Technical Sheet No. 37

Virus Detection: *Tomato yellow leaf curl virus* Isolates

Method: Multiplex Polymerase Chain Reaction

General

Virus Detected: TYLCV isolates from tomato plants.
Method: Multiplex Polymerase Chain Reaction

Developed By

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Goal

The development of a rapid multiplex PCR protocol to detect and differentiate between isolates of TYLCV from tomato tissues.

Introduction

Tomato yellow leaf curl disease (TYLCD) is one of the most damaging diseases of tomatoes worldwide. Several virus species belonging to the genus Begomovirus of the family Geminiviridae have been reported to cause TYLCD (Rybicki et al., 2000). Two TYLC virus species are known to cause TYLCD in the Western Hemisphere: *Tomato yellow curl virus* (TYLCV, formerly TYLCV-Israel) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV, formerly TYLCV-Sardinia). Both species occur in Spain and Italy (Kheyr-Pour et al., 1991; Moriones et al., 1993; Navas-Castillo et al., 1999; Accotto et al., 2000; Sánchez-Campos et al., 2002) and recently TYLCSV was reported in Morocco (K. El Mehrach and D. P. Maxwell, personal communication). Identification of the virus isolates at the molecular level is a prerequisite to developing effective control measures against the most severe isolates of this virus. Therefore, the aim of this study was to develop a fast, simple, reliable and one-step PCR technique to detect two isolates of TYLCV and one isolate of TYLCSV.

Materials and Methods

Nucleic Acid Extraction

Total nucleic acid was extracted from infected tomato leaves according to the Dellaporta heat extraction method as described previously by Potter *et al.* (2003). In brief, 50-100 mg of tomato leaves were ground in liquid nitrogen using sterilized Kontes micropestle (Kontes Glass, Vineland, NJ, USA). To each tube, 500µl of Dellaporta extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM 2-β-mercaptoethanol) was added. The tubes were incubated at 65°C for 10 min. After centrifugation at 14,000 rpm for 10 min, the supernatants were transferred into new tubes and used for PCR.

Design of isolate-specific PCR primer pairs

To develop isolate-specific primer pairs, the sequence of three TYLCV isolates (TYLCV, X15656, TYLCSV-ES[2], L27708 and TYLCV-Mld, X76319) were analyzed using the DNAMAN software program (Lynnon BioSoft, Que., Canada). The consensus sequence was analyzed as a total genome and for all ORF of the virus. Different dissimilarity regions of the genome were used to generate isolate-specific primer pairs to anneal to the different virus isolates. The sequences of the generated primer pairs are shown in table 1 and the location of each primer on the genome of TYLCV is shown in Fig. 1. Before use, the concentration of all primers was adjusted to 10 µM with MilliQ water.

Table 1. Specific Polymerase Chain Reaction (PCR) primer pairs designed to differentiate among isolates of *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV).

Isolates	Primers	nt. Position	Sequence (5'-3')	Size (bp)
TYLCSV-ES[2]	Almv2516	2516	TTTTATTTGTTGGTGTTTGTAGTTGAAG	433
	Almc115	115	ATATTGATGGTTTTTTCAAAACTTAGAAG	
TYLCV	TYv2337	2337	ACGTAGGTCTTGACATCTGTTGAGCTC	634
	TYc138	138	AAGTGGGTCCCACATATTGCAAGAC	
TYLCV-Mld	TYm2664	2664	ATTGACCAAGATTTTTACACTTATCCC	316
	TYc138	138	AAGTGGGTCCCACATATTGCAAGAC	

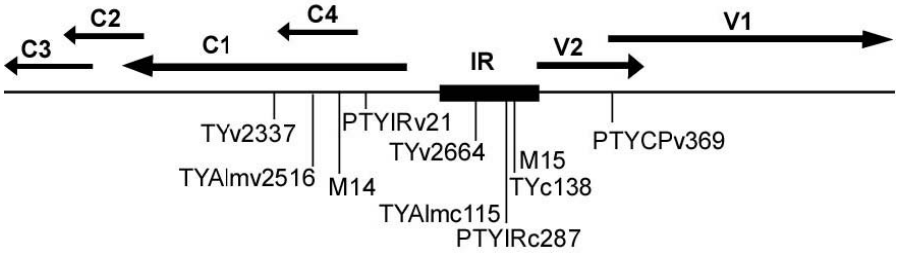

Figure 1. Genome organization of *Tomato yellow leaf curl virus* (TYLCV). Open reading frames (ORFs) are shown as black arrows. The ORFs of plus-strand (virion strand) polarity are designated *V1* and *V2*, ORFs of minus-strand (complementary strand) are designated *C1* through *C4*. IR indicates the intergenic region, the position of the developed primers is indicated.

