DEVELOPMENT OF A REAL-TIME PCR FOR TOMATO YELLOW LEAF CURL SARDINIA VIRUS

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Summary. – Recently, tomato yellow leaf curl disease has become important for the tomato grown both in greenhouse and field conditions in Tunisia. Here, we describe a rapid, specific, reliable, and sensitive real-time PCR, based on TaqMan® chemistry, for Tomato yellow leaf curl Sardinia virus (TYLCSV). This method proved suitable for the detection and quantification of this virus in tomato, pepper and bean plants. It detected the virus even in the samples that were negative by conventional assays.

Key words: coat-protein gene; real-time PCR; TaqMan® chemistry; Tomato yellow leaf curl Sardinia virus; SYBR® Green I chemistry

Introduction

In the Mediterranean region, several monopartite begomoviruses are associated with epidemics of tomato yellow leaf curl disease. Tomato yellow leaf curl virus (TYLCV) is most frequently encountered in Israel, Jordan, Egypt (Czosnek and Laterrot, 1997; Navot *et al*., 1991; Nakhla *et al*., 1993; Nakhla and Maxwell, 1998), Spain (Navas-Castillo *et al*., 1999; Sanchez-Campos *et al*., 1999), Morocco (Peterschmitt and Garnier, 1999), the Americas and the Caribbean region (Salati *et al*., 2002). A mild strain of TYLCV, TYLCV-Mld (Antignus and Cohen, 1994) has been found in Mediterranean countries (Navas-Castillo *et al*., 2000), TYLCSV in western Mediterranean and North African countries (Kheyr-Pour *et al*., 1991), and Tomato yellow leaf curl Malaga virus (TYLCMalV) in Spain (Monci *et al.,* 2002). Recently, tomato yellow leaf curl disease has become important also in Tunisia; and preliminary evidence indicates that its causal agent is a TYLCSV (Fekih-Hassen *et al*., 2003).

There is a large number of viruses causing tomato yellow leaf curling; these viruses belong to 6 different species within the genus *Begomovirus*, family *Geminiviridae*. For example, TYLCSV belongs to the species *Tomato yellow leaf curl Sardinia virus*. It has a circular single-stranded DNA genome of 2.8 kb that is encapsidated in a twin-isometric particle (Stanley, 1985; Navot *et al*., 1992). It is transmitted by the whitefly *Bemisia tabaci* in a persistent circulative manner to tomato, pepper (Cohen and Nitzany, 1966) and bean plants (Navas-Castillo *et al*., 1999; Sanchez-Campos *et al*., 1999; Gorsane *et al*., 2004).

TYLCSV and similar viruses are currently detected by several methods including DAS-ELISA (Harrison *et al*., 1991; Macintosh *et al*., 1992), squash blot hybridization (Nakhla *et al*., 1993), dot-blot hybridization (Czosnek and Laterrot, 1997; Accotto *et al*., 2000), PAGE (Accotto *et al*., 2000), and PCR (Navot *et al*., 1992; Nakhla and Maxwell, 1998; Fekih-Hassen *et al*., 2003).

Recently, a novel method, real-time PCR, has been applied to plant pathogens ranging from Tomato spotted wilt

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Abbreviations: CP = coat protein; TYLCV = Tomato yellow leaf curl virus; TYLCSV = Tomato yellow leaf curl Sardinia virus

virus (Boonham *et al*., 2002), Cymbidium mosaic virus, Odontoglossum ringspot virus (Eun *et al*., 2000), and Sugarcane yellow leaf virus (Korimbocus *et al*., 2002). For example, a version of this method based on TaqMan® chemistry, consists of hydrolysis of a target DNA-bound oligonucleotide probe, labeled with a reporter fluorescent dye at its 5'-end and with a quencher at its 3'-end, by the 5'-nuclease activity of *Taq* DNA polymerase (Heid *et al*., 1996). In fact, the emitted fluorescence is measured in dependence on the PCR cycle number and the latter at which the fluorescence passes the threshold (Ct) is determined. Since Ct is inversely proportional to the target DNA quantity, it allows its determination. Consequently, this method is very sensitive and suitable for large scale assaying of the number of viral genome copies in various materials.

