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*SHORT NOTES*

# Serological and molecular identification of *Tomato yellow leaf curl virus* in Khuzestan province of Iran

Shahrokh MALEKZADEH[1, 2], Kaveh BANANEJ[1] and Aisan VAHDAT[1]

[1]Plant Virus Research Department, Iranian Research Institute of Plant Protection (IRIPP), P.O. Box-19395-1454, Tehran, Iran
[2]Department of Plant Protection, College of Agriculture, Shahid Chamran University, Ahwaz, Iran

**Summary.** A survey was conducted from 2006 to 2007 to identify the causal agent of leaf curling of tomato in eight major tomato-growing areas of Khuzestan province in southwest of Iran. Tomato leaf samples showing leaf curling, yellowing, and stunting were collected and screened for the presence of *Tomato yellow leaf curl virus* (TYLCV) by TAS-ELISA. Further confirmation was completed using graft transmission onto healthy tomato plants and PCR. Results confirmed that TYLCV is a causal agent of tomato yellow leaf curl disease (TYLCD) and is widely distributed in all the major tomato growing areas in southwest of Iran. The nucleotide sequences of the coat protein (CP) gene of four isolates (Dezfoul, Shoush, Behbahan, and Ramhormoz) were determined and deposited in GenBank (EF199814-7). Phylogenetic analysis of the CP gene further showed that all four Iranian isolates have very close relationship and formed a compact cluster together with previously sequenced Iranian TYLCV isolates.

**Key words**: *Solanum lycopersicum*, polymerase chain reaction, sequencing.

## Introduction

Tomato yellow leaf curl disease (TYLCD) is one of the most important diseases of tomato (*Solanum lycopersicum* L.) worldwide (Czosnek and Laterrot, 1997). This disease was first identified in Israel in 1930 and has since the 1960s become the most important tomato viral disease in the Mediterranean region, sub-Saharan Africa, Caribbean islands, Australia, United States, and in several Asian countries like China, India, Japan, and Iran (Czosnek *et al*., 1990; Cohen and Antignus, 1994; Hajimorad *et al*., 1996; Czosnek and Laterrot, 1997; Nakhla and Maxwell, 1998; Polston *et al*., 1999; Bananej *et al*., 2009). The estimated yield losses of TYLCD in tomato fields are between 30–100%. In the Dominican Republic, yield losses due the disease were estimated to be 50 million US$ and 32,000 ha of tomato fields were damaged in Egypt (Nakhla *et al*., 1993).

Viruses associated with TYLCD are single stranded DNA viruses of the genus *Begomovirus* (family *Geminiviridae*) that severely constrain crop production and continue to emerge worldwide (Varma and Malathi, 2003; Stanley *et al*., 2005). The family *Geminiviridae* is divided into four genera (*Mastrevirus*, *Curtovirus, Topocuvirus* and *Begomovirus*), based on genome organization and biological properties (Stanley *et al*., 2005). Tomato yellow leaf curl disease is caused by several viruses belonging to different species which together are referred to as "Tomato yellow leaf curl

Corresponding author: K. Bananej
Fax: +98 21 22403691
E-mail: k_bananej@yahoo.com

viruses". Taxonomically they belong to at least six species and 15 strains of viruses (Czosnek, 2007).

In Iran, TYLCV was first reported in 1996 from central and southern provinces of Iran (Kerman, Hormozgn, Khuzestan, Bushehr, and Sistan-Baluchestan) (Hajimorad *et al*., 1996). In total in Iran, TYLCV has been reported from ten different provinces (Hajimorad *et al*. 1996; Bananej *et al*., 2009).

The main objective of the present study was to determine if TYLCV or a related begomovirus was the causal agent of tomato leaf curling, associated with TYLCD in Khuzestan province in Iranian-Iraqi border.

## Materials and methods

### Survey and sample collection

In 2006 and 2007, tomato leaf samples (n=322) showing leaf curling, yellowing, and stunting symptoms (Figure 1) were collected from 38 fields in eight major tomato-growing areas of Khuzestan province in southwest of Iran, including: 1) Behbahan, 2) Ramhormoz, 3) Dezfoul, 4) Andimeshk, 5) Shoush, 6) Shoushtar, 7) Sousangerd, and 8) Ahwaz (Figure 2). The samples were screened for the presence of TYLCV using serological, graft transmission and molecular techniques.

