

MOLECULAR IDENTIFICATION OF SPECIES OF THE TOMATO YELLOW LEAF CURL VIRUS COMPLEX IN JORDAN

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SUMMARY

The distribution of the Tomato yellow leaf curl virus complex was investigated in five locations in Jordan. Hybridization results of samples collected in 2002 showed that *Tomato yellow leaf curl virus* (TYLCV) occurred in all surveyed areas, while the incidence of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was restricted to Al-Mafraq and the central Jordan Valley. Both virus species could be detected in all areas surveyed in 2003. Using specific PCR primers, TYLCV, TYLCSV and TYLCV-Mild (TYLCV-Mld) could be detected in symptomatic tomato plants. Alignment analysis of the amplified-PCR fragments showed that the sequence of the intergenic region (IR) of TYLCSV from Jordan (TYLCSV-Jo1) shared high sequence homology with TYLCSV-ES[1] and TYLCSV-ES[2] from Spain. In addition, TYLCV-Jo1 had sequence identity of 95% and 93% with TYLCV-[Alm] and TYLCV-[DO], respectively.

Key words: tomatoes, *Tomato yellow leaf curl virus*, *Tomato yellow leaf curl Sardinia virus*, PCR, dot blot hybridization, Jordan.

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) is one of the most damaging diseases of tomato worldwide. Yield losses of 100% are common, particularly when plants are infected early in development (Nakhla and Maxwell, 1999). Several virus species belonging to the genus *Begomovirus* of the family *Geminiviridae* were reported to cause TYLCD (Rybicki *et al.*, 2000). Begomoviruses have a circular, single-stranded DNA genome encapsidated in a paired particle (Navot *et al.*, 1991). These viruses are transmitted by the whitefly, *Bemisia tabaci*, in a circulative manner (Cohen and Harpaz, 1964). Symptoms induced by TYLC viruses consist of foliar curling

and yellowing, reduced leaflet area, plant stunting and reduced fruit size and yield (Nakhla and Maxwell, 1999). TYLC viruses cause serious losses in tomato crops in the Mediterranean basin, tropical Africa, South-east Asia, North America, South America and the Caribbean region (Makkouk, 1978; Czosnek and Laterrot, 1997; Moriones and Navas-Castillo, 2000). Three TYLC virus species are known to cause TYLCD in the Western Hemisphere: *Tomato yellow Leaf curl virus* (TYLCV, formerly TYLCV-Israel), *Tomato yellow leaf curl Sardinia virus* (TYLCSV, formerly TYLCV-Sardinia) and *Tomato yellow leaf curl Malaga virus* (TYLCMaV) (Fauquet *et al.*, 2003). These virus species occur in Spain and Italy (Kheyri-Pour *et al.*, 1991; Navas-Castillo *et al.*, 1999; Accotto *et al.*, 2000; Sánchez-Campos *et al.*, 2002).

In Jordan, TYLCD was first reported in 1978 (Makkouk, 1978). Since then, TYLCD has spread to all vegetable-producing regions in Jordan, where it has become the limiting factor for tomato production during the summer and fall, causing up to 100% yield loss. Since the geographical distance between the Jordan Valley, where tomatoes are commonly grown, and Israel does not exceed few kilometers, it was assumed that TYLCV is the most dominant species affecting tomatoes in Jordan. However, this assumption has never previously been confirmed by sequencing of the virus genome. Therefore, the aims of this work were to study the distribution of viruses causing TYLCD in Jordan and to characterize the most prevailing isolates at the molecular level.

MATERIALS AND METHODS

Samples collection. Survey studies were conducted in three regions (Jordan Valley, Al-Mafraq and Amman) where tomatoes are commonly grown in Jordan. A total of 78 and 259 leaf samples with leaf curling, yellowing and stunting disease symptoms were collected in 2002 and 2003, respectively, from 58 farms in 5 locations and kept at -80°C until use.

Nucleic acid extraction. Total nucleic acids were ex-

Table 1. Description of primers and polymerase chain reaction (PCR) conditions used for amplification of *Tomato yellow leaf curl virus* (TYLCV).

