CZU: 632.9:635.64

<https://doi.org/10.53040/gppb7.2021.20>

QPCR DETECTION AND QUANTIFICATION OF '*Candidatus phytoplasma solani*' IN TOMATO WITH PRIMERS TARGETING CPN60 GENE

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Abstract

Tomato is one of the most economically important crops in Republic of Moldova. However, it is affected by a number of pathogens. One of the wide spread diseases is stolbur caused by the infection agent '*Candidatus Phytoplasma solani*'. Accurate diagnostics of the disease at an early stage is essential for successful control of the disease. In this work, we describe detection and quantification of '*Candidatus Phytoplasma solani*' in tomato by real-time PCR, as well as suitability of the method for assessing resistance of different tomato varieties to *Phytoplasma* infection.

Key words: '*Candidatus Phytoplasma solani*', stolbur, tomato, real-time PCR

Introduction

Tomato is one of the most economically important crops in Moldova. However, it is affected by a number of diseases, which reduce fruit quality and yield. One of the important diseases causing significant yield loss and negatively affecting fruit quality is stolbur. It is caused by the infection agent '*Candidatus Phytoplasma solani*' (Favali et al.). Accurate diagnostics at an early stage is essential for successful control of the disease. However, the diagnostics is often difficult due to non-specific symptoms, similar to some viral and fungal diseases. Besides, many symptoms manifest in the later stage of the infection, when it is too late for the disease control measures to be effective. The impossibility of cultivation of phytoplasmas *in vitro* makes diagnostics even more complicated. Thus, accurate detection of '*Ca.P. solani*' in tomato is important for efficient control of the stolbur disease. One of the ways of accurate and timely detection of *Ca. P. solani* in tomato is application of molecular genetics techniques, providing rapid and reliable diagnostics of phytoplasma disease (Marzachi et al.). The most affordable, and thus the most promising, are PCR-based techniques, which involve DNA extraction followed by amplification of a specific fragment by a pair of primers and detection of the amplified fragment. PCR-based methods include conventional PCR, nested PCR, real-time PCR. Currently, the most widely used PCR-based method for detection of '*Ca.P. solani*' is nested PCR, since it provides high sensitivity and specificity (Gundersen et al.). However, it is time consuming, requires two rounds of amplification, and is poorly suitable for quantitative analysis. A fast alternative, which gives an opportunity of quantification of the pathogen, is a real-time PCR-based method. However, this sets additional requirements to the primers, since those will have to show high specificity and sensitivity, being able to amplify the target sequence in a single round, and high efficiency which will make them suitable for quantitative analysis.

In this work, we detected and quantified '*Ca. P. solani*' DNA in tomato DNA samples by SYBR-Green based real-time PCR. We developed specific primers for real-time PCR detection of '*Ca.P. solani*', tested them, and quantified the number of copies of '*Ca.P. solani*' DNA in the DNA sample of plants of two tomato varieties. These varieties were previously reported to have contrasting resistance to phytoplasma infection, so one could expect that they would have different number of copies of '*Ca.P. solani*' chaperonin gene, with less resistant variety having more copies of '*Ca.P. solani*' DNA than the more resistant one.

Materials and methods

The tomato varieties, used in this study, Elvira and Cerasus, were bred in the Institute of Genetics, Physiology and Plant Protection (Chisinau, Moldova). Elvira was previously reported to have a higher sensitivity to phytoplasma infection, than Cerasus (Zamorzaeva et al.). For DNA extraction, 12 plants of each variety were pulled, and DNA was extracted by DNA-zol (Thermofisher) method, as described in

the Manufacture's protocol. The concentration of purified DNA was measured by Nanodrop, and 9 ng of purified DNA was used per reaction. For real-time PCR analysis, we developed 2 pairs of primers from the unique chaperonin-60 (cpn60) gene of '*Ca. P. solani* (Dumoncaux et al.)'. Primers met the requirements for real-time PCR primers: no more than 2 GC pairs on the 3' end, 5' and 3' self-complementarity no more than 5. Primer pairs used in this study are shown in table 1:

Table 1. Primer pairs used for real-time PCR analysis

name	Sequence	Temp late	Length, bp	Start	Stop	Tm, °C	CG%	Self5'	Self3'
qfys5	CAGGAGCAAATCCGATGCTAGTT	plus	23	53	75	60.99	47.83	5.00	0.00
qfys6	CGCCGTTTTTGCCCACTTTT	minus	20	244	225	60.81	50.00	3.00	0.00
qfys7	GGATCGAACTTGCTGCTCAAAC	plus	22	83	104	60.41	50.00	4.0	0.0
qfys8	CCGTTTTTGCCCACTTTTCCAT	minus	23	242	220	60.99	43.48	2.0	2.0

This table indicates primer name, sequence, orientation, length. It gives its location on the chaperonin-60 (cpn60) gene(KJ939980.1), number of start and stop nucleotide for primers, CG content, and 5' and 3' self-complementarity. PCR conditions were as recommended by SybrGreen producer (Applied Biosystems) -initial incubation at 50°C for 2 minutes, initial denaturation at 95°C for 2 minutes, and alternation of 95°C for 15 sec and 60°C for 1 minute for 40 cycles. The reactions were performed in 96-well plates (BIORAD) in BIORAD CFX96 touch real-time PCR machine. The detection was done at SYBR channel. Real-time PCR primer efficiency was calculated using qPCR efficiency calculator:

