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Molecular evidence of *Tomato yellow leaf curl virus*-Sicily spreading on tomato, pepper and bean in Tunisia

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Summary. Unusual symptoms including yellowing, stunting, curling, crumpling and plant size reduction were observed in tomato fields and green houses in Tunisia. These symptoms, generally associated with the *Tomato yellow leaf curl virus* (TYLCV) complex, have become increasingly common in recent years. In order to ascertain the molecular characteristics of Tunisian isolates by PCR, both the coat protein gene and the intergenic region of eleven isolates were amplified using specific primers, and sequenced. The PCR procedure also allowed the amplification of viral DNA fragments using a bean total DNA as a template. Phylogenetic analysis suggested that these Tunisian isolates clustered with a Sicilian isolate of TYLCSV-Sic. This is the first report of the involvement of this viral species in *Phaseolus vulgaris* disease.

Key words: TYLCSV, surveys, CP gene, IR region, sequence variability.

Introduction

Tomato yellow leaf curl virus (TYLCV), is the causal agent of severe epidemics reducing tomato yields from fields and green houses in many tropical, subtropical and temperate regions of the world (Markham *et al*., 1994; Czosnek and Laterrot, 1997). TYLVC belongs to the *Geminivirus* species in the genus Begomovirus (Briddon and Markham, 1995) a group of small plant viruses with a circular single-stranded DNA genome encapsidated in twinned isometric particles (Harrisson, 1985). These viruses are transmitted by whitefly insects and infect a large variety of crop plants throughout the world. Molecular data indicate that TYLCV has identical monopartite genome organization with two transcription units containing six open reading frames (ORFs). The two virion sense ORFs (V1 coding for the pre-coat and V2 for the coat protein) and the four complementary sense ORFs (C1 coding for the Rep protein, C2, C3 and C4) are interspaced by the intergenic region including regulatory elements for the replication and transcription of the viral genome (Kheyr-Pour *et al*., 1991; Antignus and Cohen, 1994; Crespi *et al.,* 1995).

Tomato yellow leaf curl (TYLC) disease is associated with TYLCV–Sardinia (TYLCSV-Sar) and

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TYLCV-Israel (TYLCV-Is) species that coincide with the western and eastern Mediterranean clusters respectively (Fauquet and Mayo, 1999). Although TYLCV caused severe tomato losses in many Mediterranean countries, its presence in Tunisia was not confirmed until recently. Early diagnosis of TYLCV was made on the basis of unususal symptoms such as leaf yellowing, and curling, and plant size reduction in tomato crops. Molecular analysis assessed by hybridization and PCR-RFLP (*Ava* II) of the coat protein gene were the tools of choice to identify TYLC disease and to cluster the Tunisian isolates with the Sardinian viral species (Fekih-Hassan *et al*., 2003). Since sequence analysis of parts of the viral genome makes it possible to identify and discriminate between different geminiviruses and between strains of the same virus (Rybicki, 1994; Padidam *et al*., 1995), it seemed interesting to determine the nucleotide sequence of the Tunisian TYLCV isolates and to establish their relationship with other TYLCV strains in Mediterranean countries. To do this, we used the PCR technique coupled with sequence analysis to characterize both the coat protein gene and the intergenic region of the Tunisian TYLCV genome. Here we present an updated report on the spread of TYLCV in Tunisian crops with the molecular evidence that Tunisian isolates are members of the TYLCSV-Sicilian (Sic) group. In addition, this is the first report of the natural occurence of TYLCSV-Sic on bean.

Materials and methods

Sample collection

Surveys were undertaken in the main Tunisian tomato growing areas in southern Tunisia, the Sahel (central eastern), central Tunisia, Cap-Bon (north-eastern) and northern Tunisia in 2001 and 2002. Approximately, 60 samples with either TYLC-like or virus-like symptoms were harvested. At each site, TYLCV occurrence was also looked for by selecting symptomatic leaves from pepper coexisting with tomato cultivars, as well as from bean. All samples belonged to local Tunisian varieties. Plant material was stored at -80°C until use. A tomato sample infected with an Egyptian isolate belonging to the Israeli viral species was used as a control (Nakhla *et al*., 1993).