Primer	Sequence (5'-3')	Nt. position	Size (bp)	Target region	TYLCV isolate	PCR cycling conditions	References
TYAlmv2516 TYAlmc115	TTTTATTTGTTGGTGTGTTGTTAGTTGAAG ATATTGATGGTTTTTTCAAAACCTTAGAAG	2516-2544 115-86	433	IR	TYLCSV-ES[2]	94°C/5 min, 30 X (94°C/1 min, 62°C/45 sec., 72°C/1 min.), 1X (94°C/1 min, 56°C/1 min., 72°C/10 min).	This study
TYv2337 TYc138	ACGTAGGTCTTGACATCTGTTGAGCTC AAGTGGGTCCCACATATTGCAAGAC	2337-2364 138-125	634	IR	TYLCV	94°C/5 min, 30X(94°C/1 min, 62°C/45 sec., 72°C/1 min.), 1X (94°C/1 min, 56°C/1 min., 72°C/10 min).	This study
TYv2664 TYc138	ATTGACCAAGATTTTTTACACTTATCCC AAGTGGGTCCCACATATTGCAAGAC	2664-2691 138-125	316	IR	TYLCV-Mld	94°C/5 min, 30X(94°C/1 min, 62°C/45 sec., 72°C/1 min.), 1X (94°C/1 min, 56°C/1 min., 72°C/10 min).	This study
MA14 MA15	TGCATTTATTTGAAAACG AAAGGATCCCACATATTG	2587-2615 163-145	378	IR	TYLCSV-ES[2]	94°C/1min, 30X(94°C/1min, at 50°C/1 min, 72°C/1 min) 72°C/5 min.	Sánchez-Campos <i>et al.</i> , 2002
PTYIRv21 PTYIRc287	AACTCTGCAGTTGAAATGAATCGGTGTCCC ATATCTGCAGTTGCAAGACAAAAAAGTTGGGACC	2636-2666 123-89	389	IR	TYLCV	30 X (94°C/1 min, 55°C/2 min, 72°C/2 min), 72°C/3 min.	Nakhla <i>et al.</i> , 1993
PTYCPv369 M15	ACGCCCG(T/C)CTCGAAGGTTCCG AAAGGATCCCACATATTG	360-380 163-145	261 2	V1, V2, C1, C2, C3, C4, IR	TYLCV-Mld	30X(94°C/1 min, 56°C/1 min., 72°C/1 min.) plus 4 sec every cycle), 72°C/10 min.	This study

tracted from symptomatic and healthy tomato plants using a modified procedure of the Dellaporta heat extraction method as described by Potter *et al.* (2003).

Probe preparation and dot blot hybridization. The intergenic region (IR) of the begomovirus genome was selected to generate two sets of specific DNA probes and clones of TYLCSV from Morocco and TYLCV from Egypt (AY594174) were used as target DNA. Probe set (A) was generated using PCR primer pair MA14/MA15 that was used to detect TYLCSV isolates in Spain and Morocco (Navas-Castillo *et al.*, 1999; Monci *et al.*, 2000; Sánchez-Campos *et al.*, 2002) and the PTYIRv21/PTYIRc287 primer pair, previously described to amplify the IR of TYLCV (Nakhla *et al.*, 1993). Probe set (B) was generated using primer pairs TYAlmv2516/TYAlmc115 and TYv2337/TYc138 (Table 1) that amplify the IR of TYLCSV-Spain (TYLCSV-ES[2]) (L27708) (formally called TYLCSV-Alm) and TYLCV from Egypt (AY594174), respectively. The position of each primer is shown in Fig. 1. Dot blot hybridization was performed using the Alk Phos Direct Hybridization kit (Amersham Pharmacia, Piscataway, NJ, USA) according to the manufacturer's instructions.

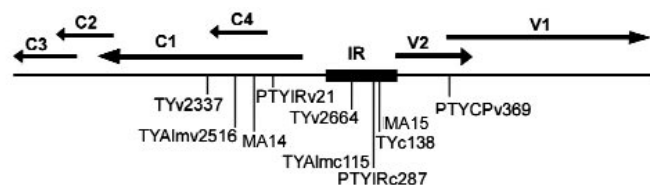


Fig. 1. Genome organization of *Tomato yellow leaf curl virus* (TYLCV). Open reading frames (ORFs) are shown as black arrows. IR indicates the intergenic region and the positions of the primer pairs employed in this study are indicated.

