAWGGBCCTTCACA
PBL1 <sup>V</sup> 2040	30	degenerate	GCCTCTGCAGCARTGRTCKATCTTCATACA
PCR <sup>C</sup> 1	29	degenerate	CTAGCTGCAGCATATTTACRARWATGCCA
PARI <sup>C</sup> 496	27	degenerate	AATACTGCAGGGCTTYCTRTACATRGG
PAL1 <sup>V</sup> 1978	30	degenerate	GCATCTGCAGGCCACATYGTCTTYCCNGT
TYLCV-[Ab] 349 <sup>V</sup>	20	349-368	CTCGAAGGTTCCGCGAAGGC
TYLCV-[Ab] 1997 <sup>C</sup>	22	1976-1997	CGCGGCCATGGAGACCTAATAG
TYLCV-[Ab] 2333 <sup>V</sup>	28	2333-2358	CCGAGCTCTTAGCTGCCTGAATGTTCCGG
TYLCV-[Ab] 2338 <sup>C</sup>	28	2313-2338	CCGAGCTCAACAGATGTCAAGACCTACG
TYLCV-[Ab] 1015 <sup>V</sup>	29	1015-1041	CTGCATGCCTCTAATCCAGTGTATGCAAC
TYLCV-[Ab] 1020 <sup>C</sup>	29	994-1020	CTGCATGCGTACATGCCATATACAATAAC

<sup>a</sup> Nucleotides at degenerate positions are represented by a single letter of the IUPAC ambiguity code: B = C, G; K = G, T; R = A, G; W = A, T; Y = C, T.

<sup>b</sup> GAGCTC = *SacI* restriction site, GCATGC = *SphI* (*PaeI*) restriction site

<sup>c</sup> Complementary-sense strand primers

<sup>v</sup> Virion-sense strand primers



**Fig. 1** Yellowing and leaf curling symptoms in tomato from Abadeh (Fars province) greenhouse (A) and in tomato plants graft-infected with scion of naturally infected tomato samples from (B) Firouzabad (Fars province), (C) Borazjan (Boushehr province) and (D) Yasouj (Kohgiluyeh and Boyer-Ahamd province).

**Table 2. Full-length genome similarity of TYLCV-[Ab] with other T(Y)LCV isolates from Iran and elsewhere**

Virus	Abbreviation <sup>a</sup>	Genomic sequence accession number	Total nucleotide similarity (%) with TYLCV-[Ab]
Tomato yellow leaf curl virus-Israel [Spain:Almeria:Pepper:1999]	TYLCVIL[ES:Alm:Pep:99]	AJ489258	95.2
Tomato yellow leaf curl virus-Israel [Dominican Republic]	TYLCV-IL[DO]	AF024715	94.8
Tomato yellow leaf curl virus-Israel [Cuba]	TYLCV-IL[CU]	AJ223505	94.5
Tomato yellow leaf curl virus-Iran [Iran:Iranshahr:1998]	TYLCV-IR[IR:Ira:98]	AJ132711	92.4
Tomato yellow leaf curl virus-Mild [Portugal:2:1995]	TYLCV-Mld[PT:2:95]	AF105975	91.2
Tomato yellow leaf curl virus-Mild [Spain:72:1997]	TYLCV-Mld[ES:72:97]	AF071228	90.9
Tomato yellow leaf curl virus-Israel [Israel:Rehovot:1986]	TYLCV-IL[IL:Reo:86]	X15656	89.3
Tomato yellow leaf curl virus -[Kahnouj]	TYLCV-[Kahnouj]	EU635776	88.8
Tomato yellow leaf curl virus-[Iran 2]	TYLCV-IR2	EU085423	86.8
Tomato yellow leaf curl virus-Mild [Israel:1993]	TYLCV-Mld[IL:93]	X76319	86.6
Tomato yellow leaf curl virus-Mild [Japan:Shizuoka]	TYLCV-Mld[JR:Shz]	AB014346	86.4
Tomato leaf curl Karnataka virus-Bangalore [India:Bangalore:1993]	ToLCKVBan[IN:Ban:93]	U38239	75.8
Tomato leaf curl Bangladesh virus-[Bangladesh:2]	ToLCBDV-[BD:2]	AF188481	75.7
Tomato leaf curl Karnataka virus-Iran [Iran:Iranshahr]	ToLCKV-IR[IR:Ira]	AY297924	74.9
Tomato leaf curl Taiwan virus-A [Taiwan]	ToLCTWV-[TW]	U88692	74.6
Tomato leaf curl Bangalore virus-B [India:Bangalore 5]	ToLCBV-B[IN:Ban5]	AF295401	74.0
Tomato yellow leaf curl Thailand virus-A [Thailand:1]	TYLCTHV-A[TH:1]	X63015	70.3
Tomato leaf curl New Delhi virus-India [India:Lucknow]	ToLCNDV-IN[IN:Luc]	Y16421	69.4
Tomato leaf curl New Delhi virus-India [India:New Delhi:Mild:1992]	ToLCNDVIN[IN:ND:Mld:92]	U15016	68.6
Tomato leaf curl Palampur virus-Iran [Iran:Jiroft], DNA A	ToLCPMV-IR[IR:Jir]	FJ660444	67.3
Abutilon mosaic virus-[USA:Hawaii]	AbMV-[US:Haw]	U51137	55.7