Molecular evidence of the presence and spread of TYLCSV in Tunisia was recently shown by dot-blot assay hybridization and a qualitative PCR targeting coat protein (CP) gene. These results together with those of restriction fragment length polymorphism analysis allowed the assignment of the obtained isolates to TYLCSV (Fekih-Hassen *et al*., 2003).

Interestingly, the abovementioned isolates showed typical TYLCSV symptoms but were negative for begomoviruses by conventional detection methods. Therefore, a gel-free real-time PCR for TYLCSV has been developed and applied to samples collected throughout Tunisia.

Materials and Methods

Plant material. Leaves of tomato, pepper and been plants showing tomato yellow leaf curl disease symptoms were collected from both open fields and greenhouses in 2002–2003 (Table 1). Freshly collected samples were stored at -80°C until tested.

Total DNA was extracted from plant samples by a modification of the method described by Dellaporta *et al*. (1983). Briefly, leaf tissue (5 mg) was frozen in liquid nitrogen, ground, mixed with an extraction buffer (0.5 ml) (50 mmol/l EDTA, 100 mmol/l Tris-HCl, 500 mmol/l NaCl, 10 mmol/l β-mercaptoethanol), vortexed and allowed to stand at 65°C for 10 mins. Then, 5 mol/l potassium acetate pH 8 (130 µl) was added, chilled on ice for 10 mins, clarified by centrifugation, precipitated with isopropanol (1:1), and redissolved in sterile water (100 µl per pellet). After treatment with RNAse A (1 U per sample) for 2 hrs at 37°C, DNA was extracted first with phenol/chlorophorm/isoamylalcohol (25:24:1) and chlorophorm/isoamyl alcohol (24:1), and ethanol-precipitated (3:1). DNA was finally dissolved in sterile water. Its concentration was estimated by spectrophotometry.

Real-time TaqMan® PCR. The primers and probes were used in concentrations of 900 nmol/l and 100 nmol/l, respectively. Cycling conditions were the same as in the SYBR® Green I real-time PCR. The emitted fluorescence was measured and plotted versus the cycle number. From this plot, Ct was determined. The viral genome copy number was then read from standard curve, i.e. the plot of the logarithm of viral genome copy number versus the Ct value.

SYBR® Green I real-time PCR. The reaction mixture (25 µl) consisted of 200 µmol/l each of dATP, dCTG and dGTP, 400 µmol/l dUTP, 1.25 U AmpliTaq Gold®, 0.5 U Amprase® UNG, 3 mmol/l $MgCl_2$; 100 ng DNA, and 300 nmol/l of each primer in 1x SYBR® Green I buffer. AmpErase® UNG prevented carry-over contamination of hydrolysis products containing dUTP before amplification. The reaction was performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

It consisted of 50°C/2 mins (AmpErase®UNG treatment), 95°C/10 mins (AmpliTaq Gold® activation) and 40 cycles of 95°C/15 secs and 60°C/1 min.

Cloning and sequencing of TYLCSV CP gene. To obtain a positive control for the real-time PCR, a 650 nt-fragment of the CP gene of Bn isolate of TYLCSV (Fekih-Hassen *et al*., 2003) was cloned, following purification with the QIAquick™ PCR Kit (Qiagen), in the pGEM-T Easy vector (Promega). The cloned fragment in the resulting recombinant vector pTnTY-Bn was sequenced in an automated sequencer using the Ready Reaction Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The obtained sequence showed a 95% identity with that of the TYLCSV Sicily strain (Gorsane *et al*., 2004).

Results

The probe

The Primer Express Software™ (Applied Biosystems) was used to select the probe specific for the conserved region of TYLCSV CP gene (Table 2). For this purpose, the sequence of the cloned fragment in pTnTY-Bn was employed (Fekih-Hassen *et al*., 2003), Theoretically, a probe should have a T_m by about 10°C higher than that of the primers and, the length below 30 nucleotides, no single nucleotide repetition (prevention of secondary structures), and no G at the 5'-end

Table 1. Samples collected from open fields and greenhouses in Tunisia in 2002–2003

Plant	No. of samples collected in regions					
	North	Cap Bon	Sahel	Middle	South	Total
Tomato	1	15	17	3	15	51
Pepper	0	0	3	0	3	6
Bean	0	2	1	0	0	3