DNA extraction

Total DNA was extracted from leaf samples as described by Dellaporta *et al*., (1983) with a few modifications. Five mg of leaf tissue was ground with 1 ml of extraction buffer (50 mM EDTA, 100 mM Tris-HCl, 500 mM NaCl, 10 mM β-mercaptoethanol), vortexed and allowed to stand at 65°C for 10 min. After adding potassium acetate (5 M, pH 8), the mixture was incubated on ice for 10 min and clarified by centrifugation at 16,000 *g* for 20 min at 4°C. An equal volume of isopropanol was added to the supernatant, and the solution was incubated for 10 min at -20°C and centrifuged for 10 min at 16,000 *g*. The pellet was resuspended in sterile water and subjected to RNAse extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). After precipitation of the supernatant with three volumes of absolute alcohol for 30 min at -20°C and centrifugation for 10 min at 16,000 *g*, DNA was washed with 70% ethanol and resuspended in sterile water.

PCR amplification

Degenerate primers amplifying the coat protein gene were designed by analysing sequence data of several strains of TYLCV so that the primers (CPv and CPc) annealed to conserved nucleotide sequence within both the Israeli and the Sardinian genome types.

The sense CPv primer sequence was: 5'-ACGCCCG(T/C)CTCGAAGGTTCG-3' and the complementary CPc primer sequence: 5'-GTACA(T/A)GCCATATACAATAACAAGGC-3'.

Two species-specific sets of primers were also designed for amplification of the intergenic region (IR), one for the Sardinian, and one for the Israeli viral species (Nakhla *et al*., 1993). The first set included the sense M14 primer: 5'-TGGATTTATTTGAAACG-3' and the complementary M15 primer: 5'-AAAGGATCCCACATATTG-3'. The second set included the sense IRv 21 primer: 5'- GTTGAAATGAATCGGTGTCCC-3' and the complementary IRc 287 primer: 5'- TTGCAAGACAAAAAACTTGGGACC-3'.

PCR amplification was carried out with 150 ng of each sample extract DNA. The presumed viral DNA was amplified using the following conditions: 3 min of denaturation at 95°C followed by 35 cycles at 95°C for 50 s, 55°C for 50 s and 72°C for 1 min with a final extension step of 72°C for 10 min.

Amplified fragments were subjected to electrophoresis in 1% agarose gel and stained with 0.5 μg ml^{-1} ethidium bromide.

Sequencing

For each positive sample, three PCR amplicons of the considered region were column purified using the Qiaquick kit (Qiagen, Madison, WI, USA) and sequenced on both strands using PCR primers and the involving "ready reaction big dye terminator cycle sequencing" kit.

The nucleotide sequence of the coat protein fragment and the intergenic regions of eleven isolates were fully aligned, compared with each other and with sequences of known isolates from gene bank databases: TYLCSV-Sic (Z28390), TYLCSV-Sar (X61153), TYLCV (X76319, X15656). Data were used to draw phylogenetic trees using the DNAMAN software package program (Lynnon BioSoft, Vaudreuil, Quebec, Canada).

Results

TYLCV detection

A novel TYLC like-disease was observed in Tunisian tomato open fields and plastic houses. Based upon symptom observation, TYLC incidence was up to 60% in some fields in the Sahel. In the 2001–2002 growing seasons, tomato samples were collected in fields and greenhouses from plants showing leaf curling, yellowing and necrosis. Infected plants always presented fruit malformation and size reduction. In addition, some samples were also collected in neighbouring fields from pepper and bean plants exhibiting yellowing, crumpling, mosaic and/or mottling. TYLCV was thought to be involved in causing these symptoms so that all these samples were subjected to PCR, which is commonly used for TYLCV disease diagnosis. For the PCR, a Dellaporta DNA extraction was performed on each plant sample and 150 ng of the DNA obtained was subjected to coat protein gene PCR amplification using broad-spectrum degenerate primers. Of the 60 samples tested in this way, 11 showed a single DNA fragment of the expected size (673 bp) revealing TYLCV. An analougous viral amplicon was obtained with the Egyptian isolate used as a positive control and as a source of TYLCV-Is (Fig. 1). No fragment was detected when using healthy leaf extract. Among the positive samples, two were from pepper, eight from tomato and one from bean. All positive samples were geographically clustered in the Sahel and Southern Tunisia, while PCR assays for samples from central and northern Tunisia and Cap-Bon were negative (Table 1). Any symptoms in the older symtomatic samples were probably caused by other viruses, or by unfavourable growth and climate conditions.