<sup>a</sup>Assigned abbreviations are as Fauquet *et al.* (2008)

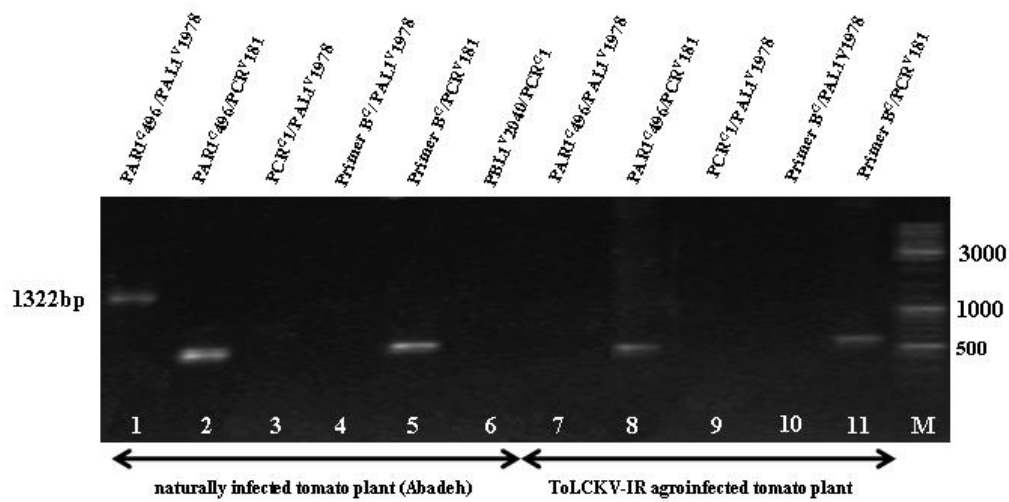


Fig. 2 Electrophoresis pattern of PCR product of TYLCV-[Ab] and ToLCKV-IR amplified from total nucleic acid extracts of infected tomato plants using WTGs degenerate primers as outlined at the top each lane. M= Marker (DNA ladder Mix, Fermentas).

