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Molecular characterization of a Moroccan isolate of *Tomato yellow leaf curl Sardinia virus* and differentiation of the *Tomato yellow leaf curl virus* complex by the polymerase chain reaction

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Summary. The polymerase chain reaction (PCR) was used to identify an isolate of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) from southwestern Morocco and to detect the members of the *Tomato yellow leaf curl virus* (TYLCV) complex. Thirty-five tomato samples with typical TYLCV symptoms were collected from infected tomato fields in the Souss-Massa region. PCR was performed with a general primer pair based on the coat protein (*Cp*) gene of the TYLCV complex, as well as with specific primer pairs for TYLCV and TYLCSV. Of the 35 samples tested, 29 generated a viral DNA product with the general primer pair, 29 samples gave a viral DNA product with the TYLCV-specific primers, and of these, 9 also gave a product with the TYLCSV primer pair; 6 samples did not give any PCR product with either primer pair. The full-length genome of TYLCSV was amplified with overlapping primers at the unique *Nco*I site in the TYLCSV genome (GenBank accession number X61153). The full-length genome of the TYLCSV isolate from Morocco is 2,777 nucleotides long (accession number AY702650) and is almost identical (97% nucleotide identity) to a TYLCSV isolate from Murcia, Spain (accession number Z25751). A PCR-based diagnostic method was developed to distinguish between TYLCV and TYLCSV in Morocco. To diagnose the TYLCV/TYLCSV complex a general primer pair was designed that anneals to a conserved region of the *Cp* gene. To diagnose TYLCSV exclusively, two primer pairs were designed to anneal specifically to the replication-associated protein gene (*Rep*) of TYLCSV from Morocco. To detect TYLCV exclusively, a primer pair previously described to amplify the intergenic region (IR) of TYLCV was used. The PCR primers were tested for their effectiveness using DNA clones of the TYLCSV from Morocco and of the TYLCV from the Dominican Republic. PCR using these primers offers a rapid means to detect the TYLCV complex and to distinguish between the two TYLCV species present in Morocco.

Key words: geminivirus, begomovirus, TYLCV, PCR, detection.

Introduction

The *Tomato yellow leaf curl virus* (TYLCV) is a geminivirus that seriously limits tomato production in Morocco (Petershmitt *et al*., 1999; Monci *et al*., 2000; Jebbour and Abaha, 2002; Sedegui *et al*., 2002) and throughout the Mediterranean Basin (Czosnek and Laterrot, 1997; Fauquet *et al*., 2005). The TYLCV complex belongs to the genus *Begomovirus* (Fauquet and Mayo, 1999). This virus infects many economically important agricultural crops as well as ornamentals and weeds. TYLCV is transmitted in a circulative and persistent manner by the whitefly *Bemisia tabaci* (Gennadius)

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(Bedford *et al*., 1994; Cohen and Antignus 1994; Ghanim *et al*., 2001). Disease symptoms in tomato consist of a prominent upward curling of leaflet margins, a reduction of leaflet area, a yellowing of young leaves, together with stunting and flower abortion. Infection results in a dramatic decrease of plant growth and reduces yields up to 100% if infection occurs during early growth (Moriones and Navas-Castillo, 2000).

All members of the TYLCV complex have a single genomic component consisting of covalently closed circular single-stranded DNA of approximately 2,800 nucleotides. The TYLCV genome encodes six partially overlapping open reading frames (ORFs), two on the viron-sense strand (V2 and CP), and four on the virion-sense complementary strand (Rep, REn, TrAP and C4), separated by an intergenic region (IR) of approximately 300 nucleotides (Kheyr-Pour *et al*., 1991, Navot *et al*., 1991).

Several species and strains of the TYLCV complex are known; they include TYLCV (formerly TYLCV-Israel) (X15656, Navot *et al*., 1991), TYLCV-Mild (X76319, Antignus and Cohen, 1994), *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (X61153, Kheyr-Pour *et al*., 1991), and TYLCMalV from Malaga, Spain (AF271234, Monci *et al*., 2002), as well as isolates of TYLCSV from Sicily, Italy (Z28390, Crespi *et al*., ,1995), from Murcia, Spain (Z25751, Noris *et al*., 1994), and from Almeria, Spain (L27708, Reina *et al*., 1994).

In Morocco one isolate of TYLCV was partially identified in 1999 in tomato samples from the coastal region near Casablanca (Petershmitt *et al*., 1999). This isolate was closely related to the TYLCV isolate from the Dominican Republic (TYLCV-[DO], accession number AF024715). The presence of both TYLCV and TYLCSV was subsequently reported in the Souss-Massa region but they were not fully characterized in molecular terms (Monci *et al*., 2000, Sedegui *et al*., 2002).