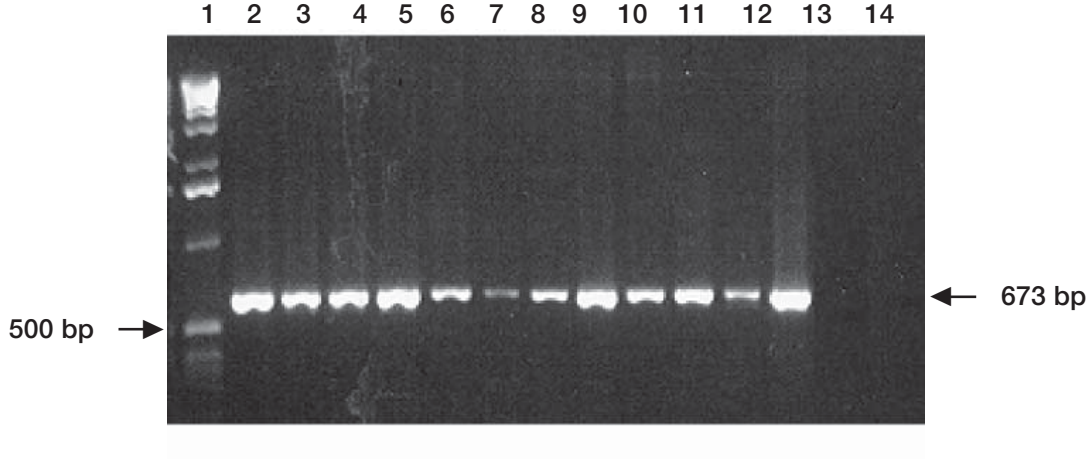

Fig. 1. Agarose gel (1%) electrophoresis of CP DNA amplicons obtained from tomato samples using CPc and CPv primers. Sources of nucleic acids are as follows: lane 1, ladder (1 kb [BRL]); lane 2, coat protein gene of the Egyptian isolate; lanes 3 to 13 correspond to TYLCV-Bn/Bz/Cb/Ch/Dk/Ge/Ha/Mk/Pim1/Pim2 and TYLC-Tb amplified CP region; lane 14, healthy control.

Table 1. Origin, field symptoms and TYLCV content of collected samples.

Area	Host	Symptoms	No. of samples	TYLCV content and strain code
North	Tomato	Curling	2	-
Cap-Bon (North-East)	Tomato	Curling	5	-
	Tomato	Curling and necrosis	6	-
	Pepper	Curling	1	-
	Pepper	Mosaic	1	-
	Pepper	Yellowing	1	-
	Bean	Mosaic and yellowing	1	-
Center	Tomato	Necrosis and yellowing	1	-
	Tomato	Curling	2	-
	Tomato	Yellowing	4	-
	Pepper	Curling	1	-
	Bean	Mosaic	1	-
Sahel	Tomato	Yellow curling	17	+ (Tb, Ge, Bn, Cb, Bz, Dk, Mk)
	Tomato	Mosaic and necrosis	7	-
	Pepper	Mosaic	1	+ (Pim2)
	Pepper	Necrosis and yellowing	1	-
	Pepper	Mottling	1	+ (Pim1)
	Bean	Yellowing, crumpling	2	+ (Ha)
South	Tomato	Curling	3	-
	Tomato	Yellow curling	1	+ (Ch)
	Tomato	Mosaic	1	-

For TYLCV detection, a species-specific set of primers was used that exclusively targeted the intergenic region of members of the TYLCSV-Sar group (by the amplification of an approximatively 348 bp viral DNA fragment from the previously selected positive samples). No viral amplicon was detected with using the primer set flanking the intergenic region of the TYLCV-Is viral group except with the Egyptian isolate (Fig. 2).

Sequence comparison of the coat protein gene region

Each analysed sequence included 553 nucleotides (Fig. 3). Despite random nucleotide changes overlapping this region, the Tunisian sequences were remarkably similar to each other, with more than 91% identity. Pepper isolate Pim2 showed the most variable sequence (91% identity) while the other pepper isolate, Pim1, had a more conserved sequence (98%). Analysis failed to reveal any correlations between isolates variability patterns, area of origin or host plant. The sequences of these isolates and those from other parts of the world were used to construct a phylogenetic tree (Fig. 4). Results revealed a specific clustering of Tunisian, Sicilian and Sardinian isolates, which shared more than 91% sequence identity. Conversely, identity was only 79% with the severe and mild Israeli isolates, which constituted a separate viral group.