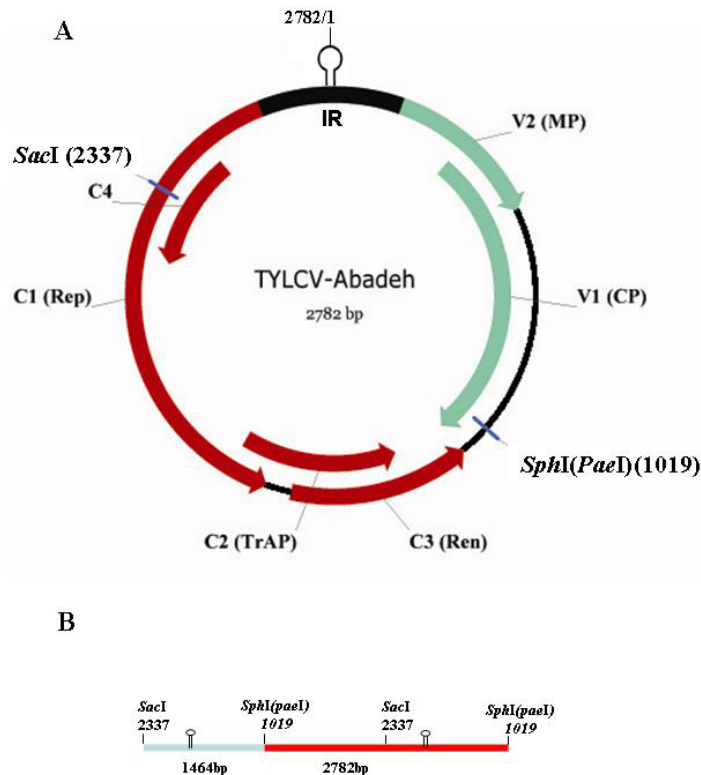


Fig. 3 (A) Proposal genome organization of TYLCV-[Ab]. ORFs on both virion-sense strand (V) and complementary-sense strand (C) are displayed by arrows. The position of the conserved stem-loop structure (  $\Omega$  ) and intergenic region (IR) are marked. The location of the two unique sites of restriction enzymes *SacI* (2337) and *SphI* (1019) used for construction of a 1.5 mer infectious clone is shown. (B) Schematic drawing of 1.5 mer TYLCV-[Ab] infectious clone inserted into pBin19 binary plasmid.

this clone with *SphI* and the backbone of the construct containing the 1464 bp *SphI/SacI* fragment of TYLCV-[Ab] designated as pTZ-0.5TYLCVAb clone. A full-length *SphI* monomeric DNA was released from pTZ-*SphI*.0TYLCVAb construct and sub-cloned into the corresponding site of the pTZ-0.5TYLCVAb to produce the pTZ-1.5TYLCVAb construct. This construct was digested with *HindIII* and sub-cloned into the corresponding site of binary vector pBin19 to create the pBin-1.5TYLCVAb. The resulting pBin construct was introduced into *Agrobacterium tumefaciens* strain C58 by electroporation with a Gene Pulser apparatus (Bio-Rad, Germany) according to the manufacturer's specifications.

### Whole plant infectivity assays

Cultures of *A. tumefaciens* harboring the pBin-1.5TYLCVAb construct were grown for 36-48 h and inoculated into the axillary buds of tomato (*Solanum lycopersicum* cv. Gross Lisse) and tobacco (*Nicotiana tabacum* cv. Turkish) plants as described previously (Dry *et al.*, 1993). The agroinoculated plants were evaluated for symptom appearance 21-45 days post-inoculation (dpi). Meanwhile, developing leaves were sampled from these plants and DNA was extracted and analyzed for the presence of the viral genome by PCR using the specific TYLCV-Ab 349<sup>V</sup>/1997<sup>C</sup> oligonucleotide primer pair (Table 1).

## Results

### Detection of T(Y)LCV like-DNAs

Naturally infected tomato plants collected from greenhouses in Abadeh showing the T(Y)LCV like-symptoms (upward leaf curling, severely reduced leaf size, yellowing of the leaf margins and veins, flower abscission and stunting of plants, Fig. 1A) were analyzed by PCR for the presence of T(Y)LCV like-DNAs using five WTGs degenerate oligonucleotide primer pairs. Both PAR1<sup>C</sup>496/PCR<sup>V</sup>181 and PCR<sup>V</sup>181/PrimerB<sup>C</sup> primer pairs amplified a PCR product of the expected size (~500 bp) from a naturally infected Abadeh tomato plant extract (Fig. 2 lanes 2 and 5). A PCR fragment corresponding to the expected DNA product was also obtained with leaf extract from a tomato plant agroinfected with an infectious construct of ToLCKV-IR [IR:Ira] (Behjatnia *et al.* 2009) (positive control) (Fig. 2 lanes 8 and 11). These results indicated that the Abadeh tomato

plants was infected with a T(Y)LCV-like DNA. A PCR product of the expected size (~1300 bp) was also amplified from the naturally infected Abadeh tomato plant extract when the PAR1<sup>C</sup>496/PAL1<sup>V</sup>1978 primer pair was used in PCR (Fig. 2, lane 1). The size of this fragment was determined to be exactly 1322 bp when cloned and sequenced.

To determine whether Abadeh tomato plant contained a bipartite begomovirus, PCR reactions were set with a pair of specific DNA-B degenerate oligonucleotide primers PBL1<sup>V</sup>2040/PCR<sup>C</sup>1 (Rojas *et al.* 1993) (Table 1). No DNA fragment was obtained (Fig. 2 lane 6) indicating that the Abadeh tomato plant was infected with a monopartite T(Y)LCV.

### Cloning and sequencing of full-length genome of TYLCV-[Ab] and its relationship with other TYLCVs

Initial comparison of the sequence of the 1322 bp DNA fragment isolated from Abadeh tomato infected plant with the nucleotide sequences in GenBank using Standard nucleotide-nucleotide BLAST program from National Center for Biotechnology Information (NCBI) indicated that this fragment was most similar (95%) to a DNA fragment of the 5' region of the C1 ORF, intergenic region and 5' region of the V1 ORF of an Israeli isolate of TYLCV (TYLCV-IL) genome.