Effective management of TYLCV epidemics depends on the rapid and accurate detection and identification of the causal agent. Thus, the present study was undertaken to identify the members of the TYLCV/TYLCSV complex present in tomato fields of the Souss-Massa region. Since it was not known whether TYLCV from Morocco is a variant of TYLCV, of TYLCSV, or a new recombinant, the virus present in the field was cloned and sequenced. The information derived from this study was used to design specific PCR primers in order to identify the members of the TYLCV/TYLCSV complex present in tomato fields in Morocco.

Materials and methods

DNA extraction

Total DNA was extracted from tomato leaf tissue showing tomato yellow leaf curl disease symptoms, using the Puregene DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN, USA). The DNA concentrations were adjusted to 10 ng μl^{-1} and the DNA samples were stored at -20°C.

PCR amplification of TYLCV and TYLCSV

Three sets of primers were used for the detection of TYLCV and TYLCSV: 1, the general primer pair PTYv369/PTYc1023 (Navot *et al*., 1991); 2, the TYLCV-specific primer pair PTYv21/PTYc287 (Nakhla *et al*., 1993), and 3, the TYLCSV-specific primer pair MA14/MA15 (Navas-Castillo *et al*., 1999). All molecular biology chemicals for the PCR were purchased from Promega Corp. (Madison, WI, USA). PCR reaction mixtures of 25 μl contained: 2.5 μl of 2.5 mM deoxynucleotide triphosphates (dNTPs); 2.5 μl 10× buffer; 2.5 μl 25 mM $MgCl_2$; 2 U *Taq* DNA polymerase; 2.5 μl each forward and reverse-sense primer at 10 μM; 5 μl sample DNA (approximately 50 ng). The PCR was carried out in a MJ DNA Engine PT200 Thermocycler (MJ Research Inc., Waltham, MA, USA). The cycling protocol was: 1 cycle of 4 min at 94°C, followed by 30 cycles of melting (1 min at 94°C), annealing (1 min at 55°C), and DNA extension (1 min at 72°C). The reaction was terminated by 5 min incubation at 72°C. The PCR products were separated by electrophoresis in a 1.5% agarose gel (Seakem LE, BioWhittaker Molecular Applications, Rockland, ME, USA) in 0.5× TBE buffer (Tris-borate EDTA) and visualized with a Kodak Gel Logic 200 Imaging System, after staining with ethidium bromide. PCR fragments were directly sequenced using the Big Dye Sequencing Kit™ (Biotechnology Center, Madison, WI, USA). Sequences were compared with known DNA sequences through the National Center for Biotechnology Information (NCBI) BLAST program and with the DNAMAN software (Lynnon Corp., Quebec, Canada).

Amplification of the full-length genome of TYLCSV from Morocco

The overlapping primer method (Patel *et al*., 1993) was used to amplify the full-length TYLCSV DNA. A DNA extract from symptomatic plants that gave a PCR product only with the TYLCSV-specific primer pair MA14/MA15, and not with the TYLCV-specific primer pair, was used as template for amplification of the full-length viral genome. The following primers were used (see sequences in Table 1): TYS-*Nco*v/TYS-*Nco*c; TYS-*Sph*v/TYS-*Sph*c; TYS-*Sac*v/TYS-*Sac*c, which overlapped the *Nco*I and *Sph*I sites in the *Cp* gene and the *Sac*I site in the *Rep* gene of TYLCSV respectively (L27708 and X61153). The PCR cycling protocol was: denaturation (94°C for 3 min), followed by 35 cycles of melting (94°C for 30 s), annealing (57°C for 30 s), and extension (68°C for 3 min). The reaction was terminated by 5 min incubation at 72°C. PCR products were ligated into plasmid pCR 2.1 (TOPO TA cloning version C, Invitrogen, Carlsbad, CA, USA). The recombinant plasmids were used to transform DH5α-T1 *Escherichia coli* cells and were identified by restriction enzyme analysis. A recombinant plasmid (pTYLCSV-MO-1), which contained the full-length TYLCSV insert of approximately 2,800 nucleotides, was selected for further characterization.

Sequence analysis of the TYLCSV clone

The full-length TYLCSV clone from Morocco was sequenced using the dideoxy chain termination method with the M13F and M13R sequencing primers, and with primers derived from the sequences obtained. TYLCSV sequences from Morocco were compared with the sequences of known TYLCV and TYLCSV isolates using the NCBI BLAST program and the DNAMAN software. The following virus entries were used in the comparison: TYLCV (X15656), TYLCV-Mild (X76319), TYLCV isolates from the Dominican Republic (AJ489258) and from Iran (AJ132711), the recombinant TYLCMalV from Malaga, Spain (AF271234), and TYLCSV isolates from Sardinia, Italy (X61153), Sicily, Italy (Z28390) and Murcia, Spain (Z25751).