Sequence comparison of the intergenic region

Nucleotide sequence analysis of the Tunisian isolates showed features characteristic of *Begomoviruses*, including the perfect conservation of the 5'TAATATTC3' sequence with the T/AC cleavage site at the point where rolling circle replication is initiated (Heyraud-Nitschke *et al*., 1995). We noticed several point mutations situated mainly in the first half of this region (Fig. 5). In order to investigate sequence variability in the intergenic

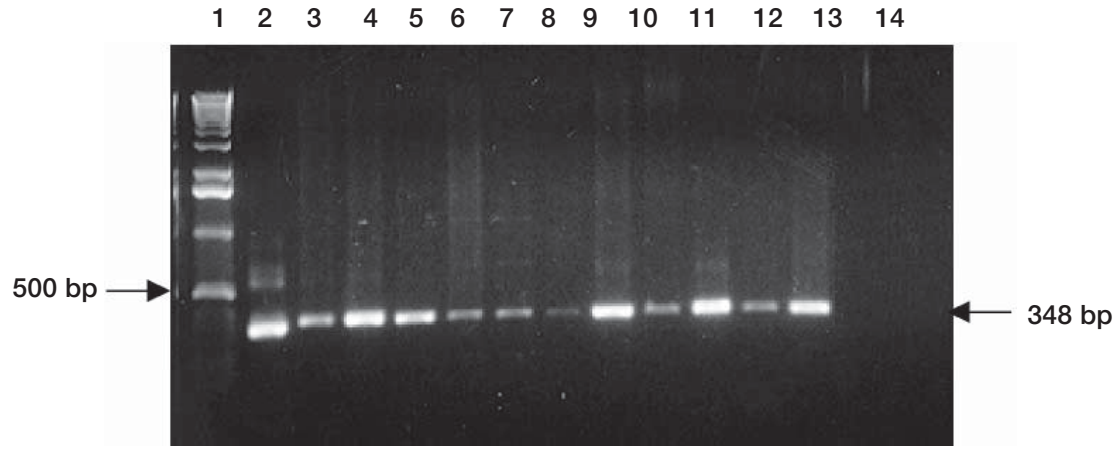

Fig. 2. Agarose gel (1.5%) electrophoresis of IR DNA amplicons obtained from tomato samples. Lane 1, 1 kb ladder (BRL); lane 2, intergenic region of the Egyptian isolate amplified using IRv 21 and IRc 287 primers; lanes 3 to 13 correspond, respectively, to TYLCV-Bn/Bz/Cb/Ch/Dk/Ge/Ha/Mk/Pim1/Pim2 and TYLC-Tb amplified IR region using M14-M15 primers; lane 14, healthy control.

region, this region was divided into two fragments and compared with the corresponding regions of known isolates. The first region (IR/1) encompassing the left hand side of the stem loop structure, corresponded to the end part of the genome. The second, IR/2, started with the nick-site and corresponded to the begining of the viral genome.

Phylogenetic trees (Fig. 6a and 6b) based on either the IR/1 or the IR/2 sequence, provided further support for clustering Tunisian isolates with the a TYLCSV-Sic group, while the Israeli isolates belonged to another, clearly distinct group. The Tunisian IR/2 sequences are thus highly homologous with the Sicilian sequence, with which it sometimes shares a 99% sequence homology (Mk isolate). They are slightly different from the Sardinian group (93% homology) and distantly related to the Israeli group (67%). The pepper isolate Pim2 had a sequence perfectly identical to the Bn, Dk, Ch, Bz, and Tb tomato isolates. The IR/1 region did not follow a similar pattern. Here the deduced phylogenetic tree showed three main branches. The first comprised the Tunisian and Sicilian isolates with highly homologous sequences (more than 95% identity) and with the isolates from tomato (Tb and Ge) and bean (Ha) having identical sequences. The second branch included the mild and severe Israeli isolates, with sharing 73% of sequence homology, and the last branch was the Sardinian TYLCV. The isolate sequences of the first branch were distantly separated from those of the two branches consisting of the Israeli and Sardinian TYLCV, with 59 and 73% of sequence homology respectively.

Discussion

By the end of the nineties, Tunisian tomato cultures seemed to be extensively affected by TYLCV. This paper is the first study of the extent of this TYLC-like disease in Tunisia based on symptom observation, as a first step in viral diagnosis, then on PCR identification and sequence analysis. The epidemiological study detected TYLC in Southern Tunisia and the Sahel, but not in northern or central Tunisia or Cap-Bon. The absence of TYLC in such closely related geographical areas may be explained by the occurrence of a local biotype of *Bemisia tabaci* in those areas that poorly vectors this virus. As no information is available about the genetic variability of whiteflies in Tunisia, this suggestion must await further research to discover whether a specific relationship exists between certain whitefly biotypes and TYLC. The relatively cold winters in northern Tunisia and the absence of extensive bean cultivation could also be a reason for the absence of massive infestations of *B. tabaci*. TYLCV in tomato crops is a matter for con-