The sequence of the 1322 bp DNA fragment was used to design two specific PCR primers, TYLCV-Ab349<sup>V</sup> /1997<sup>C</sup>, which allowed amplification of the rest of the genome. These primers amplified a 1648 bp DNA fragment. After cloning and sequencing of this fragment, the complete sequence of the virus was determined by assembly of sequences of 1322 bp and 1648 bp clones using Seqman Program (from DNASTAR group). The size of assembled full-length sequence containing the regions selected for the degenerate primers is shown to be 2782 bp. Based on the sequence data, two adjacent primer pairs TYLCV-[Ab] 2333<sup>V</sup>/2338<sup>C</sup> and TYLCV-[Ab] 1015<sup>V</sup>/1020<sup>C</sup> (Table 1), flanking the naturally occurring *SacI* and *SphI* restriction sites, respectively, were designed and used to amplify and clone the full-length genome of the virus. The sequences of both strands of two independent clones (pTZ-*SacI*.0TYLCVAb and pTZ-*SphI*.0TYLCVAb) were obtained using M13 universal primers to confirm the sequences of regions selected for the degenerate primers. The

size of full-length genome was confirmed to be 2782 bp. The nucleotide sequence data have been deposited in GenBank under accession number [FJ355946](#).

The features of the TYLCV-[Ab] genome are similar to those of the other monopartite TYLCV isolates which have two overlapping ORFs, V1 and V2 on the virion-sense strand and four ORFs C1–C4 on the complementary-sense strand. ORFs C1–C3 partially overlap while ORF C4 is contained within ORF C1 (Gronenborn 2007) (Fig. 3A). A 313 nt-long intergenic region (IR) of the virus embodies a stem-loop structure characteristic of begomoviruses. The stem is 11 bp long and the loop contains the invariant TAATATTAC sequence with the T/AC cleavage site where rolling circle replication is initiated (Heyraud-Nitschke *et al.* 1995).

Comparison of complete DNA nucleotide sequence of this virus with those of other T(Y)LCV isolates from Iran and different parts of the world for which complete genome information were available (Table 2) indicated that this TYLCV isolate is most similar to Almeria isolate of TYLCV-IL [ES:Alm:Pep:99] with 95.2% identity at nucleotide level. According to ICTV criteria, it can be classified as a new strain of Israeli group of TYLCV. Among five T(Y)LCV isolates reported from Iran (Table 2), for which complete genome information are available, TYLCV-[Ab] is more similar to TYLCV-IR[IR:Ira:98], their genome sharing 92.4 % homology. It was more distantly related to ToLCPMV-IR[IR:Jir], with which genome shares only 67.3 % homology (Table 2).

### Taxonomic position of TYLCV-[Ab]

A dendrogram obtained by phylogenetic analysis with ClustalX program indicates three main groups and some subgroups of T(Y)LCV isolates used in this analysis (Fig. 4). The first main group (Group I, Fig. 4) is represented by Israeli isolate of TYLCV and closely related isolates spread across the globe. This group is divided into two subgroups, represented by different isolates of Tomato yellow leaf curl virus – Israel (TYLCV-IL) and Tomato yellow leaf curl virus – Mild (TYLCV-Mld) species, respectively. TYLCV-IR[IR:Ira:98] and TYLCV-[Ab] appear to be severe forms of TYLCV, grouping with TYLCV-IL while the TYLCV-IR2 and TYLCV-[Kahnouj] are two mild isolates of TYLCV as they grouped with TYLCV-

Mld group. The second main group (Group II, Fig. 4) is represented by both monopartite and bipartite isolates of T(Y)LCVs from Southern India and East Asia including TLCV-Bangalore 2 to 5, TYLCTHV-A[TH:1] and ToLCTWV-[TW]. ToLCKV-IR[IR:Ira] is in this group and shows more similarity to monopartite ToLCV isolates from Southern India. Bipartite TYLCTHV-A[TH:1] and ToLCTWV-[TW] isolates constitute a subgroup distinct from ToLCV isolates from Southern India and Iran. The third main group (Group III, Fig. 4) is represented by DNA A of bipartite ToLCV isolates from Northern India and ToLCPMV-IR[IR:Jir] from Jiroft, Iran, are quite different from those of Southern India.