Primers designed to discriminate between the TYLCV species

General and species-specific primer sets were designed based on the sequence of the Moroccan TYLCSV isolate and on the sequences of two TYLCV species: TYLCSV from Malaga, Spain (AF271234) and TYLCV from the Dominican Republic (AJ489258). Sequences were aligned using DNAMAN software. One general primer set and two Morocco TYLCSV-specific primer sets were designed. A general primer set, PTYGv568/PTYG1236, was designed to anneal to the conserved regions of the *Cp* gene, and two specific primer pairs, PTYMv1887/PTYMc2322 and PTYMv1887/PTYMc2596, were designed to anneal to the *Rep* gene of the TYLCSV isolate from Morocco. The primer pair PTYIRv21/PTYIRc287, previously designed to amplify the IR of TYLCV (Nakhla *et al*., 1993), was used to detect TYLCV. The number associated with each primer refers to the nucleotide position on the genome from which the

Table 1. Overlapping primers used in the polymerase chain reaction to amplify fragments and full-length TYLCSV from Morocco.

Primers	Nucleotide sequence and position on the TYLCSV genome (from 5' to 3')
TYS-*Nco*v	180 ATTACCATGGTCTCCGATGCATGCTCG 206
TYS-*Nco*c	192 ATTACCATGGACTGAATCAGGAAATTCATTTAATAATGG 155
TYS-*Sph*v	194 ATTAGCATGCTCGCAATTAAATAT TTGCAGC 204
TYS-*Sph*c	203 AATTGCATGCATCGGAGACCATGGACTG 179
TYS-*Sac*v	2210 TTTGCGTAAGCGTCGTTGGCTGTCTG 2235
TYS-*Sac*c	2611 AAGATCAGGTCGTTTTAGTATCAAGGC 2585
MA14	2587 TGCATTTATTTGAAAACG 2604
MA15	162 AAAGGATCCCACATATTG 145

TYS = TYLCSV-specific primers designed by us for this study. MA14/MA15: TYLCSV-specific primers designed by Navas-Castillo *et al*., (1999); v = viral sense primer or c = complementary sense primer.

primer was derived; v anneals to the viral genome sense strand and c to the viral genome complementary strand. The PCR primers were tested using the full-length TYLCSV genomic clone from Morocco and a full-length TYLCV genomic clone from the Dominican Republic. Each PCR reaction mixture of 25 μl contained: 2.5 μl of 10 mM deoxynucleotide triphosphates (dNTPs); 2.5 μl 10× buffer; 2.5 μl 25 mM $MgCl_2$; 0.1 μl *Taq* DNA polymerase; 1 μl of each 10 μM primer; 5 μl of plasmid DNA at different concentrations. Cycling conditions were: 4 min of denaturation at 94°C, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, terminated by an extension step at 72°C for 5 min.

Results

Characterization of PCR products from infected tomato samples

The primer pairs designed were tested using previously characterized full-length clones of TYLCV and TYLCSV. Using DNA from infected tomato as template, PCR products of the expected sizes were obtained confirming the usefulness of the primers: a 698 bp product was obtained using the general primer pair PTYv369/PTYc1023, a 312 bp product using the TYLCV-specific primer pair PTYv21/PTYc287, and a 338 bp product using the TYLCSV-specific primer pair MA14/MA15. No PCR product was obtained when DNA extracts from non-infected tomatoes were used as template.

The primers were then used to analyze tomato samples collected from infected fields. Of the 35 samples analyzed, 29 gave virus-specific PCR products with the general CP primer pair and with the TYLCV-specific primer pair. Nine of these samples also gave PCR fragments with the TYLCSV-specific primer pair, and thus contained mixed infections. Six samples did not yield virus-related PCR fragments with the general CP primers or with either of the specific primer pairs.

The PCR-amplified DNA fragments were identified by sequencing. The results validated the specificity of the primer pairs used. The 312 bp DNA fragment presented 100% nucleotide (nt) identity with the homologous TYLCV IR (AF024715) and 57% nt identity with TYLCSV (X61153). The 338 bp DNA fragment presented 98% and 97% nt identity with the IRs of the TYLCSV isolates from Almeria (L27708) and Murcia (Z25751) respectively, and 54% nt identity with the IR of TYLCV. These results confirmed the presence of both TYLCV and TYLCSV in Morocco, as previously reported (Monci *et al*., 2000, Sedegui *et al*., 2002).