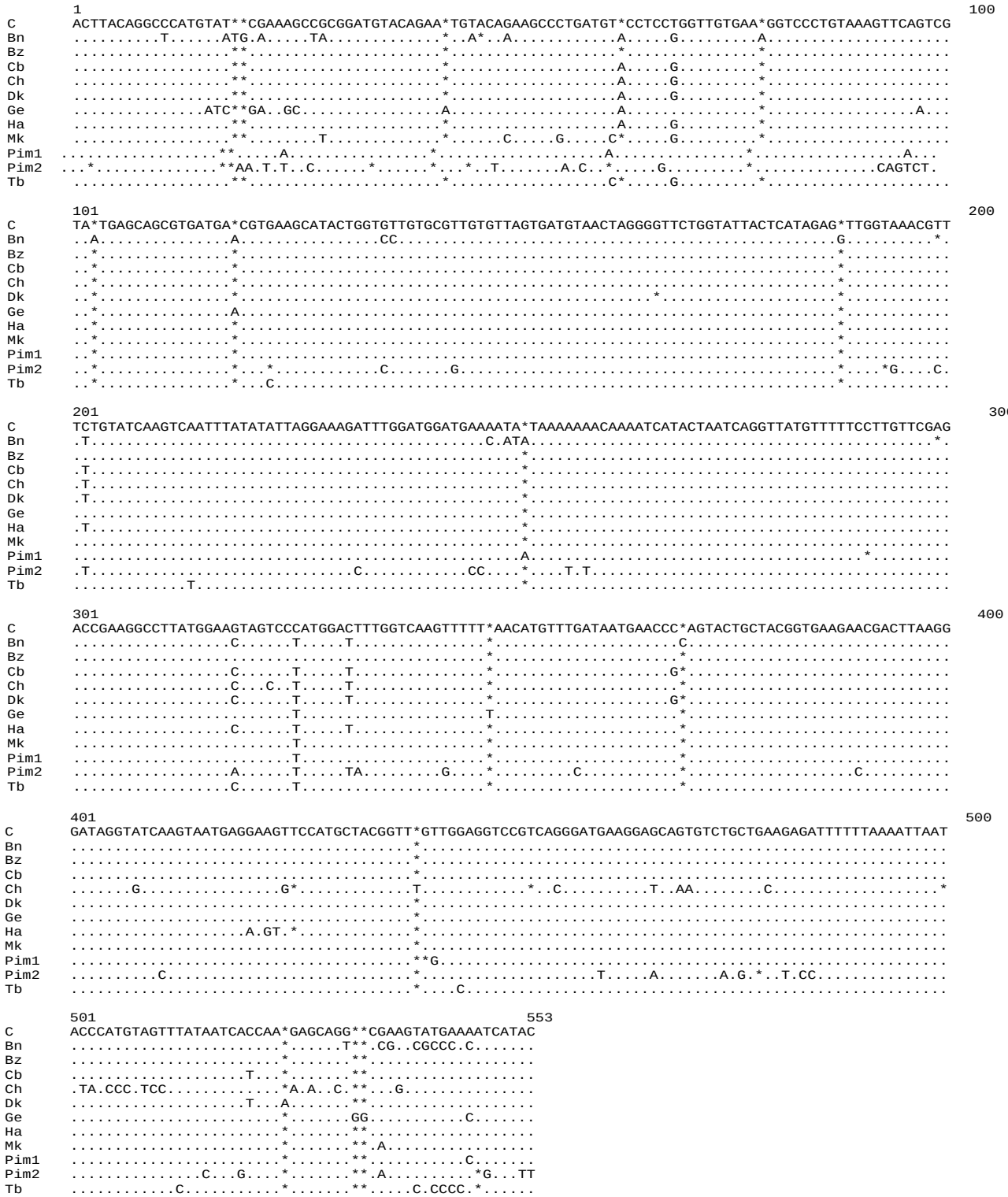

Fig. 3. Multiple alignment of CP nucleotide sequences of Tunisian TYLCV isolates. C indicates consensus sequence. Asterisks show deletions. Dots indicate nucleotides identical to the consensus.