Table 2. Characteristics of the probe and the primers tested for the real-time PCR

		% GC	T_m (ºC)	Position of CP gene (5'-3')	Sequence (5'-3')
Probe		63	69	192–215	CCTCCGGGTTGTGAAAGGTCCCTG
Forward Primers	PTYLCS1	40	58	154–178	GCTACGGATGTACAGAATGACAAAA
	PTYLCS2	42	59	142–174	CAAAAGCTACGGATGTACAGAATGAC
Reverse primers	PTYLCR1	50	58	217–238	CGCTGCTCATTACGACTGAACT
	PTYLCR2	43	58	239–261	ACCAGTATGCTTCACGTTCATCA

(prevention of reporter quenching). Such a probe was synthesized with a reporter dye (FAM, 6-carboxyfluorescein) at its 5'-end and a quencher (TAMRA, 6-carboxytetramethylrhodamine) at its 3'-end (Applied Biosystems). The probe was tested at two concentrations, 100 and 200 nmol/l, in real-time Taqman® PCR. As these two concentrations did not give significantly different amplification, the lower one, being less costly, was chosen as optimal.

The primers

The Primer Express Software™ was also used to design the primers specific for TYLCSV CP gene. Two reverse and two forward primers, non-overlapping the probe, with GC content over 40% and T_m at least 10°C lower than that of the probe were synthesized (Table 2).

The four combinations of the two forward and two reverse primers were tested for amplification of the TYLCSV CP gene by SYBR® Green I real-time PCR using pTnTY-Bn. As optimal primer pair showing the lowest Ct and highest ΔRn values was evaluated PTYLCS2/PTYLCR1 (Table 3). Besides, it gave reproducible results. When this primer pair was tested at three different concentrations, 100, 300 and 900 nmol/l, the highest one was found as optimal (data not shown).

The optimized assay

The optimized real-time TaqMan® PCR with 900 nmol/l primers and 100 nmol/l probe was tested on 5 TYLCSV-positive and 5 TYLCSV-negative samples. Fluorescence was obtained with positive (Ct <37) but not with negative samples (Ct ≥37).

To test the quantification capability of this assay, it was run with different concentrations of pTnTY-Bn in duplicate. A linear dependence of Ct values on the logarithm of initial plasmid DNA copy numbers was established (Fig. 1). The slope of the line was –3.25 with $R^2 = 0.99$, indicating a PCR efficiency of nearly 100% and a detection range of 6 logarithm units. The Ct value of 37 refers to the number of amplification cycles required to consider a tested sample positive for TYLCSV. The threshold sensitivity of the assay was 5 copies of the virus genome.

Table 3. Selection of optimal primer pair

pTnTY-Bn concentration	Primers							
	S1R1		S1R2		S2R1		S2R2	
	ΔRn	Ct	ΔRn	Ct	ΔRn	Ct	ΔRn	Ct
NTC	–	40	–	40	–	39,28	–	40
	–	40	–	40	–	39,29	–	40
NTC	–	40	–	40	–		–	40
	–	40	–	40	–	39,23	–	40
54.10^{-4} µg/µl	$5,06.10^{-1}$	29,80	$5,12.10^{-1}$	31,62	$6,97.10^{-1}$	28,85	$5,32.10^{-1}$	31,62
	$5,65.10^{-1}$	29,31	$5,01.10^{-1}$	31,50	$7,69.10^{-1}$	27,88	$4,98.10^{-1}$	32,01
54.10^{-5} µg/µl	$4,33.10^{-1}$	32,84	$3,69.10^{-1}$	34,25	$5,79.10^{-}$	32,13	$3,52.10^{-1}$	34,61
	$4,05.10^{-1}$	32,58	$3,69.10^{-1}$	34,96	$5,38.10^{-1}$	31,80	$2,88.10^{-1}$	35,45
54.10^{-5} µg/µl	$1,60.10^{-1}$	38,02	$1,46.10^{-1}$	38,79	$3,42.10^{-1}$	35,67	$1,16.10^{-1}$	39,39
	$2,07.10^{-}$	37,23	$9,77.10^{-2}$	39,73	$2,92.10^{-1}$	36,20	$4,44.10^{-2}$	40
54.10^{-7} µg/µl	$2,06.10^{-2}$	40	$-1,58.10^{-2}$	40	$8,50.10^{-2}$	39,92	$1,03.10^{-1}$	39,31
	$6,61.10^{-2}$	40	$4,75.10^{-2}$	40	$1,25.10^{-2}$	38,80	$4,06.10^{-2}$	40
54.10^{-8} µg/µl	–	–	$-4,49.10^{-3}$	40	$2,38.10^{-2}$	40	$4,73.10^{-2}$	40
	$-3,69.10^{-3}$	40	$9,74.10^{-2}$	39,51	$2,93.10^{-2}$	40	$3,07.10^{-2}$	40