Sequence analysis of a full-length clone of TYLCSV from Morocco

Considerable variation has been reported among isolates of the TYLCV complex (Fauquet *et al*., 2005). In addition recombinants have been described within this group (Monci *et al*., 2002). Hence it was essential to determine whether the Moroccan isolate was identical to other isolates of TYLCSV or whether it had a recombined genome. The full-length TYLCSV from Morocco was amplified with the primer pair TYS-*Nco*v/TYS-*Nco*c using DNA from infected tomato plants as template (Fig. 1, Lane 5). The 2,800 nucleotide

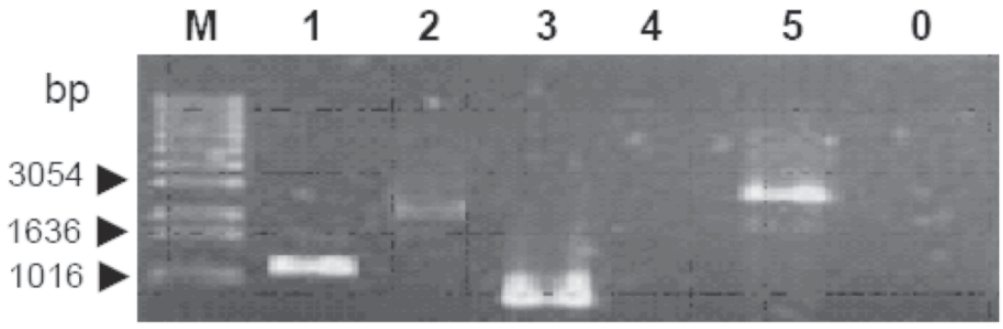

Fig. 1. PCR amplification of TYLCSV DNA fragments from infected tomato plants, using several primer pairs. Lane 1, MA14/PTYc1023; lane 2, PTYv369/MA15; lane 3, TYS-*Sac*v/TYS-*Sph*c; lane 4, TYS-*Sph*v/TYS-*Sph*c; lane 5, TYS-*Nco*v/TYS-*Nco*c. Lane 0, no DNA template. M, molecular marker, 1 Kbp ladder (Invitrogen). Note that the full-length genome of TYLCSV from Morocco was amplified in the sample in lane 5.

product was ligated into PCR 2.1-Topo and introduced into *E. coli* cells. Recombinant plasmids were identified by *Eco*RI restriction analysis: three bands were obtained, one of approximately 3,900 bp which corresponded to the plasmid vector, and two of approximately 1,600 and 1,200 bp which corresponded to the virus insert. This result confirmed the cloning of a full-length TYLCSV isolate and showed the presence of an *Eco*RI restriction site in the viral genome. The recombinant plasmid which contained the full-length TYLCSV clone of approximately 2,800 bp was named pTY-MO-1. This plasmid was characterized further.

The complete nucleotide sequence of the full-length TYLCSV clone from Morocco was determined using the overlapping primer method. The seven sequences obtained with different primers were assembled using Chromas and DNAMAN softwares. The full-length genome of the clone TYLCSV isolate from Morocco consisted of 2,777 nt. It was deposited in GenBank and was assigned the accession number AY702650. Six ORFs encoding putative proteins of approximately 10 KDa or greater were identified, which corresponded to the ORFs previously identified for other monopartite TYLCV (Navot *et al*., 1991) and TYLCSV isolates (Kheyr-Pour *et al*., 1991; Noris *et al*., 1994). Table 2 shows a comparison of the nucleotide and amino acid sequences between the TYLCSV isolate from Morocco (ORFs and IR) and other TYLCSV/TYLCV isolates. Regardless of the region examined, the TYLCSV isolate from Morocco was 97% identical with the TYLCSV isolates from Murcia (Z25751) and from Almeria (L27708). In contrast, the levels of homology with TYLCSV isolates from Italy were significantly smaller, especially in the IR. Thus TYLCSV from Morocco (AY702650) is not a new recombinant and appears to have been introduced from Spain.

Differentiation of TYLCV and TYLCSV by PCR

Sequences of three members of the TYLCV complex: TYLCSV from Morocco (AY702650), TYLCSV from Malaga, Spain (AF271234) and TYLCV from the Dominican Republic (AJ489258), were compared to identify conserved regions suitable for the design of general primers allowing detection of the three viruses and of specific regions suitable for the design of primers specific for detecting the TYLCSV isolate from Morocco.