Polymerase chain reaction (PCR). Five samples that were infected with more than one virus isolate were selected for PCR. Primer pairs MA14/MA15 and PTYIRv21/PTYIRc287 were used to amplify the IR of

TYLCSV and TYLCV as previously described by Navas-Castillo *et al.* (1999) and Nakhla *et al.* (1993), respectively. Primers designed in this study (TYAlmv2516/TYAlmc115, TYv2337/TYc138 and TYv2664/TYc138) were used to amplify the IR of TYLCSV, TYLCV and TYLCV-Mld, respectively. The parameters for the PCR reaction were optimized for 25 μ l. All components of the PCR reaction were obtained from Promega Co. (Madison, WI, USA). The final concentrations of reaction components were: 0.25 mM deoxynucleotide triphosphate (dNTPs), 1x *Taq* DNA polymerase buffer, 0.25 mM MgCl₂, 0.5 units *Taq* DNA polymerase, 2.5 μ M of each complementary and virus-sense primers and 2.5 μ l of DNA. PCR cycle parameters were as described in Table 1. The primer pair (PTYCPv369/M15) was used to amplify most of the TYLCV-Mld genome. All PCR reactions were performed in a programmable thermal controller (model PTC-200; MJ Research Inc., Watertown, MA, USA) and PCR products were resolved by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Cloning, sequencing and alignment analysis. To confirm the identity of the amplified fragments, PCR products were ligated to the pGEM-T Easy Vector (Promega Co., Madison, WI, USA) and cloned according to the manufacturer's instructions. Clones of TYLCSV, TYLCV and TYLCV-Mld were sequenced at Biotechnology Center (Madison, WI, USA) using the Big Dye sequencing kit (Applied Biosystems, Foster City, CA, USA). Alignment analysis was carried out by means of the DNA-MAN software program (Lynnon BioSoft, Vendreuil Quebec, Canada).

RESULTS

Distribution of TYLCV and TYLCSV in Jordan. Dot blot hybridization technique was used to investigate the distribution of TYLCV and TYLCSV in Jordan. Probe set (A) was used, in 2002, to analyze symp-

Table 2. Detection of *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES) and *Tomato yellow leaf curl virus* (TYLCV) by dot blot hybridization using DNA probe set A from tomato samples collected in 2002.

Site	No. of fields	No. of samples	No. of positive samples (%)		
			TYLCSV-ES	TYLCV	TYLCSV-ES + TYLCV
Al-Mafraq	14	33	2 (6)	25 (76)	2 (6)
Central Jordan Valley	4	11	3 (27)	8 (73)	3 (27)
North Jordan Valley	9	23	0 (0)	3 (13)	0 (0)
North Amman	2	4	0 (0)	3 (75)	0 (0)
South Amman	3	7	0 (0)	5 (71)	0 (0)

tomato samples for the presence of TYLCV and TYLCSV. Hybridization results showed that plants in all surveyed areas were infected with TYLCV (Table 2). The highest rate of TYLCV infection was recorded in Al-Mafraq (76%), whereas, samples collected from the northern Jordan Valley showed the lowest disease incidence (13%). On the other hand, low incidence of TYLCSV (6% and 27%) could be detected only in samples obtained from Al-Mafraq and the central Jordan Valley, respectively (Table 2). In 2003, probe sets (A) and (B) were used to analyze samples for infection with TYLCV and TYLCSV. Hybridization results with probe set (A) indicated that 255 samples (98%) were infected with TYLCSV. However, only 91 samples (35%) reacted with probe specific to TYLCV (Table 3). All samples that were infected with TYLCV were also found to be infected with TYLCSV. The highest rate of TYLCSV infection was in Al-Mafraq area (98%) and 81% of samples from the southern Jordan Valley were infected with both virus species (Table 3, column A). Using probe set (B) (Table 3, column B), 99% of samples from the northern Jordan Valley were found to be infected with TYLCSV-ES. However, 40 samples (56%) from the same area were infected with TYLCV. Similar results show that 97 and 3% of samples from the southern Jordan Valley were infected with TYLCSV-ES and TYLCV, respectively. No mixed infection in these samples could be detected. On the other hand, samples from Al-Mafraq, the central Jordan Valley, the northern Jordan Valley and south Amman were found to be infected with both virus species.

