MOLECULAR EXAMINATION OF TOMATO PLANTS WITH *TYLCV*- LIKE SYMPTOMS

Deaghileva Angela, Mitin Valentin, Pasha Lilia, Tumanova Lidia

Institute of Genetics, Physiology and Plant Protection of Academy of Sciences of Moldova

Rezumat

Îngălbenirea şi răsucirea frunzelor de tomate este una din cele mai distructive boli ale tomatelor în zonele tropicale şi subtropicale determinând pierderea completă a culturilor în cazul infectării la etapele timpurii de dezvoltare a acestora. Agentul care determină dezvoltarea bolii – virusul îngălbenirii şi răsucirii frunzelor de tomate (*Tomato Yellow Leaf Curl Virus – TYLCV*) este transmis de *Bemisia tabaci.* Dat fiind faptul că pe loturile experimentale ale Institutului de Genetică, Fiziologie şi Protecţie a Plantelor al AŞM au fost depistate plante de tomate cu simptome de îngălbenire şi răsucire a frunzelor, a fost efectuat screening-ul molecular al acestora în vederea *PCR*-identificării infecţiei cu *TYLCV.* În acest scop a fost realizat design-ul a şase primeri oligonucleotidici specifici, omologi fragmentelor conservative din genomul complet al *TYLCV* din *GenBank.* În rezultatul analizelor nested-PCR efectuate, în mostrele de tomate studiate nu au fost identificate secvenţe omoloage *TYLCV*, fapt ce ar indica asupra lipsei virusului în acestea.

Cuvinte-cheie: tomate, *Tomato yellow leaf curl disease,* boala îngălbenirii şi răsucirii frunzelor de tomate, *Tomato Yellow Leaf Curl Virus* - virusul îngălbenirii şi răsucirii frunzelor de tomate, *PCR*, *nested-PCR*.
Depus la redacţie 11 iulie 2016

Adresa pentru corespondenţă: Deaghileva Angela, Institutul de Genetică, Fiziologie şi Protecţie a Plantelor al Academiei de Ştiinţe a Moldovei**,** str. Pădurii 20, MD – 2002 Chişinău, Republica Moldova; e-mail: show2003@yandex.ru; tel.: +373 22 660434

Introduction

Tomato yellow leaf curl disease (*TYLCD*) is the most destructive disease of tomato in tropical and subtropical zone of the world. *TYLCD* symptoms are manifested in curling and yellowing of leaves and in a general stunting of the plants. At the early stage of plant development the infection can due to yield losses up to 100 % [2]. Presently assumed, that 6 species and 15 strains of *TYLCV*-like viruses are involved in *TYLCD* development [6]. They all are transmitted by *Bemisia tabaci* and belonge to the genus *Begomovirus*, the family *Geminiviridae.* All members of the genus *Begomovirus* have the same morphology, so their visual identification is difficult. Serological methods, such as ELISA, also do not allow reliable diagnostic of the *TYLCV*-like viruses. Molecular methods based on the polymerase chain reaction (PCR) are used for the efficient detection of causative factor of *TYLCD* [1, 2]. The essential viral agent of *TYLCD* was defined on the basis of accumulated molecular data and named *Tomato yellow leaf curl virus*-Israel. Another strain associated with *TYLCD* was sequenced later and named *Tomato yellow leaf curl virus*-Mild [3, 11].

These two strains are accepted as a basis for the molecular identification of *Tomato yellow leaf curl virus* (*TYLCV*). In Moldova, as well as in the European Union, *TYLCV* is listed as quarantine organism [4, 7].

The aim of this work was molecular testing of tomato from the experimental fields of IGPPP of ASM with symptoms of *TYLCD.* Screening of the tomato plants for the molecular identification of *TYLCV* was performed.