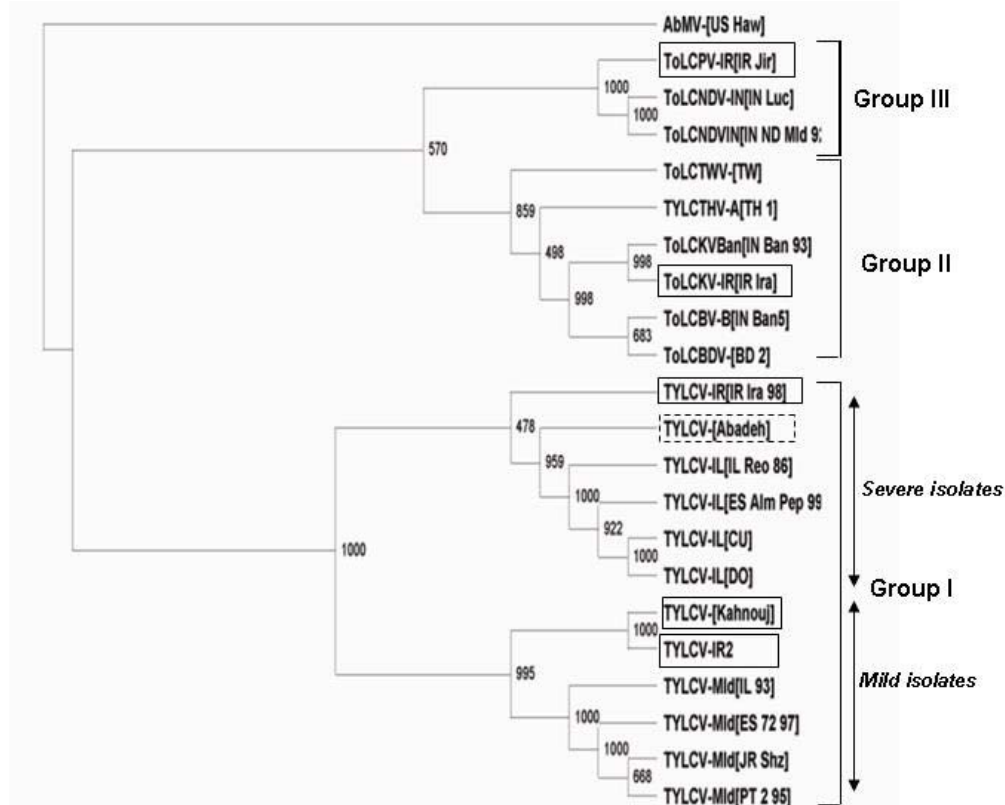
### Characterization of a TYLCV-[Ab] defective DNA

As mentioned above, two adjacent primer pairs: TYLCV-[Ab] 2333<sup>V</sup>/2338<sup>C</sup> and TYLCV-[Ab] 1015<sup>V</sup>/1020<sup>C</sup> (Table 1) were used to amplify the full-length genome of the virus. The Primer pair TYLCV-[Ab] 1015<sup>V</sup>/1020<sup>C</sup> (Table 1) gave a single PCR product of the size predicted for the full-length genome of the virus (Fig. 5A lane 1). However, the primer pair TYLCV-[Ab] 2333<sup>V</sup>/2338<sup>C</sup> (Table 1), in addition to a very faint full-length DNA band, amplified an 897 bp fragment (Fig. 5A lane 2). After cloning and sequencing, this fragment was found to be a viral defective DNA about one third of the full-length of genome. The part of the genome from which defective DNA is derived is shown in Fig 5B. This is the first report of generating a subgenomic DNA in a naturally infected T(Y) LCV plant in Iran.

### Infectivity of the cloned genome of TYLCV-[Ab]

To test the infectivity of TYLCV-[Ab] genome, a partial dimeric (1.5 mer) clone (Fig 3B) was constructed in a binary vector to form pBin-1.5TYLCVAb. Twenty two tomato (*S. lycopersicum* cv. Grosse Lisse) and 12 tobacco (*N. tabacum* cv. Turkish) plants were agroinoculated each with 75 µl of bacterial suspension containing the pBin-1.5TYLCVAb construct. Symptoms including leaf curling and mild yellowing were observed on newly developed leaves of all agroinoculated tomato plants 25–30 days post inoculation (dpi) (Fig. 6A left). Subsequently, TYLCV typical symptoms including severe leaf