### Serological assays

All samples were tested for the presence of TYLCV by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) (Clark and Adams, 1977), using a commercial kit (Bioreba, AG, Switzerland) following the manufacturer's instructions. Two wells were used per sample. Samples were considered to be positive if the $A_{405}$nm values were greater than three times those of healthy control.

### Graft transmission

Representative TAS-ELISA positive samples (five samples from each region) were grafted on healthy tomato plants in 3 replications (n=120) (cv. Red-Cloud) at 4–6 leaf stage and were kept in greenhouse conditions until symptoms appeared. Evaluations were carried out visually and confirmed using TAS-ELISA, 40–45 days post infection.

Figure 1. Symptoms produced by TYLCV in a naturally infected tomato plant.

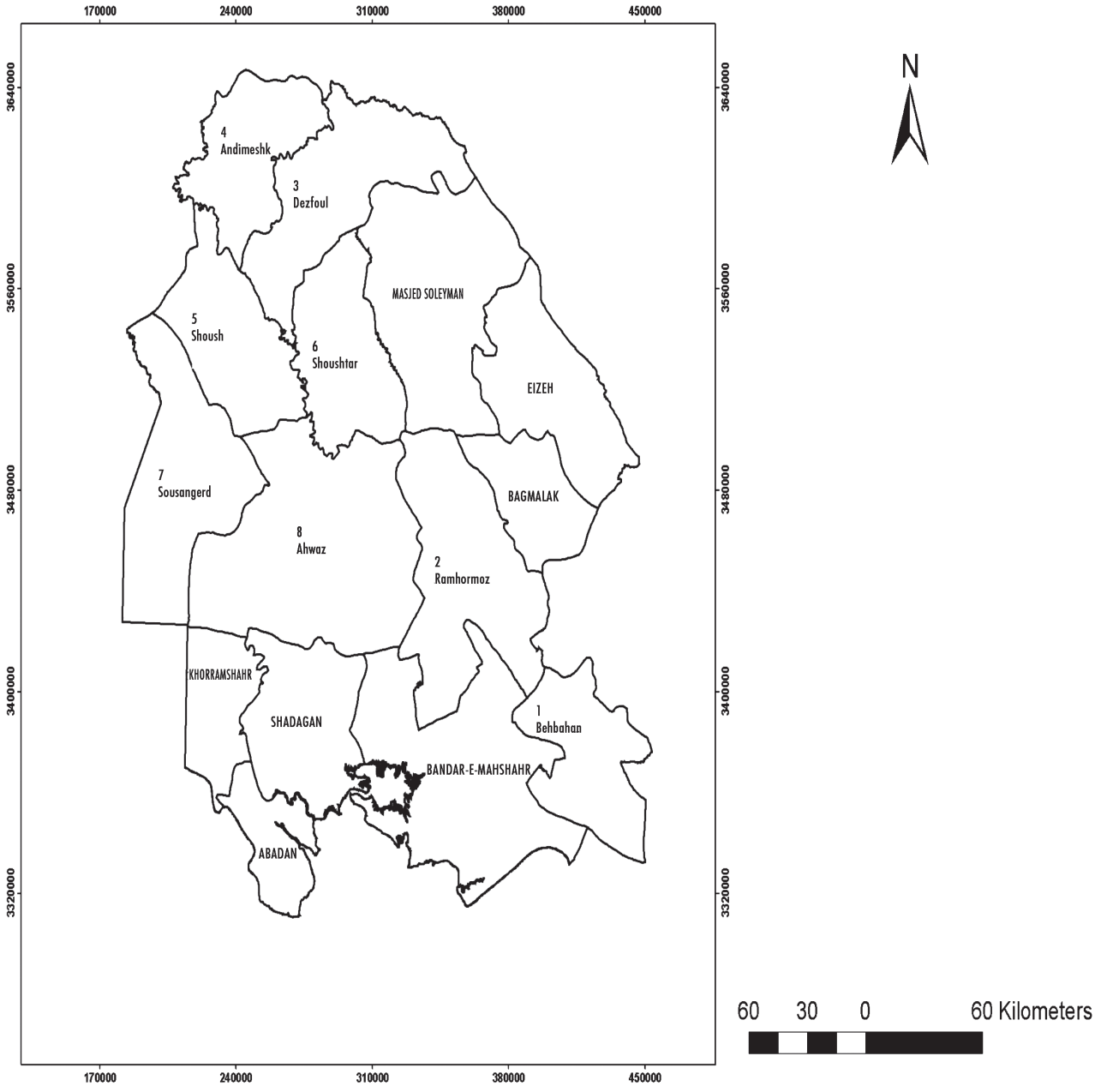


Figure 2. Distribution of TYLCV in tomato growing areas determined using TAS-ELISA and/or PCR, in the following surveyed regions of Khuzestan province: 1) Behbahan, 2) Ramhormoz, 3) Dezfoul, 4) Andimeshk, 5) Shoush, 6) Shoushtar, 7) Sousangerd and 8) Ahwaz.

**DNA extraction and PCR assays**

Total DNA was extracted from infected samples (those that reacted positively to the serological test) using the protocol described by Bendahmane *et al*. (1995). DNA pellets were resuspended in 25 µL of sterile distilled water and stored at -20°C. PCR was used to amplify fragments from the extracted DNA of samples using one set of primers: TY1(+):5'-GCCCATGTA(T/C)-CG(A/G)AAGCC-3' and TY2(-):5'-GG(A/G)TTAGA(A/G)GCATG(A/C)

GTAC-3' designed to TYLCD-associated viruses present in the Mediterranean basin, that would produce a DNA fragment approximately 580 bp of the encoding coat protein (CP) gene (Accotto *et al*., 2000). Amplification was performed in an automated thermal cycler (Eppendorf Mastercycler® 5330 [Eppendorf, Hamburg, Germany]) programmed for the following thermo-cycling conditions: 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, and a final extension for 7 min at 72°C. Aliquots of the PCR products were analyzed by electrophoresis on agarose gel (1%) in TBE buffer (90 mM Tris borate, 2 mM EDTA) at 80 V for 60 min. The amplified DNA fragments were stained with ethidum bromide (0.5 μg $mL^{-1}$), visualized and photographed using the Gel DOC IMAGO System (B&L Systems, the Netherlands). The size of fragments was determined by comparison with GeneRuler 1 kb DNA Ladder (Fermentas, Tehran, Iran).