Materials and methods

Different symptomatic tomato plants with curling and yellowing leaves were tested. Total DNA from leaves and pathogens were isolated together from the infected plants by a modified CTAB method [9]. Internal control primers have been designed on the basis of evolutionarily conserved sequences of 18S ribosomal RNA gene from *GenBank* [10]. The sequences of the primers are as follows: 5'-ttcgggaccggagtaatgatt-3' ($n64^+$), 5'-caaccataa- acgatgccgacta-3' ($n65^+$), 5'-gcccttccgtcaattccttt-3' ($n66^-$), 5'-catgcaccaccacccataga-3' ($n67^-$).

The PCR mixture, in a volume of 25 µl, contained: 66 mM tris-HCl (pH-8,4), 16 mM $(NH_4)_2SO_4$, 2,5 mM $MgCl_2$, 0,1% Tween 20, 7% glicerol, 100 µg · ml^{-1} *Bovin Serum Albumin*, 0,2 mM dNTPs of each, 1,25 U *Taq DNA* polymerase (*Fermantas*), 5 pM of each primers, and 5-10 ng DNA. The parameters for the PCR were optimized as follows: 1 cycle: 95^{0}C – 4 min, 60 ^{0}C – 1 min, 72^{0}C – 1 min; 34 cycles: 95^{0}C –1 min, 60^{0}C – 1 min, 72^{0}C – 1 min. The first round of the *nested*-PCR included 1 cycle: 95 ^{0}C – 4 min, 55^{0}C – 2 min, 72^{0}C – 1 min; 4 cycles: 95^{0}C – 1 min, 55^{0}C – 2 min, 72^{0}C – 1 min; followed by 30 cycles: 95^{0}C –1 min, 60^{0}C – 1 min, 72^{0}C–1 min. The second round conditions of the *nested*-PCR were: 30 cycles: 95^{0}C–1 min, 60^{0}C – 1 min, 72^{0}C – 1 min.

The products of amplification were divided into 1,5 % agarose gel by electrophoresis (5-8 V/cm) in a migration buffer of Tris-HCl/borate/EDTA (pH 8.0) with ethidium bromide, viewed in the UV (302 nm) and photographed.

Results and discussion

About 25 leaf samples from tomato plants with *TYLCV*-like symptoms as curling, yellowing and stunting were collected from the experimental fields of IGPPP of ASM. Design of 6 specific primers was implemented to identify the viral DNA in infected plants by PCR analysis. Description of primers used in this study for the amplification of *TYLCV* is presented in table 1.

Table 1. Description of primers for the amplification of *Tomato yellow leaf curl virus*

Primer	Sequence (5' → 3')	Orientation	Genome region
cv1	CCAATCAAATTGCATCCTCAAACG	Forward	IR
cv2	CTGTTCACGGATTTCGTTGTATGT		V2
cv3	TGGGCCACGATTTAATTAGGGAT		V2
cv4	ACATGGGCCTTCACATCCAC	Reverse	V1
cv5	TGACCTGATTAGTGTGATTCTGCT		V1
cv6	GTTGCGGTACTGGGCTCAT		V1

Primer design niceties are essential for a successful PCR reaction. Highly conserved regions of *TYLCV* complete genome sequence from *GenBank* databases for primer creation were selected: intergenic region, which contains motifs for viral replication and transcription, areas of V1 and V2 genes [12]. The V1 gene is involved in the virus movement, the V2 gene encodes the coat protein of the virus [5]. Specificity of PCR depends on the melting temperature (T_m) of the primers. So, the T_m of all created primers is similar and close to the 60^0 C. Primer sizes are from 19 to 24 bases, they contain 40-60% GC. The 3'-end of each primer includes no more than three G or C bases in avoidance of nonspecific annealing of the primer.

Primers position on the complete genome sequence of *TYLCV* (*GenBank*: *Tomato yellow leaf curl virus* – Mild, ACCESSION EF054894) are indicated on figure 1.