cern since this virus consistently causes more severe symptoms than the previously existing potyviruses or cucumoviruses. Previous studies based either on ultrastructural changes in infected tomato cells (Cherif and Russo, 1983) or using a wide broad spectrum probe (Czosnek *et al*., 1997) suggested that TYLCV occurred in Tunisia, but none of these studies investigated the molecular or biological characteristics of the Tunisian isolates of TYLCV. Recently TYLCV tomato isolates were detected and typed with molecular means using dot-blot hybridization and PCR-RFLP of the CP gene (Fekih-Hassan *et al*., 2003). In that study to characterize the Tunisian isolates of TYLCV genetically, we conducted a molecular investigation based on PCR amplification of the coat protein gene and the intergenic region followed by sequencing of both regions. Using these two highly specific components of the viral genome, we found a consistent sequence-based clustering of TYLCV isolates. Sequence comparison of parts of the geminivirus genome has established critera to discriminate between isolates from different strains, and isolates belonging to the same strain have more than 90% sequence identity. The sequence identity of the CP gene of viruses from different geographical areas ranges from 65 to 80%, while isolates of the same strain spreading in the same region have a homology greater than 80%. This is because IR isolates of the same strain have more than 90% sequence homology wheras different geminiviruses have nucleotide identities of less than 85% for the same region (Rybicki, 1994, 1998; Padidam *et al*., 1995).

Within the viral CP gene region, sequences were fairly well conserved among Tunisian iso-

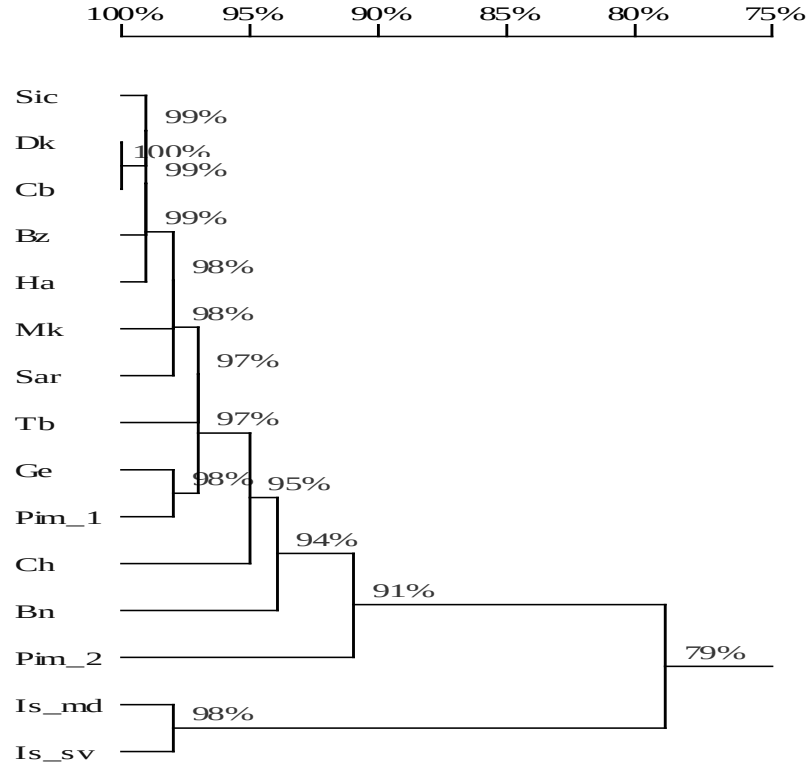

Fig. 4. Phylogenetic tree showing relationships among coat protein genes of Tunisian isolates and other known TYLCV strains. Numbers above the branches indicate percentage of homology. Horizontal branches are proportional to sequence homologies.

lates, suggesting that they were the same viral entity regardless of the host plant. On the other hand, these same isolates also had more than 91% homology with viruses from Italy (Sardinia and Sicily) indicating that all these isolates have a common origin. Comparison of the viral intergenic region revealed a more stringent clustering of Tunisian isolates into the TYCSV-Sic group (more than 95% identity). The Israeli strains formed a separate group (less than 67% of sequence identity), and the relative position of the TYLCSV-Sar isolate varied when different parts of the IR region were used as the basis of analysis. The end part of TYLCSV-Sar genome was homologous to the Tunisian isolates (93% identity) whereas its begining showed more differences (73% identity). This result is not surprising since the IR is the part of the genome that shows the greatest amount of variation (Harrison and Robinson, 1999). In several cases, it seems that, apart from the conserved nonanucleotide sequence, different parts of this region evolve independently. Thus, that bean and pepper isolates display sequences which strongly resembled those of tomato isolates with no significant nucleotide changes reflecting any biological properties associated with the original host.