**RFLP of a CP gene fragment**

Larger PCR reactions (50 μL) were performed and then purified using Wizard PCR Preps DNA Purification System, according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). The purified PCR products were digested with the restriction enzyme *Ava* II (Accotto *et al*., 2000), according to manufacturer's instruction (Fermentas). Digested DNA (5 μL) was finally analyzed on 3% agarose gel (Sigma Co., St. Louis, MO, USA).

**Sequencing of amplified PCR products**

PCR products (CP) were purified using the Wizard PCR Preps DNA Purification System, according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA) and the two strands were directly sequenced at MWG-Biotech AG (Ebersberg, Munich, Germany). Complete and partial sequences of TYLCV used for comparison were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) including sequences previously found in Iran. Sequence analyses and comparisons were performed using the ClustalX (Thompson *et al*., 1997), GeneDoc version 2.6.002 (http://www.psc.edu/biomed/genedoc/). The phylogenetic tree was constructed using the neighbor joining method implemented in ClustalX program. Phylogenetic trees were drawn by tree view program version 1.6.6 (http://taxonomy. zoology.gla.ac.uk/rod/rod.html) and subjected to bootstrap (using 1000 replicates) options.

## Results

**Virus survey**

TAS-ELISA results revealed that 124 out of 322 (39%) symptomatic tomato samples obtained from eight different regions of Khuzestan province were infected with TYLCV (Table 1). Grafted tissue from TAS-ELISA positives developed yel-

Table 1. Geographical distribution of TYLCV in the eight major tomato growing areas of Khuzestan province, Iran from a field survey in 2006–7.

| Area | Symptom[a] | No. of fields surveyed | No. of infected samples/No. of tested samples | Percentage infection (%) |
|---|---|---|---|---|
| Ahwaz | M,VC,VY | 4 | 12/30 | 40 |
| Sousangerd | Y | 2 | 7/16 | 44 |
| Ramhormoz | LC,Y,LR,VY | 1 | 15/26 | 58 |
| Behbahan | M,Y,LR,LC | 6 | 27/72 | 38 |
| Andimeshk | M,Y,VC | 6 | 11/42 | 26 |
| Dezfoul | LR,LC,VY | 7 | 12/50 | 24 |
| Shoush | M,Y,LR,LC | 4 | 21/46 | 46 |
| Shoushtar | LR,LC,Y,M | 8 | 19/40 | 48 |
| Total | - | 38 | 124/322 | 39 |

[a] LC, leaf curling; LR, leaf rolling; M, mosaic; Y, yellowing; VY, vein yellowing; VC, vein clearing.