Regarding the specificity of the assay it did not detect TYLCV.

The suitability of the real-time TaqMan® PCR for detection and quantification of TYLCSV was tested on 60 plant samples. Twenty-seven tomato and one pepper sample tested positive with viral load ranging from 1.5 to 26,070 viral genome copies/µg DNA, while all bean samples tested negative. No correlation between disease symptoms and viral load was observed. When the same 60 samples were tested by dot-blot hybridization using a broad-range probe for TYLCSV CP at low stringency, only 5 samples were positive (data not shown).

Discussion

The technology of real time PCR has revolutionized plant virus diagnostics. This study describes application of this technology to TYLCSV, a widespread viral pathogen attacking tomato, pepper, and bean plants in Tunisia. Using the sequence of the CP gene of the TYLCSV Bn isolate, the only isolate reported from Tunisia, the corresponding primers and probe were designed for a real-time PCR, based on TaqMan® chemistry. An optimal primer pair and its concentrations were selected by means of a SYBR® Green I real-time PCR.

Using the optimized real-time PCR, based on TaqMan® chemistry, 28 of 60 plant samples were found positive for TYLCSV. When the same samples were tested by dot-blot hybridization, only 5 samples were positive. This proved the superiority of the developed assay in terms of sensibility as compared to a conventional detection method.

All the samples showing typical TYLCSV infection symptoms were also positive by the real-time PCR. Some samples showing only atypical symptoms (e.g. light yellowing) were also positive by this assay. However, no correlation between the symptoms and the viral load was found. This observation may be explained by the fact that the symptom expression depends on several factors such as viral strain, plant cultivar, time-point of viral infection, co-infection with other viruses, environmental conditions, etc. In fact, many viruses are known to infect tomato crops in Tunisia (Ben Moussa *et al*., 2000). Of the begomoviruses causing tomato yellow leaf curl disease in Tunisia only TYLCSV was detected.

Bean samples showing yellowing symptoms, tested in this study, were negative for TYLCSV despite this virus was reported to infect beans in Tunisia (Gorsane *et al*., 2004). The observed symptoms could be explained by other virus infections or environmental conditions.

The sensitivity threshold of the assay was estimated at 5 copies of viral genome. This value is comparable to those reported for real-time PCR for other viruses, in particular Sugarcane yellow leaf virus (Korimbocus *et al*., 2002), Tomato spotted wilt virus (Roberts *et al*., 2000), Cymbidium mosaic potexvirus and Odontoglossum ringspot tobamovirus (Eun *et al*., 2000). Such a threshold will allow virus detection before appearance of symptoms and then discarding of infected plants to stop the virus spread.

A real-time PCR, in general, is highly specific for the target virus, provided optimal probe and primers are used. As an accurate quantification of viral load is possible already at the early stage of the amplification and gel electrophoresis of the products is not necessary, a real-time PCR is faster, simpler and more reliable as compared to an end-point PCR (Heid *et al*., 1996). Moreover, in contrast to the qualitative character of the latter assay, the former assay enables quantification of viral DNA. Similarly to a multiple qualitative end-point PCR, in which several probes detect several viruses simultaneously (Anfoka *et al*., 2005), a multiple quantitative real-time PCR may be developed in future.

The real-time PCR seems to be the most useful tool available for TYLCSV detection and quantification, since it combines sensitivity, specificity and rapidity. It has a potential to replace hybridization and qualitative end-point PCR assays for large scale TYLCSV monitoring. The protocol described here was developed using one isolate of TYLCVS (Tunisian Bn isolate), but it is expected to be applicable to other TYLCSV isolates and strains.

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