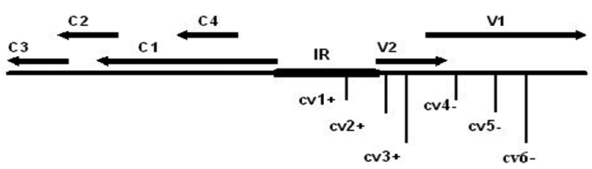

Figure 1. Primers position on the complete genome sequence of *Tomato yellow leaf curl virus* (2791 bp). IR - intergenic region. V1, V2, C1, C2, C3, C4 – viral genes. Arrows show open reading frames in viral sequence.

An internal positive control was used to test for the presence of PCR inhibitors in DNA samples. Primer pairs were used to amplify specific fragments: the fragment of 315 bp was amplified using the primer pair n64-n66, 436 bp – n64-n67, 138 bp – n65-n66 and 259 bp – n65-n67. The result of application of these primer pairs on the tomato DNA is shown on figure 2. Consequently, the fragments of expected sizes were revealed for all tomato DNA samples.

In spite of the application of various PCR conditions (annealing temperatures were 50°C, 55°C, and 60°C, different combinations of primer pairs were used), sequences homologous to *TYLCV* in the studied samples of tomato were not detected. Thus, the

internal control is determined, but the target sequence is not identified. This result indicates that the reaction of amplification is successful and that the target sequence is absent. To substantiate this result, the epidemiological situation in Europe was analyzed. According to the EFSA data, *TYLCV* is presented only in 7 countries from the 28 Member States of EU: Cyprus, France, Greece, Italy, Malta, Portugal, and Spain [1, 6]. Due to the fact, that *TYLCV* is adapted to tropical and subtropical climates, low temperatures lead to abolition of the infection.

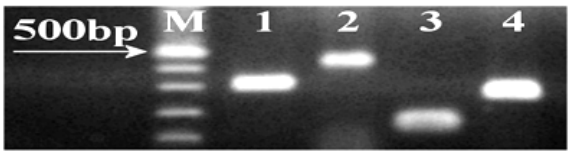

Figure 2. PCR-amplified products of the tomato DNA using primers to 18S ribosomal RNA gene. 1. n64-n66, 2. n64-n67, 3. n65-n66, 4. n65-n67. M. 100 *bp Ladder DNA marker.*

On the other hand, symptoms like leaf yellowing, curling and deformation, flower and fruit abscission, general stunting of the plant are inherent not only to *TYLCD*. So, the same tomato DNA samples were examined for the presence of other pathogens. PCR analysis with primers to *Alternaria* spp. and *Fusarium* spp. was performed. The set of primers, applied in this study, is shown in table 2.

Table 2. Primers to *Alternaria* spp. RNA polymerase II second largest subunit (rpb2) gene and *Fusarium* spp. final elongation factor 1a (tef1) gene.

Primer	Sequence (5' → 3')	Orientation
Alternaria		
ac3	GTGTCTGGGTTGGTGTCCAT	Forward
ac4	ACGGCCAGCATCTGTGAAG	Reverse
Fusarium		
fc3	CCATCGAGAAGTTCGAGAAGGTT	Forward
fc4	CCCAGGCGTACTTGAAGGAA	Reverse

In the studied tomato plants with symptoms of leaf yellowing, curling and deformation *Alternaria* spp. infection was detected. Mixed infection of both pathogens (*Alternaria* spp. and *Fusarium* spp.) was found in some samples. The results of PCR via primers to *Alternaria* spp. and *Fusarium* spp. are shown on figure 3.

Alternaria spp. was detected in all samples of DNA from five symptomatic tomato plants. The primer pair ac3-ac4 was used, which amplified a 145 bp nucleotide fragment: lines 1-5 on figure 3. Presence of *Fusarium* infection in the same samples was detected for two instances: lines 6 and 9 (Figure 3). A fragment of 300 bp was obtained as a result of the primer pair fc3-fc4. Sizes of both amplicons are as expected.