Detection of TYLCV, TYLCSV and TYLCV-Mld isolates by PCR. DNA of the expected sizes, 378 bp and 389 bp, of TYLCSV and TYLCV, respectively, could be amplified from symptomatic tomato plants using the primer pairs MA14/MA15 and PTYIRv21/

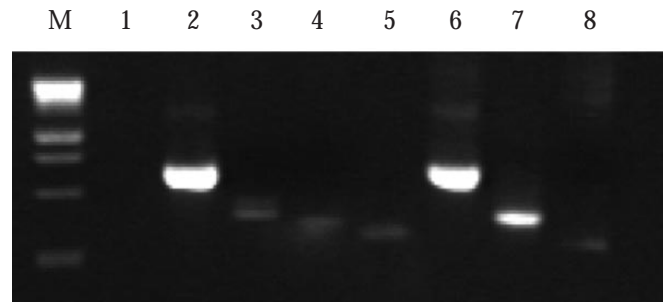


Fig. 2. Detection of the TYLCV, TYLCSV-ES and TYLCV-Mld isolates by polymerase chain reaction (PCR). Lane 1: healthy control; lanes 2-5: isolate TY-Jo1 amplified using TYv2337/TYc138, TYAlmv 2516/TYAlmc115, MA14/MA15 and TYv2664/TYC138 primer pairs, respectively; lanes 6-8: DNA from TYLCV, TYLCSV-ES and TYLCV-Mld clones amplified using the same primer pairs. M: 1 kb DNA marker (Promega, Madison, USA).

PTYIRc287, respectively. An isolate (hereafter called TY-Jo1) obtained from Al-Mafraq was also subjected to PCR analysis using the primer pairs designed in this study (Table 1). Fig. 2 shows that different fragment sizes could be amplified from plants infected with TY-Jo1 when primer pairs specific for TYLCV, TYLCSV-ES[2] and TYLCV-Mld were used. Viruses that have been amplified using MA14/MA15 and TYv2337/TYc138 primer pairs were, hereafter, called TYLCSV-Jo1 and TYLCV-Jo1, respectively. In addition, a band of 2612 bp, hereafter called TYLCV-Mld [Jo1], was detected in TY-Jo1 when the primer pair M15/PTY-CPv369 was used (not shown).

Sequencing and alignment analysis. Sequences of the amplified PCR fragments were deposited in the GenBank under accession no. AY193715 for TYLCSV-Jo1, AY646681 for TYLCV-Jo1, and AY594175 for TYLCV-

Table 3. Detection of *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES) and *Tomato yellow leaf curl virus* (TYLCV) by dot blot hybridization from tomato samples collected in 2003^a.

Site	No. of fields	No. of samples	No. of positive samples (%)					
			Probe set A			Probe set B		
			TYLCSV-ES	TYLCV	TYLCSV-ES + TYLCV	TYLCSV-ES	TYLCV	TYLCSV-ES + TYLCV
Al-Mafraq	8	79	77 (98)	19 (24)	19 (24)	77 (98)	32 (41)	32 (41)
Central Jordan Valley	6	58	58 (100)	20 (35)	20 (35)	58 (100)	37 (64)	37 (64)
South Jordan Valley	3	36	35 (97)	29 (81)	29 (81)	35 (97)	1 (3)	0 (0)
North Jordan Valley	8	72	71 (99)	20 (28)	20 (28)	71 (99)	40 (56)	40 (56)
South Amman	1	14	14 (100)	3 (21)	3 (21)	14 (100)	7 (50)	7 (50)

^a DNA probe set A was generated using primer pairs M14/M15 specific for TYLCSV-ES and PTYIRv21/PTYIRc287 specific for TYLCV. DNA probe set B was generated using primer pairs TYAlmv2516/TYAlmc115 specific for TYLCSV-ES and TYv2337/TYc138 specific for TYLCV.