A general primer set was designed to amplify a region common to the three viruses located in the *Cp* gene. To differentiate the TYLCSV isolate from

Table 2. Comparison between TYLCSV from Morocco and other members of the TYLCV complex. Percent nucleotide (nt) identities and derived amino acid (aa) between open reading frames (V1 and V2, C1 to C4) and intergenic region (IR).

Viruses	CP (V1)		V2		Rep (C1)		C2		C3		C4		IR
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	
TYLCSV-[ES-Mur] (Z25751) [a]	99	99	99	99	98	97	99	98	99	98	99	98	97
TYLCSV-[ES-Can] (AJ519675)[b]	99	98	99	99	98	97	99	95	99	87	98	97	97
TYLCMalV (AF271234)[c]	99	99	99	98	83	80	90	66	87	73	80	74	59
TYLCSV-[ES-Alm] (L27708)[d]	98	98	98	94	98	97	99	99	99	100	98	97	97
TYLCSV-[IT-Sic] (Z28390)[e]	93	98	95	98	93	91	92	92	89	84	94	85	81
TYLCSV-[IT-Sar] (X61153)[f]	93	98	95	99	92	97	93	98	90	87	91	79	67
TYLCV-Mld (X76319)[g]	82	89	83	83	80	80	78	67	78	66	77	59	59
TYLCV-[DO] (AJ489258) [h]	82	88	82	81	86	89	75	66	82	66	73	41	53
TYLCV-[IL] (X15656)[i]	81	88	83	83	77	76	77	66	74	67	71	41	54
TYLCV-R[IR] (AJ132711)[l]	82	87	83	83	78	78	77	67	76	66	71	47	61

[a], TYLSCV: *Tomato yellow leaf curl Sardinia virus* from Murcia, Spain; [b], TYLCSV isolate from the Canary Islands, Spain; [c], recombinant TYLCSV from Malaga, Spain; [d], TYLCSV isolate from Almeria, Spain; [e], TYLCSV isolate from Sicily, Italy; [f], TYLCSV isolate from Sardinia, Italy; [g], a mild isolate of TYLCV from Israel; [h], a TYLCV from the Dominican Republic; [i], a TYLCV isolate from Israel; [1l], a recombinant TYLCV isolate from Iran. The GenBank accession number of the various isolates is given in parentheses. Virus nomenclature is according to Fauquet and Stanley (2005).

Table 3. Primer sets designed to amplify DNA of TYLCV and TYLCSV from infected tomato plants in Morocco.

Primer	Sequence 5' to 3'	Detected TYLCV species	Amplicon size
PTYGv568	CGTTGTGTTAGTGATGT(T/A) ACT(A/C) G(G/T)GG	General primer	692 pb
PTYG1236	ACGAC(A/C)TTACAG(T/C)CTC(A/C)GACTGGTC		
PTYSMv1887	TCCACACAAATAATTATGTG GGCCTGAG	TYLCSV	PTYMv1887/PTYSMc2322 462 bp
PTYSMc2322	TCCGAACATTCAGGGAGCTA AATCAAGC		PTYMv1887/PTYSMc2596 734 bp
PTYSMc2596	TGACCAAATGGCTCAGCCTA AGCG		
PTYIRv21	GTTGAAATGAATCGGTGTCCC	TYLCV	312 pb
PTYIRc287	TTGCAAAGACAAAAAACTTGGGACC		

Morocco, two specific primer sets were derived from the *Rep* gene of this virus. The PTYIRv21/ PTYIRc287 primer pair, previously described as amplifying the IR of TYLCV (Nakhla *et al.*, 1993), was used to detect the TYLCV isolates. The selected primers, their sequences and targets, as well as the size of the expected PCR amplicons are presented in Table 3.

To confirm the specificity of the primers to identify the TYLCSV isolate, we used clones of TYLCSV from Morocco (AY702650) and TYLCV from the Dominican Republic (AY594174) as template. In addition, a virus DNA pool, prepared by mixing the clones of TYLCSV from Morocco and TYLCV from the Dominican Republic, was used to test the efficiency of the primers to detect mixed infections. Several DNA concentrations were prepared in order to test the efficiency of detection in low and high viral infection levels.