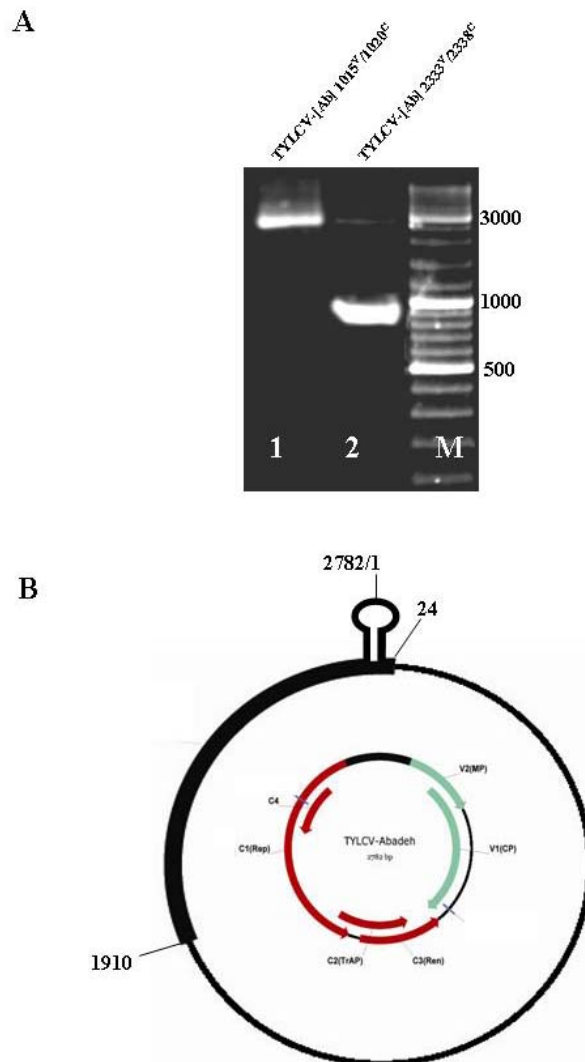
**Fig. 4** Phylogenetic tree obtained from the alignment of nucleotide sequences of 20 T(Y)LCV isolates and AbMV as out-group using ClustalX program. The bootstrap score shows the viability of tree. ( — ) Iranian isolates of T(Y)LCV. ( - - - ) TYLCV-Abadeh. See table 2 for abbreviation of virus names.

curl and yellowing, markedly smaller misshapen leaflets and severe leaf cupping were observed on all aerial parts of the inoculated tomato plants (Fig. 6A and B). Such plants were markedly stunted compared to healthy plants although their shoots elongated in a spindly form. No symptoms were observed on any of the agroinoculated tobacco plants even three months postinoculation. Presence of viral DNAs in agroinfected plants was analyzed 21 dpi using specific TYLCV-[Ab] 349<sup>V</sup> /1997<sup>C</sup> primers. The expected 1648 bp DNA fragment was amplified from both agroinoculated tomato and tobacco plants (Fig. 7) indicating that the single DNA component of TYLCV-[Ab] is sufficient for infectivity and that tobacco is a symptomless host of TYLCV-[Ab].

#### **Widespread occurrence of TYLCV-[Ab] in southern Iran**

Symptomatic field and greenhouse tomato plants in three major tomato growing regions in southern

Iran were tested for the presence of TYLCVs using PCR with the primer pair PCR<sup>V</sup>181/primerB<sup>C</sup>. These primers amplified a 533 bp DNA fragment from leaf extract of tomato plants graft-infected with scion of naturally infected tomato samples from Firouzabad (Fars province), Borazjan (Boushehr province) and Yasouj (Kohgiluyeh and Boyer-Ahmad province) (Fig. 1B, C & D). An amplified DNA from each region was cloned and sequenced. This DNA fragment extends from the invariable nonanucleotide (TAATATTAC) in the conserved stem-loop region to the sequence encoding eight invariable amino acids (CEGPKVQ), present in the N-terminal region of the coat protein. Comparison of the sequence of the 533 bp DNA fragments isolated from three above-mentioned regions with the sequences in GenBank indicated that this DNA is most similar (97%) to corresponding region of T(Y)LCV-[Ab]. It seems that the TYLCV-[Ab] is the main component of



**Fig. 5 (A)** Predicted PCR product (2782 bp, lanes 1 and 2) and smaller product than that expected (897 bp, lane 2) amplified from DNA extract of a TYLCV-[Ab] naturally infected tomato plant using either TYLCV-[Ab] 1015<sup>V</sup>/1020<sup>C</sup> (lane 1) or TYLCV-[Ab] 2333<sup>V</sup>/2338<sup>C</sup> (lane 2) primers (Table 1) expected to amplify the full-length viral genome (2782 bp). **(B)** Schematic drawing of 897 bp DNA fragment on viral genome recognized as a defective DNA. The region of TYLCV-[Ab] DNA present in defective DNA is shown by thick arc in outer circle (1910-2782 and 1-24) and genome organization of TYLCV-[Ab] (as in Fig. 3) is shown in inner circles.

tomato leaf curl disease in southern Iran.