Mld [Jo1]. Results of the alignment analysis indicated that TYLCSV-Jo1 shared high degree of nucleotide identity (>98%) with TYLCSV-ES[2] (L27708) and TYLCSV-Spain[1] (TYLCSV-ES[1]) (Z25751). However, the sequence identity between TYLCSV-Jo1 and TYLCSV from Sardinia (X61153) did not exceed 67%. In addition, TYLCSV-Jo1 shared 81 and 84% identity with TYLCMaV from Malaga (AF271234) and TYLCSV-Sic from Sicily (Z28390), respectively. Sequence identities of 93 and 95% were also observed between TYLCV-Jo1 (AY646681) and TYLCV-[DO] from Dominican Republic (AF024715.2) and TYLCV-[Alm] from Almeria (AJ489258), respectively (not shown). Furthermore, alignment analysis of TYLCV-Mld [Jo1] revealed 97% identity with the mild isolate of TYLCV from Israel (TYLCV-Mld) (X76319) (not shown).

DISCUSSION

Since it was first reported in Israel (Cohen and Harpaz, 1964), many species and isolates of the TYLCV complex have been described. Although TYLCV has been reported to occur in Jordan more than twenty-five years ago (Makkouk, 1978), no reports on the most prevalent members of the TYLCV complex are available. In addition, no attempts have been made to characterize members of the TYLCV complex at the molecular level. Therefore, molecular characterization and distribution of members of the TYLCV complex in Jordan was the main aim of this study.

The IR of begomovirus genome was chosen to generate specific DNA probes and PCR primers because DNA sequence in this region of the virus genome is highly specific for a given virus (Padidam *et al.*, 1995). Results of the survey studies demonstrated that TYLCV was widely dispersed throughout the main tomato-growing regions in Jordan. These findings are in accordance with previous report that showed the distribution of TYLCV all over the Mediterranean basin (Czosnek and Laterrot, 1997). Data presented in this study demonstrate the distribution of the strain "Spain" of TYLCSV (TYLCSV-ES) in different regions in Jordan. To our knowledge, this is the first report on the occurrence of TYLCSV-ES in the Eastern part of the Middle East. When probe set (A) was used to analyze tomato samples collected in 2002, TYLCSV-ES could be detected only in samples from the central Jordan Valley and Al-Mafraq. This virus species could not be detected in other locations and this may have been due to the low number of samples collected from these areas. However, TYLCSV-ES could detect in approximately 98% of symptomatic samples collected in 2003 (Table 3).

Although the farms where TYLCSV-ES was detected in the Jordan Valley are only a few kilometers from the border with Israel, this virus species is not known to oc-

cur in Israel. A possible explanation is that TYLCSV-ES was first introduced into the eastern regions of Jordan (Al-Mafraq) and with time, *via* infected planting materials or the whitefly vector, the virus reached the Jordan Valley. It is expected that in the near future it might be detected in Israel and other neighboring countries.

Analysis of samples by dot-blot hybridization with probe set (A) under high stringency showed that approximately 80% of samples from the southern Jordan Valley were mixedly infected with TYLCV and TYLCSV-ES. However, when the same samples were tested using probe set (B), none of these samples was found to have mixed infection and 97% of them were infected with TYLCSV-ES only. These results suggest that probe set (B) is more specific to these virus species than probe set (A).

Sequencing results with TYLCSV-Jo1 showed a high degree of identity with TYLCSV-ES[1] and TYLCSV-ES[2]. However, sequence similarity between TYLCSV-Jo1 and TYLCSV, from Sardinia (X61153), was limited to 67%. These results demonstrate that the TYLCSV isolate from Jordan belongs to the strain "Spain" of this species.

Breeders in Jordan should take the results of this study into consideration in the development of their breeding programs for TYLCD resistance because differences in the behavior of breeding lines against different members of the TYLCV complex isolates have been previously reported (Fargette *et al.*, 1996).

ACKNOWLEDGMENT

This work was partially supported by the Middle East Research and Cooperation (MERC) project M21-037. Authors express their appreciation to Prof. D.P. Maxwell, University of Wisconsin-Madison, for reviewing this manuscript.

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Received 13 September 2004

Accepted 7 January 2005