<https://www.thermofisher.com/ro/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html>

For real-time PCR standard, a fragment was amplified by conventional PCR using primer pair qfys7 and qfys8. Then the fragment was visualized on the gel, excised and purified as described (Abraham et al.). The DNA concentration of the purified DNA fragment was determined by Nanodrop spectrophotometer. Given the fragment size 160 bp, the copy number per 1 ng of fragment DNA was determined, using an on-line calculator:

<https://www.thermofisher.com/ro/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html>

Results and discussions

First, the primers were experimentally tested to assess their specificity and efficiency, and their optimal concentration in the reaction. For specificity testing, the dissociation curve was obtained for both primer pairs. Figure 1 shows the dissociation curve of the fragment, amplified by the primer pair qfys5-qfys6, with the DNA of tomato infected with '*Ca. P. solani*' as a template. For primer and DNA optimal concentration testing, the fragment was amplified in the PCR reaction containing 200 nM (1a, 1b) and 400 nM (1c, 1d) of each primer, and no dilution (1a, 1c) and 10-fold dilution (1b, 1d) of the template DNA. As one can see, the higher primer concentration resulted in higher fluorescence, yet did not cause the formation of primer dimers (Figure 1c), so we can observe a single sharp pronounced peak. However, this primer pair was not able to amplify the fragment from a more diluted template (Figure 1b,d).

Figure 1. The dissociation curve of the fragment, amplified by the primer pair qfys5-qfys6. Primer concentration: 200 nM each (a, b); 400 nM each (c,d). Template dilutions: non-diluted (a,c), 10-fold dilution (b,d).

Figure 2 shows the dissociation curve of the fragment, amplified by the primer pair qfys7-qfys8, with the DNA of tomato infected with '*Ca. P. solani*' as a template. For primer and DNA optimal concentration testing, the fragment was amplified in the PCR reaction containing 200 nM (2a, 2b) and 400 nM

(2c, 2d) of each primer, and no dilution (2a, 2c) and 10-fold dilution (2b, 2d) of the template DNA. The higher primer concentration resulted in higher fluorescence, yet did not cause the formation of primer dimers (Figure 2c), so we can observe a single sharp pronounced peak. This primer pair was able to amplify the fragment even in the reaction with the diluted template (Figure 2b, 2d). So, this primer pair was used for further analysis, at a concentration of 400nM each.

Figure 2. The dissociation curve of the fragment, amplified by the primer pair qfys7-qfys8. Primer concentration: 200 nM each (a, b); 400 nM each (c,d). Template dilutions: non-diluted (a,c), 10-fold dilution (b,d).

Then, the efficiency of the primer pair qfys7-qfys8 was evaluated. The DNA fragment was amplified by the same primer pair in a conventional PCR, and diluted to be used as a template for the qPCR reaction. Four dilutions (10-fold, 100-fold, 1000-fold and 10000-fold) of DNA of the initial PCR reaction were made, used as a template in real-time PCR reaction, Ct values were measured, and the concentration curve was built for calculating primer efficiency.

The primer efficiency graph built from the serial dilutions is shown in Figure 3a. The slope of -3.3183 indicates almost 100% primer efficiency, therefore this primer pair can be used in real-time PCR assays for measuring '*Ca.P.solani*' DNA concentrations.

Figure 3. The primer efficiency graph for primer pair qfys7-qfys8 and the relationship between the initial copy number of the template in the reaction and the Ct value.

So, the primer pair qfys7-qfys8 was used for quantifying '*Ca.P.solani*' DNA in tomato samples. For this, serial dilutions of DNA standard with known copy number was analyzed by real-time PCR, the Ct values were recorded and used to build a standard curve. Figure 3b shows the dependence of the Ct value from the initial number of copies of the template in the reaction.

Using the relationship between the number of fragment copies and the Ct value, the number of copies of '*Ca.P. solani*' in each sample was calculated. Figure 4 shows the results of the quantitative analysis of phytoplasma in 2 tomato genotypes: Elvira and Cerasus.

As one can see from the figure, the number of copies of '*Ca.P.solani*' DNA in the DNA sample of Elvira variety is twice as high as in that of Cerasus. This is consistent with previous findings that Cerasus is more resistant to phytoplasma infection than Elvira.

Figure 4. Quantification of 'Ca.P. solani' in tomato varieties Elvira and Cerasus.

Conclusions

As a result of this work, we developed a pair of primers for quantification of 'Ca.P. solani' in tomato, set the optimal conditions, and tested them using DNA of two tomato varieties with contrasting resistance to phytoplasma infection. The real-time PCR method can be successfully used for detection of phytoplasma infection and for its quantification as well as for comparing the resistance of tomato varieties to phytoplasma infection. There is a potential for using these primers for detection and quantification of 'Ca.P. solani' in crops other than tomatoes.

Acknowledgements

We would like to express our sincere gratitude to Zgardan D., Sturza R., Technical University of Moldova for providing us with laboratory facilities (real-time PCR).

This work was funded by the bilateral project STCU #6378 «Development of a new technique for assessing the resistance of tomatoes to the phytoplasma», funded by the European Union and the Republic of Moldova, and the State Project 20.80009.5107.11 «Long-term ex situ conservation of plant genetic resources in the Gene Bank using the methods of molecular biology for plant germplasm health testing», financed by the National Agency for Research and Development.

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