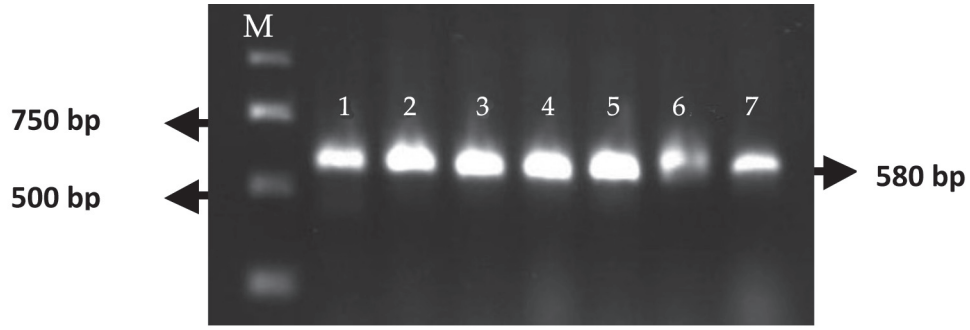


Figure 3. 1% Agarose gel electrophoresis of PCR amplification of the coat protein, using TY1 (+) and TY2 (-) primers described by Accotto *et al*., (2000), from 7 TYLCV isolates in tomato. 1) Ahwaz; 2) Behbahan; 3) Ramhormoz; 4) Dezfoul; 5) Shoush; 6) Shoushtar; and 7) Andimeshk. M, 1Kb Ladder DNA marker (Fermentas).

low leaf curl symptoms in 54 out of 120 of grafted tomato plants, after 40–45 days. Infection by TYLCV was further confirmed in 46 out of 54 grafted symptomatic tomato samples using TAS-ELISA

**PCR and CP sequence comparisons**

Using TY1 (+) and TY2 (-) primers, a band corresponding to the expected PCR product size (~580 bp) (Accotto *et al*., 2000) was amplified in the seven positive TAS-ELISA samples taken from all surveyed regions (Figure 3).

Following digestion of PCR amplified fragments (580 bp) with *Ava* II restriction enzyme, all samples from Shoush, Ramhormoz, Behbahan, Dezfoul, and Shoushtar; produced the Is-pattern (fragments of 302 and 277 bp) (Figure 4), like those described by Accotto *et al*., (2000). The nucleotide sequences of the CP gene of four TYLCV isolates