DNA of the expected size (692 bp) was amplified both from TYLCSV from Morocco and from TYLCV from the Dominican Republic, using the general primer pair PTYGv568/ PTYG1236. No PCR product was obtained with the negative control (Fig. 2). These results confirmed the ability of these primers to detect infection with TYLCV/ TYLCSV isolates. The specific primers designed to detect TYLCSV from Morocco, PTYMv1887/ PTYSMc2322 and PTYMv1887/PTYSMc2596, resulted in PCR products of 462 bp and 734 bp respectively, when using a mixture of clones of TYLCSV from Morocco and of TYLCV from the

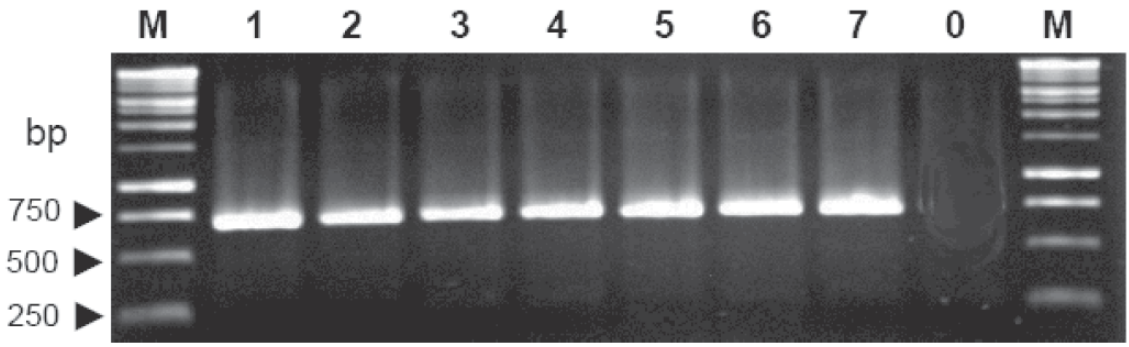

Fig. 2. PCR products obtained with the general primer pair PTYGv568-PTYG1236, using cloned full-length genomic DNA of TYLCSV from Morocco (pTYLCSV-MO-1) and TYLCV from the Dominican Republic (TYLCV-[DO]) as template. Lane 1, pTYLCSV-MO-1 (1 ng DNA); lane 2, pTYLCSV-MO-1 (0.1 ng); lane 3, TYLCV-[DO] (0.1 ng); lane 4, TYLCV-[DO] (0.01 ng); lane 5, mixture of pTYLCSV-MO-1 (1 ng) and TYLCV-[DO] (0.01 ng); lane 6, mixture of pTYLCSV-MO-1 (0.1 ng) and TYLCV-[DO] (0.01 ng); lane 7, mixture of pTYLCSV-MO-1 (1 ng) and TYLCV-[DO] (0.1 ng); lane 0, no DNA template; M, 1 Kbp ladder (Promega).