### Discussion

Like those of other WTGs (Argüello-Astorga *et al.* 1994), the intergenic region of TYLCV-[Ab] contains short nucleotide repeats. The sequence AATCGGTGT in the virion-sense strand at nucleotides 2626-2634 is repeated at nucleotides 2653-2661. These repetitive sequences, known as

iterons are postulated to act as specific binding sites (the 5' and the 3' Rep-binding motifs, respectively) for C1 proteins during replication (Argüello-Astorga *et al.* 1994; Chatterji *et al.* 2000). Interestingly, the sequence AATCGGTGT is conserved in the 3' Rep-binding motif of all TYLCV-IL isolates, whereas the putative 5' Rep-binding motif in some isolates of TYLCV-IL, such

as Sinaloa (EF523478), Culiacan Mexico (DQ631892), USA (EF539831) and China (Beijing3, GU983859), but not in TYLCV-[Ab] and many other TYLCV-IL isolates, has the AATTGGTGT sequence in which the C residue at nucleotide position number four is replaced by a T residue. On the basis of this difference the wide range of TYLCV-IL isolates (at least 24 isolates, Fauquet *et al.* 2008) are divided in two groups. The Rep of each geminivirus specifically binds to its own Rep-binding motifs and it has been shown that even one nucleotide change in each of the Rep-binding motifs will result in independently replicating variants (Behjatnia *et al.* 2001; Chatterji *et al.* 1999). It is, therefore, possible that one nucleotide difference in the 5' Rep-binding motif sequence of some isolates of TYLCV-IL results in independent replication of each group isolate by its own Rep.

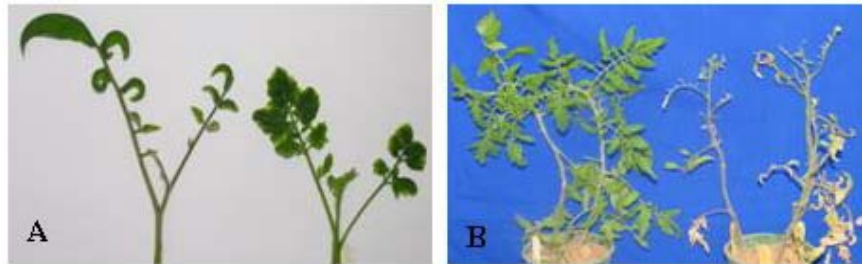
In addition to the full-length DNA genome, distinct subgenomic DNA species known as defective (df) DNA molecules have been found in plants infected with geminiviruses (Behjatnia *et al.* 2007; Frischmuth and Stanley 1991; Stanley *et al.* 1990). A df DNA derived from TYLCV-[Ab] genome, found to be associated with natural infection of tomato plants in Abadeh, contains a large deleted region that disrupt all of the viral genes required for replication, encapsidation and spread of the virus except for the C4 ORF. However, the viral origin of replication (*ori*), including the Rep binding domains, was present in this df DNA. Mutational analysis showed the minimum sequence requirements for the replication of df DNAs associated with TYLCV-Au infection was the intergenic region containing the *ori* and the Rep-binding domains (Behjatnia *et al.* 2007). It has been supposed that the df DNAs may act to reduce virus replication levels in host plants leading to enhanced survival of the virus-infected host (Patil & Dasgupta 2006). It would be of interest to study the role of TYLCV-[Ab] df DNA in viral replication, systemic spread and symptom amelioration.