Polymerase Chain Reaction

All components of the PCR reaction were from Promega, Co., Madison, WI, USA, unless otherwise stated. The parameters for the PCR reaction were optimized for 25 µl and the final concentrations of reaction components were: 25 µM deoxynucleotide triphosphate (dNTPs), 2.5 µl of 10X PCR buffer, 2.5 mM $MgCl_2$, 5 units *Taq* DNA polymerase, 0.5µl of 10 µM each complementary and viral-sense primers (1µl of primer TYc138) and 3 µl of six dilutions of

DNA (520 µg/ml, 52 µg/ml, 5.2 µg/ml, 520 ng/ml, 52 ng/ml, 26 ng/ml 13 ng/ml and 6.5 ng/ml) were used as target templates. PCR cycle parameters were as follows: one cycle at 94°C for 2 min; 30 cycles at 94°C for 1 min, 62°C for 90 sec, and 72°C for 1 min, followed by one cycle at 94°C for 1 min, 62°C for 1 min, and 72°C for 5 min. All PCR reactions were performed in a programmable thermal controller (model PTC-200, MJ Research Inc., Watertown, MA, USA).

Results and Discussion

Different PCR primer pairs were developed to differentiate between isolates of TYLCV and TYLCSV. To test the specificity of the generated primer pairs DNA from clones of different TYLCV and TYLCSV-ES[2] isolates were used as target template for the PCR reaction. As shown in Fig. 2, the developed primer pairs were able to specifically amplify the expected sizes from each virus isolate.

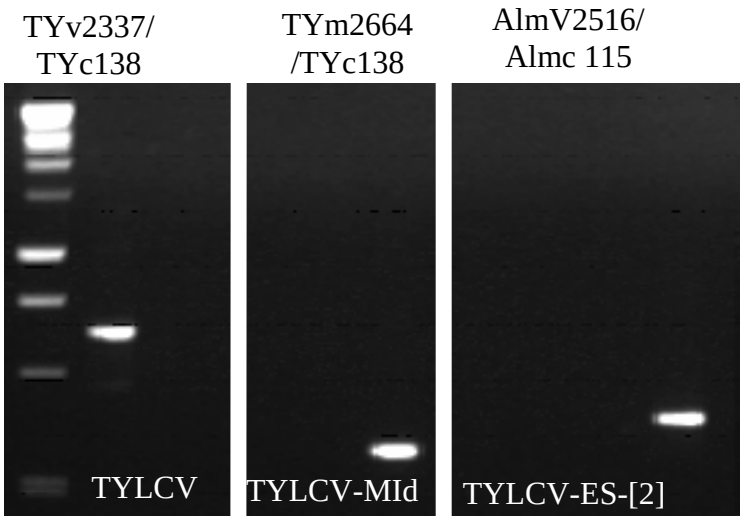

Figure 2. Specific amplification of two isolates of TYLCV and one isolate of TYLCSV using specific primer pairs by one-step multiplex polymerase chain reaction (PCR). Lane 1, 1 Kb DNA marker; lanes 2, 5 and 8, TYLCV; lanes 3, 6 and 9 TYLCV-Mld; lanes 4, 7 and 10 TYLCSV-ES[2].

To determine the detection level (sensitivity) of the multiplex PCR protocol developed using the specific primer pairs, different dilutions (52 µg/ml, 5.2 µg/ml, 520 ng/ml, 52 ng/ml, 26 ng/ml and 13 ng/ml) of the template DNA were used in the PCR reaction mixture. As shown in Fig. 3, multiplex PCR is very sensitive and could detect TYLCV and TYLCV-Mld at a dilution level of the template DNA of 6.5 ng/ml.