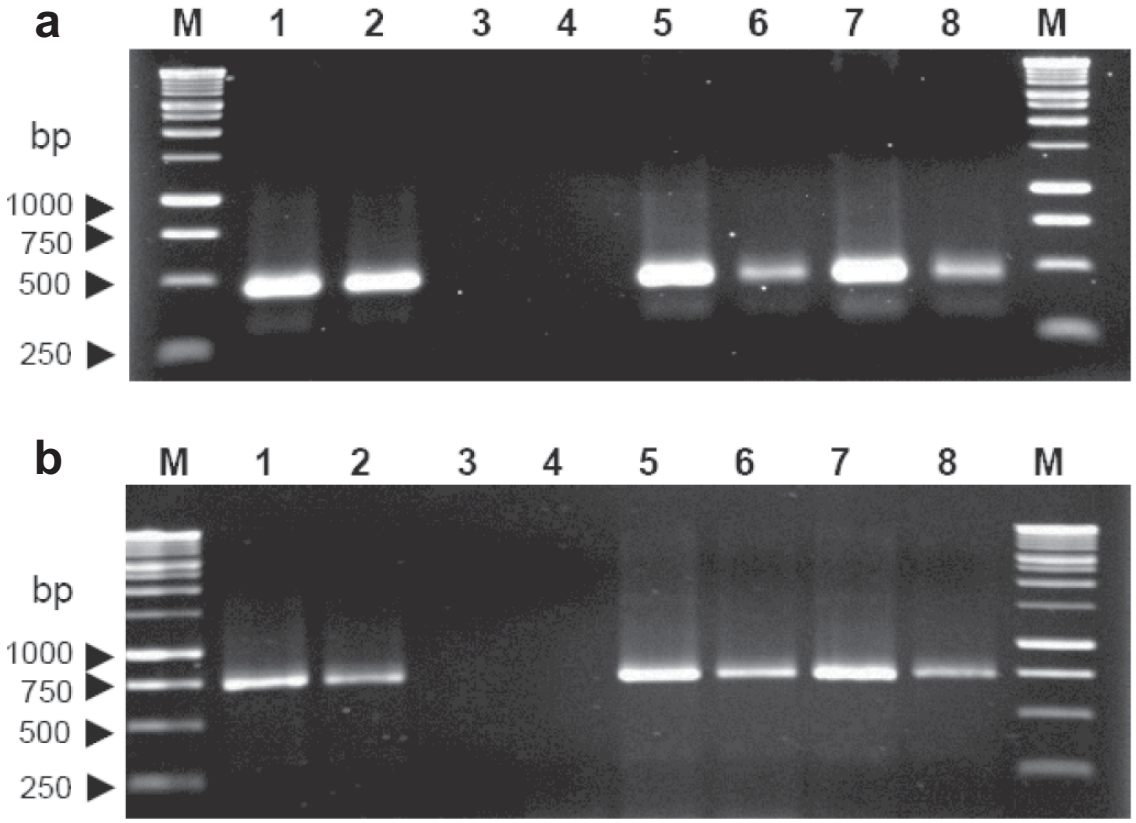

Fig. 3. PCR performed with primers specific to the TYLCSV isolate from Morocco, using cloned full-length genomic DNA of TYLCSV from Morocco (pTYLCSV-MO-1) and of TYLCV from the Dominican Republic (TYLCV-[DO]) as template. (a) with primer pair PTYMv1887-PTYMc2322 (462 bp product); (b) with primer pair PTYMv1887-PTYMc2596 (734 pb product). Lane 1, pTYLCSV-MO-1 (1 ng DNA); lane 2, pTYLCSV-MO-1 (0.1 ng); lane 3, TYLCV-[DO] (0.1 ng); lane 4, TYLCV-[DO] (0.01 ng); lane 5, mixture of pTYLCSV-MO-1 (1 ng) and TYLCV-[DO] (0.01 ng); lane 6 mixture of pTYLCSV-MO-1 (0.1 ng) and TYLCV-[DO] (0.01 ng); lane 7, mixture of pTYLCSV-MO-1 (1 ng) and TYLCV-[DO] (0. 1 ng); lane 8, mixture of pTYLCSV-MO-1 (0.1 ng) and TYLCV-[DO] (0.1 ng); M, 1 Kbp ladder (Promega).

Dominican Republic. Amplification failed when the template consisted of TYLCV from the Dominican Republic only, confirming the high specificity of the primers and the absence of any interference in the cases of mixed infection (Fig. 3, a and b).

To detect the TYLCV species, we used the specific primer pair PTYIRv21/PTYIRc287 (Nakhla *et al.,* 1993). A 312 pb product was obtained when a mixture of clones of TYLCV from the Dominican Republic and of TYLCSV from Morocco were used as template (Fig. 2). No product was obtained when only the clone of TYLCSV from Morocco was used as template. These results pointed to the high specificity of the TYLCV primer pair.

Discussion

In Morocco TYLCV has been a major constraint to tomato production since 1997, causing considerable yield losses (Petershmitt *et al*., 1999; Hanafi, 2000, Jebbour and Abaha, 2002). The disease is caused by whitefly-transmitted geminiviruses belonging to the TYLCV/TYLCSV complex. Although sequence analyses have shown a considerable diversity among members of this virus complex (Fauquet and Stanley, 2005), it is almost impossible to correlate specific symptom manifestation with a particular virus strain. Although all TYLCV/TYLCSV isolates are associated with similar yellowing, curling and stunting symp-