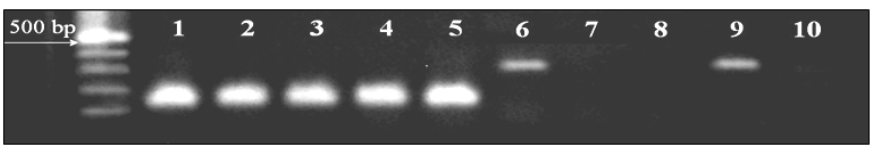

Figure 3. Electrophoresis analysis of *PCR*-amplified products of the total tomato DNA, using primer pairs to *Alternaria* spp.: ac3-ac4 (1-5); *Fusarium* spp.: fc3-fc4 (6-10). M -100 *bp Ladder DNA marker.*

Conclusions

For the first time in Moldova molecular diagnosis of the tomato plants with symptoms of *TYLCD* was performed. The design of specific primer set for *TYLCV* detection was carried out. Sequences homologous to *TYLCV* in the studied samples of tomato from the experimental fields of IGPPP of ASM were not detected. *TYLCV* identification based only on symptomatology is unreliable because similar symptoms may be caused by other phytopathogens or varies greatly depending on the soil, growth conditions, and climate. However, other species of pathogens were identified in studied symptomatic tomato plants, such as *Alternaria* spp. and *Fusarium* spp.

This work was supported by the fundamental scientific research project "Technology of molecular diagnostics of phytopatogens", financed from the state budget for the years 2015-2018.

References

1. EFSA Panel on Plant Health (PLH). Scientific Opinion on the pest categorization of *Tomato yellow leaf curl virus* and related viruses causing tomato yellow leaf curl disease in Europe. //EFSA J., 2014, 12(10):3850, 27 pp.

2. Hanssen I., Lapidot M. Major Tomato Viruses in the Mediterranean Basin. //In: Adv. in Virus Research (Ed. by Loebenstein G., Lecoq H.), Academic Press is an imprint of Elsevier, 2012, vol. 84, p. 33-36.

3. Hosseinzadeh M., Garivani M. Emerging two distinct groups of the *Tomato Yellow Leaf Curl Virus* – severe strain (*TYLCV*-IL) variants in Iran. //Trakia J. of Sciences, 2014, No 2, p. 149-161.

4. HOTĂRÎRE Nr. 356 din 31.05.2012 pentru aprobarea unor acte normative privind implementarea Legii nr. 228 din 23 septembrie 2010 cu privire la protecţia plantelor şi la carantina fitosanitară.

5. Huoying C., Rongrong F., *Xiang L.* (Tomato yellow leaf virus (TYLCV): The structure, ecotypes and the resistance germplasm resources in tomato. //African J. of Biotech., 2011, vol. 10(62), p. 13361-13367.

6. Invasive Species Compendium. /Last modified 19 April 2016/, http://www.cabi.org/isc/datasheet/55402 (visited 26.05.16; 16:20)

7. NVWA list of Q organisms and other pests (Dutch): Register: Q-organismen, Versie: 2.43, 9-3-2016. http://www.bureaubiosecurity.nl/en/Biological_agents/List_of_ quarantine_ organisms_Plant_pathogens (visited 31.05.16; 14.20).

8. Péréfarres F., *Thierry M*.**,** *Becker N. et al.* Biological Invasions of Geminiviruses: Case Study of TYLCV and *Bemisia tabaci* in Reunion Island. //J. *Viruses,* 2012, vol. 4, p. 3665-3688.

9. Saghai-Maroof M., Soliman K., Jorgensen R.. et al. Ribosomal DNA spacer-length polymorphisms in barley. Mendelian inheritance, chromosomal location, and population dynamics //Proc. Natl. Acad. Sci. USA, 1984, vol. 81, p. 8014–8018.

10. Sequences of 18S ribosomal RNA gene. *htp:/www.ncbi.nlm.nih.gov.*

11. Shirazi M., Mozafari J., Rakhshandehroo M. et al. Genetic diversity, host range, and distribution of tomato yellow leaf curl virus in Iran. //J. Acta virologica, 2014, No 58, p. 128 – 136.

12. Tomato yellow leaf curl virus (*TYLCV*) complete sequence. https://blast.ncbi.nlm.nih.gov/Blast.cgi