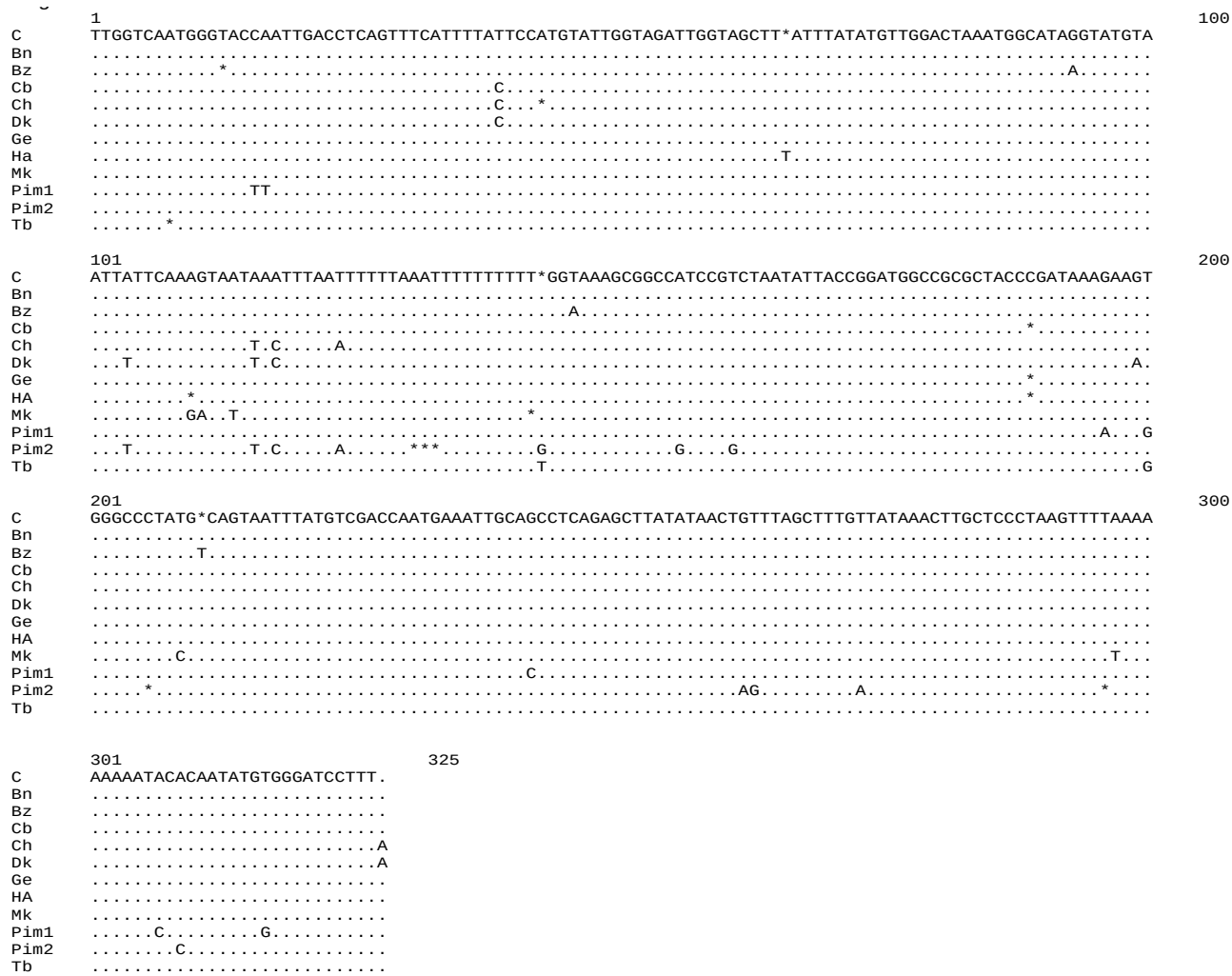

Fig. 5. Multiple alignment of IR nucleotide sequences of Tunisian TYLCV isolates. C indicates consensus sequence. Asterisks show deletions. Dots indicate nucleotides identical to the consensus. The conserved nonanucleotide motif is boxed.

The sequence data suggest that the TYLCSV-Sic isolate is spreading in Tunisia, but TYLCV-Is was not found in single infections or in association with other viral species. Such findings indicate that the geographical separation prevents the occurrence of this viral species in Tunisia, even though it has been reported in other Mediterranean countries such as Portugal, Spain and Italy (Lourou *et al*., 1996; Navas-Castillo *et al*., 1997; Accotto *et al*., 2003). Of a special interest is the fact that the Sicilian isolate was found on pepper and bean since until now only the Israeli species was thought to infect bean. In reality TYLCV-Is is associated with severe epidemics in tomato, and particularly with a new disease in common bean (Navas-Castillo *et al*., 1997, 1999). The study shows, for the first time, that bean can also host a TYLCSV-Sic isolate. Bean is known to contribute to a high level of TYLCV infection. The need to counter TYLCV by breeding of TYLCV-resistant tomatoes is clearly becoming more urgent.