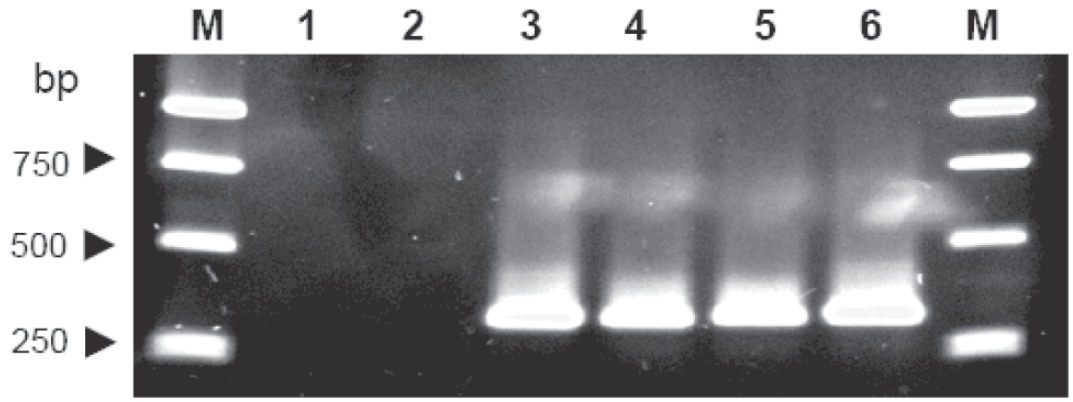

Fig. 4. PCR performed with TYLCV specific primers PTYIRv21 and PTYIRc287, using cloned full-length genomic DNA of TYLCSV from Morocco (pTYLCSV-MO-1) and TYLCV from the Dominican Republic (TYLCV-[DO]) as template. Lane 1, pTYLCSV-MO-1 (1 ng DNA); lane 2, pTYLCSV-MO-1 (0.1 ng); lane 3, TYLCV-[DO] (0.1 ng); lane 4, TYLCV-[DO] (0.01 ng); lane 5, mixture of pTYLCSV-MO-1 (1 ng) and TYLCV-[DO] (0.1 ng); lane 6, mixture of pTYLCSV-MO-1 (0.1 ng) and TYLCV-[DO] (0.01 ng); lane M, 1 Kbp ladder (Promega).

toms, their magnitude is greatly affected by environmental conditions. Thus, molecular methodologies based on the diversity of viral DNA sequences are the most powerful tools for virus identification. PCR is a rapid tool to detect and discriminate between the TYLCV/TYLCSV complexes. By designing general and specific primers, we were able to characterize the TYLCV isolates from Morocco.

The results of this study demonstrated that some tomato plants in Southwestern Morocco were infected with TYLCV and some with a local isolate of TYLCSV. Some plants also had a mixed infection of both. Although TYLCV has occurred in Morocco since 1997, no attempt has been made to characterize this TYLCSV isolate at the molecular level. The molecular characterization and differentiation of the TYLCV/TYLCSV complex in Morocco was therefore the main aim of this study.

A TYLCSV isolate was cloned and the full-length genome was sequenced. It was assigned the Genbank accession number AY702650. The nucleotide sequence of TYLCSV-Morocco had the highest homology with a TYLCSV isolate from Murcia (Z25751), indicating that the virus was originally introduced from Spain. The precise means by which TYLCSV was introduced into Morocco is not known. The virus could have been introduced by means of infected tomato seedlings or by viruliferous whiteflies carried by winds or by human transportation means (Hanafi, 2000). Clearly, it is important to carefully regulate the movement of plant materials from TYLCV-infected areas in order to avoid the inadvertent introduction of the virus into TYLCV-free tomato growing regions.

The presence of two different TYLCV isolates in the same region, and sometimes in the same plant nucleus, is a fertile ground for virus recombination (Morilla *et al*., 2004). Indeed several TYLCV isolates were found to be recombinant viruses (Navas-Castillo *et al*., 2000; Chatchawankanphanich and Maxwell, 2002; Monci *et al*., 2002; Fauquet *et al*., 2005). Recombination can lead to the evolution of new, more devastating, TYLCV strains (Monci *et al*., 2002). Therefore, knowing the precise identity of a TYLCV isolate(s) in a given region at a given time has important practical considerations in terms of developing appropriate detection methods and breeding virus-resistant cultivars by classical means and by genetic engineering.

The most effective way of managing the TYLCV/TYLCSV disease is to breed resistant tomato varieties (Friedman *et al*., 1998; Vidavski and Czosnek, 1998; Gomez *et al*., 2004). Many breeding programs have been based on the introduction of resistance from the wild tomato species *Solanum chilense*, *S. peruvianum* and *S. habrochaites* in the domesticated tomato *S. lycopersi-*

cum (Zamir *et al*., 1994; Vidavsky and Czosnek, 1998; Friedmann *et al*., 1998; Pico *et al*., 1999; Lapidot and Friedman, 2002; Gomez *et al*., 2004). In 2002, we started a breeding program for resistance to the TYLCV/TYLCSV complex in the Souss-Massa region. We are using *S. habrochaites* (Vidavsky and Czosnek, 1998) and *S. peruvianum* (Friedman *et al*., 1998) as sources for TYLCV resistance genes. This program needs to take the results of the present study into consideration since breeding lines may behave differently upon inoculation by different members of the TYLCV complex (Fargette *et al*., 1996). To support these efforts we have designed three sets of primers which can be used either in uniplex or multiplex PCR. All primers used gave similar amplification efficiencies for their respective targets. These primers will help in controlling the disease and limit the virus spread. We are also preparing an infectious clone of TYLCSV from Morocco to be used as a selection tool for resistance. This virus will be delivered to tomato plants in the laboratory by agroinoculation as is done with TYLCV (Navot *et al*., 1991).

The currently developing recombinant DNA strategies based on virus gene silencing are also dependent on the precise homologies between the silencing construct and the virus target. Therefore the future application of this technology to the development of TYLCV- resistant tomatoes must take into account the diversity of tomato-infecting geminiviruses (Abhary *et al*., 2006).

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