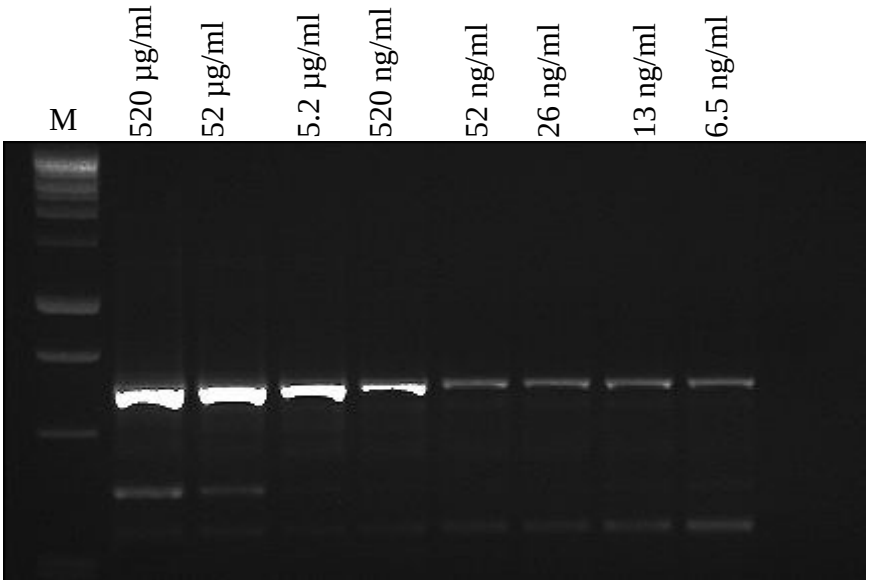

Figure 3. Detection of TYLCV, TYLCV-Mld and TYLCSV-ES[2] by multiplex PCR at different concentrations of the template DNA. M: DNA marker.

Since the main aim of developing this protocol is to be able to detect different isolates of TYLCV in infected tomato field samples in one PCR reaction, DNA from tomato plants known to be infected with two isolates of TYLCV and one isolate of TYLCSV was extracted as mentioned above and used as template in the PCR reaction. Fig. 4 shows that the developed protocol is efficient in detecting TYLCV, TYLCV-Mld and TYLCSV-ES[2] in infected tomato tissues.

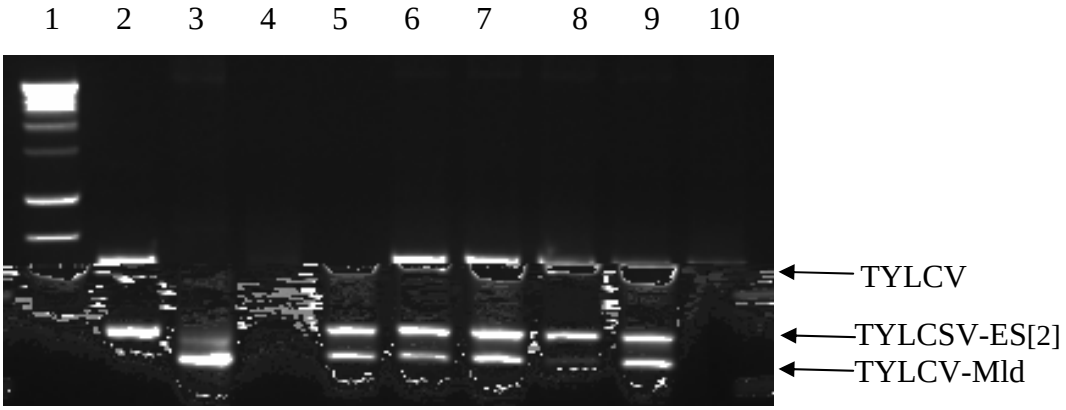

Figure 4. Agarose gel electrophoresis of multiplex polymerase chain reaction (PCR) for the detection of TYLCV isolates from symptomatic field samples. Lane 1, 1Kb DNA marker; lane 2, 3 and 4, represent DNA from TYLCV, TYLCSV-ES[2] and TYLCV-Mld clones, respectively. Lane 5; DNA extracted from healthy tomato plant, lanes 6-10; DNA obtained from symptomatic tomato plant.

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