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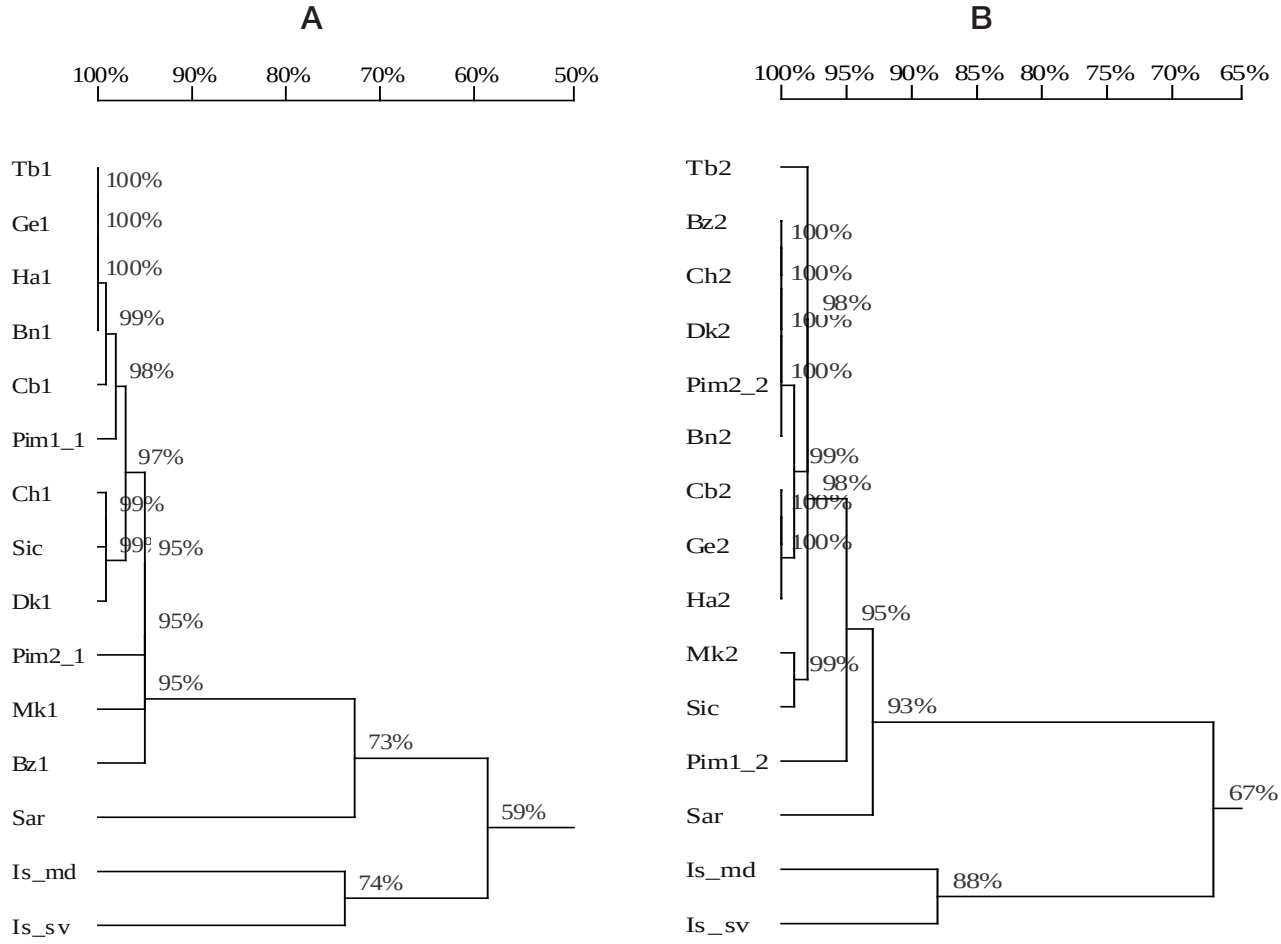

Fig. 6. Phylogenetic tree showing relationships among intergenic regions of Tunisian isolates and other known TYLCV strains. Numbers above the branches indicate percentage of homology. Horizontal branches are proportional to sequence homologies. 6a, sequence comparison of IR1 region (the end part of the genome); 6b, sequence comparison of IR2 region (the starting of the genome)

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