Since the early report of TYLCD from southern provinces of Iran (Hajimorad *et al.* 1996), this important disease has spread very rapidly and it is now quite prevalent in this country. Epidemics of TYLCD have caused devastating damage to both field and greenhouse tomato crops in recent years. Among the six T(Y)LCV isolates reported as the causal agents of TYLCD in different parts of Iran,

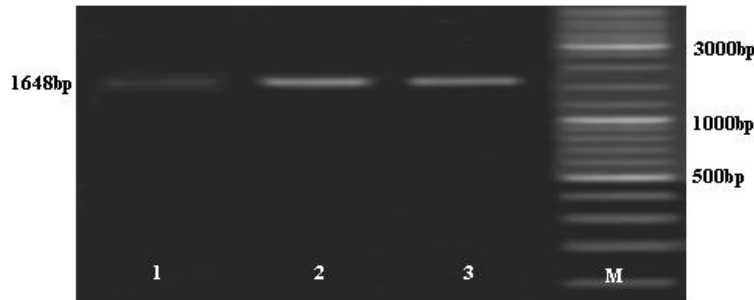
TYLCV-IR and ToLCKV-IR have been reported only from Iranshar tomato fields (Bananej *et al.* 2004; Behjatnia *et al.* 2004). ToLCKV-IR is related to ToLCV isolates from southern India (ToLCV Bangalore-2 and Bangalore-4 isolates) and may have originated from there (Behjatnia *et al.* 2004; Navas-Castillo *et al.* 2000). TYLCV-IR has a chimeric genome apparently arisen by recombination between TYLCV-IL-like and ToLCV-like ancestors (Navas-Castillo *et al.* 2000). Phylogenetic analyses conducted by Bananej *et al.* (2004) indicated that TYLCV-IR possesses a hybrid genome of which the 5' half of C1 ORF and the intergenic region were donated by ToLCKV-IR, as a minor parent, and the remaining genome sequence by TYLCV-Mid, as a major parent. On the other hand, our phylogenetic analyses showed that the TYLCV-[Ka] isolate, reported from Kahnouj (Kerman province), a region close to Iranshar (Sistan-Balouchistan province) (Fazeli *et al.* 2008), is grouped with TYLCV-Mid. These observations confirm the Bananej *et al.* (2004) conclusion that consider Iranshar region as a transition zone where West and East TYLCV isolates meet, leading to recombinations reported from this region.

Discovery of ToLCPMV-IR infecting tomatoes and cucurbits in Jiroft (Kerman province) by Heydarnejad *et al.* (2009) as a variant of Tomato leaf curl Palampur virus indicated that the fourth virus in south-eastern region of Iran is originated from bipartite ToLCV isolates from Northern India. As this virus was identified as the casual agent of TYLCD and a devastating disease of cucumber and melon in Jiroft, Heydarnejad *et al.* (2009) stated that this virus has potential to be a part of a complex that causes TYLCD in Iranian tomato crops and a major threat to cucurbit production in this country and surrounding Middle East countries. Recently, the same virus was detected in greenhouse cucumbers with a severe yellow disease in Yazd (Azarfar *et al.* unpublished data), indicating that this virus has spread to the central parts of Iran.

Among the viruses infecting tomato, TYLCV-IL isolates are the most notorious in terms of the intensity of the disease and are the most widespread isolates around the globe (Czosnek, 2007). Surveys made in tomato fields and greenhouses in four southern provinces of Iran indicated that TYLCV-[Ab] is the main component of tomato leaf curl



**Fig. 6** Symptoms induced by TYLCV-[Ab] infectious clone in experimentally agroinoculated tomato plants showing (A) leaf curling (left) and yellowing (right) of the young leaves 30dpi (left) and 40 dpi (right). (B) Tomato plants showing elongated spindly shoots, yellowing, severe leaf curling, and markedly smaller, misshaped and cupped leaflets 45 dpi compared with a healthy tomato plant on the left.



**Fig. 7** Amplification of a 1648 bp fragment in PCR using specific TYLCV-[Ab] 349<sup>V</sup> /1997<sup>C</sup> primer pair with extracted DNA from a symptomless tobacco plant (lanes 1) and a tomato plant with typical symptoms (lane 2) 21 days postagroinoculation with a TYLCV-[Ab] infectious clone . DNA extract of a naturally infected TYLCV-[Ab] was used as positive control (lane 3). M= Marker (DNA ladder mix, Fermentas).

disease in southern Iran. TYLCV-[Ab] appears to be a severe strain of TYLCV. The nucleotide sequence of TYLCV-[Ab] has a very high homology to that of TYLCV-IL isolates, indicating that TYLCV-[Ab] is a bona fide TYLCV-IL

isolate, rather than a recombinant virus, and provides further evidence that this virus has been introduced into southern tomato-growing regions of Iran from eastern Mediterranean.

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