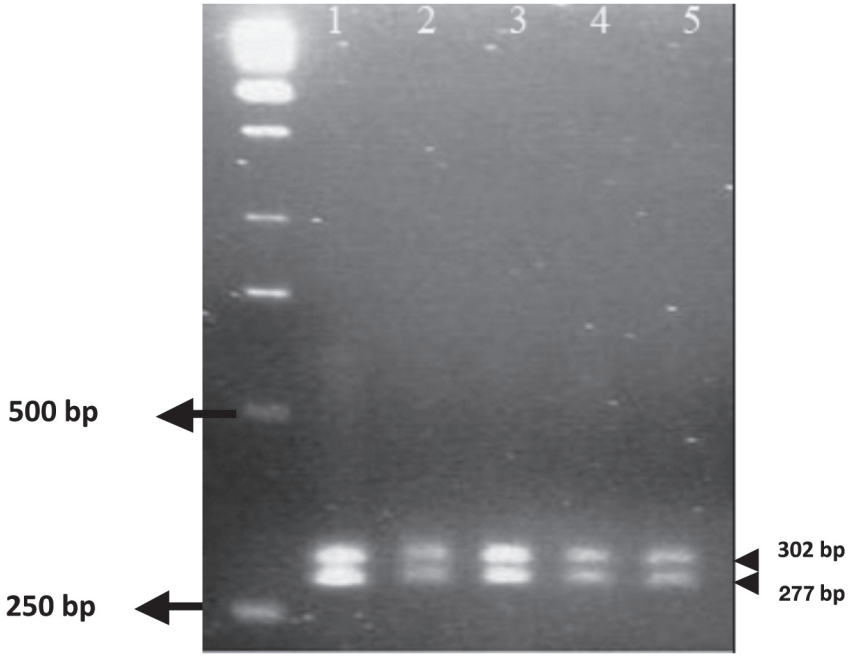


Figure 4. 3% Agarose gel electrophoresis of DNA amplicons obtained from TYLCV infected tomato samples using primers described by Accotto *et al*., (2000), and digested with *Ava* II restriction enzyme: 1) Shoush, 2) Ramhormoz, 3) Behbahan, 4) Dezfoul, and 5) Shoushtar. M, 1Kb Ladder DNA marker (Fermentas).

(Dezfoul, Shoush, Behbahan, and Ramhormoz) were determined and deposited in GenBank (Acc. No. EF199814-7).

Results of the alignment analyses of the CP of four TYLCV isolates collected in this study shared a high degree of nucleotide identity (>96%) with each others and 96–98% identity with eight Iranian TYLCV isolates (Bananej *et al*., 2009). Alignment analyses showed that of the CP indicate that TYLCV isolates in this study shared a high degree of nucleotide identity (98–100%) and close phylogenetic relationships with TYLCV-IR[IR:Ira:98] (AJ132711), TYLCV-IL[IL:Reo:86](X15656) and TYLCV-Mld[IL;93](X76319) (Bananej *et al*., 2009). However, they showed lower identity (67%) to Iranian isolate ToLCIRV-[IR:Ira](AY297924) (Behjatnia *et al*., 2004). Maximum nucleotide sequence similarity was observed between Iranian isolates and TYLCV-MId[IL;93](X76319), with 97–99% identity at nucleotide level.

Sequence similarity of Iranian TYLCV isolates and four TYLCV isolates from India: ToLCNDV-[Luc] (Y16421), ToLCNDV-Mid (U15016), ToLCKV-[IN:Ban:93](U38239), and ToLCBV-[Ban4] (AF165098) were 69–71%. The Iranian isolates represented in this study also showed 79 and 74% similarity at the nucleotide level with TYLCSV-IT[IT:Sar:88] and ToLCV-[AU], from Italy and Australia, respectively.

## Discussion

Whitefly-transmitted geminiviruses are the most important constraint to the production of vegetable crops in many countries in the world. According to Jones (2003), 114 virus species are transmitted by whiteflies (family *Aleyrodidae*), and 90% of these viruses belong to the *Begomovirus* genus. Tomato yellow leaf curl virus, the causal agent of TYLCD, is one of the most important factors limiting tomato production in many Mediterranean countries.

TAS-ELISA, grafting on healthy tomato, and PCR results confirmed that TYLCV was a causal agent of TYLCD in 39% of symptomatic plants from tomato fields of eight surveyed regions in Khuzestan province. Our results also showed that 61% symptomatic tomato samples were negative to TYLCV infection. These results may indicate further infection from different virus species. A further investigation to identify the causal agents of these symptoms is planned.

Tomato yellow leaf curl virus had previously been reported from only one area in Khuzestan province (Behbahan) in 1996 by Hajimorad *et al*. (1996). To our knowledge, our results are the first report of occurrence of TYLCV in different regions of Khuzestan province.

During this survey, relatively homogenous populations of TYLCV isolates, similar to previously partially or fully sequenced Iranian isolate AJ132711 (Bananej *et al*., 2009; Bananej et al., 2004, respectively), were detected. This is in agreement with a previous study of TYLCV variability in Iran (Bananej *et al*., 2009), showing (except in one case) that all isolates should be classified in one group based on their CP sequences.

Results obtained from this study indicate that TYLCV has now spread into new regions. This spread might be due to poor understanding by the farmers about the etiology of the disease, ineffective control measures against the whitefly vector and poor crop cultural practices. For example: growers could minimize cropping overlap, thus reducing sources of TYLCV, grow seedlings in insect-proof greenhouses, inter-plant tomatoes with whitefly 'bait' plants such as cucumber (Bananej *et al*., 2003), restrict seedling movement between provinces or introduce health certification prior to transfer between provinces. Besides these practical measures, efforts are required either to develop TYLCV-resistant varieties via classical breeding or through genetic engineering to improve crop management.

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