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Development of a real-time RT-PCR assay for detection of tomato mottle mosaic virus

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Abstract

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Tomato mottle mosaic virus (ToMMV) which is belonged to the tobamovirus genus was first identified in Mexico in 2013. According to the Turkish Ministry of Agriculture and Forestry's amendment on notification of emergency measures at World Trade Organizations (WTO), all countries are required to submit a Phytosanitary Certificate stating that the tomato and pepper seeds have been analyzed by the exporting countries using the RT-PCR method, indicating that the product is free of disease-causing agents such as ToMMV.

To obtain a reliable and specific diagnostic method to detect the virus, new primers and probe set, targeting the conserved movement protein region of the ToMMV genome, was designed. *In sillico* assessment by BLAST analysis showed a 100% match to the all ToMMV isolates in NCBI database. After RNA extraction from ToMMV infected plant material the real-time amplification protocol was developed. The protocol was tested for analytical sensitivity, analytical specificity, repeatability and reproducibility obtaining consistent results for all of the validation parameters.

This study is the validated real-time RT-PCR (reverse transcription PCR) detection method for ToMMV strains according to all the parameters required by the new diagnostic EPPO standard PM7/98 (4). This new protocol can be successfully applied at the Quarantine laboratories of the Turkish Ministry of Agriculture and Forestry for routine detection of ToMMV in pepper and tomato seeds.

Keywords: ToMMV; real-time RT-PCR; assay validation

Introduction

Plant diseases caused by bacterial, fungal and viral agents are the major source of economic loss in the agriculture industry worldwide. Monitoring of health and accurate identification of the causal agents of the diseases in plants are essential for sustainable agriculture and disease management (Sankaran et al., 2010).

Tomato (*Solanum lycopersicum* L.) is one of the world's most economically important vegetable crops. Annual tomato production has been increasing every year, but disease incidence and severity have restricted yield and quality, resulting in significant losses. Among the recognized pathogen-induced diseases, viral infections are the primary restrictions in tomato production. The potential of introducing novel viruses and their vectors into production systems increases as worldwide travel and commerce of plant materials increases. Furthermore, changing climate conditions can aid in the effective spread of the viruses (Hanssen et al., 2010).

Tomato-infecting tobamoviruses including Tomato brown rugose fruit virus (ToBRFV), Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV) are seed-borne viruses that could be easily transmitted to tomato crops. Tomato mottle mosaic virus (ToMMV) which is also belonged to the tobamovirus genus was first identified in Mexico in 2013 (Li et al., 2013). ToMMV has been detected in tomato crops in many countries across the world such as China, USA, Brazil and Iran (Sui et al., 2018). Capsicum (*Capsicum annuum*) and tomato crops are thought to be seriously affected by ToMMV through breaking resistance (Lovelock et al., 2020). In tomato crops ToMMV can cause foliar mosaic of light and dark green symptoms, chlorosis, and leaf deformation (Webster et al., 2014).

ToMMV exhibits very similar amino acid sequences to the common tobamaviruses such as ToBRFV, TMV and ToMV which can only be distinguished by sequence analysis which also results in cross-reactions between ToMMV and ToMV when using serological tests (Ambrós et al., 2017). Furthermore, in some studies, ToMMV corresponded strains were deposited in GenBank as ToMV which later re-attributed as ToMMV (EPPO Reporting Service, 2021).

In the last decades, rapid and specific serological and molecular techniques for the detection of tomato-infecting tobamoviruses have been developed. However, the widely used virus detection methods such as ELISA fail to establish a definite species-specific identification for tomato-infecting tobamoviruses (Sui et al., 2017). Due to the specific characteristics of the tobamoviruses, developing species-specific molecular-based detection techniques are essential.

It has been recently recommended by EPPO Phytosanitary Measures that the ToMMV should be included in the alert list. Thus the specific detection of ToMMV is very important and the availability of detection methods with high analytical specificity is crucial for reliable differentiation of ToMMV strains (EPPO Reporting Service, 2021).

According to the Turkish Ministry of Agriculture and Forestry's amendment on notification of emergency measures at World Trade Organizations all countries are required to submit a Phytosanitary Certificate stating that the tomato and pepper seeds have been analyzed by the exporting country using the RT-PCR method, indicating that the product is free of disease-causing agents such as Tomato apical stunt viroid (TASVd), Columnea latent viroid (CLVd), Pepper chat fruit viroid (PCFVd) and ToMMV (WTO, 2021).

In this study, we aimed to develop and validate a real-time RT-PCR protocol by the unique primers and a probe for the specific detection of ToMMV in pepper and tomato seeds.

Material and Methods

Plant materials and RNA extraction

All lyophilized virus-infected plant samples were provided from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). About 0.1 g of lyophilized and non-lyophilized leaf tissues were ground in 0.3 ml grinding buffer (0.2 M sodium acetate pH 5.2, 25 mM EDTA, and 2.5% PVP-10) (Dovas et al., 2004) in a plastic grinding bag using a hand homogenizer (Bioreba AG). Total RNA was isolated from the leaves by using a commercially available extraction kit, Plant RNA Purification Kit (Biotechrabbit, Germany).

TaqMan probe and primers design

Multiple sequence alignments of movement protein (MP) gene of different ToMMV isolates which were obtained from the public database of the National Centre for Biotechnology Information (NCBI) were first performed *in silico* using Geneious Prime alignment tool (high inclusivity). A pair of primers (ToMMVF: 5'- TGGGTTTGAGGGAGAAGATTAC-3' starting position at 5424 bp, ToMMVR: 5'- TTAGCAAGCCTGACTGACATAG-3' starting position at 5514 bp) amplifying a 112 bp long amplicon and a TaqMan probe (ToMMVP: *FAM*-5'- TGGATGACGCAGGTCCCATTGAA -3' *BHQ1* starting position at 5451 bp) were designed using online PrimerQuest Tool (https://www.idtdna.com/PrimerQuest/Home/Index).

Real-time one-step RT-PCR assay

Real-time one-step RT-PCR reaction contained: 1x Reaction buffer (Roche Diagnostics), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5 µl of RNA template from ToMMV DSMZ PV-1267 and molecular grade water to a final volume of 25 µl. The samples were placed in a 96-well plate and amplified in a LightCycler® 480 II PCR system (Roche Diagnostics). The PCR conditions were as follows: 8 min at 56°C (Reverse transcription), 30 s at 95°C, followed by 45 cycles: 5s at 95°C and 30 s at 60°C.

Assay validation

For the validation of a diagnostic protocol, the developed method was tested for its analytical sensitivity, analytical specificity, repeatability and reproducibility according to the European and Mediterranean Plant Protection Organization (EPPO) standards (EPPO, 2019).

The analytical sensitivity of Real-time one-step RT-PCR assay was evaluated using seven 10-fold (from 10^{-1} to 10^{-7}) serial dilutions which were prepared by using a starting amount of 40 ng of total RNA extract from ToMMV DSMZ

PV-1267. Three replicates of each dilution were used to generate a standard curve. The limit of detection (LOD) was recorded as the last dilution with a positive result (Loconsole et al., 2010). The slope of the standard curve was used to calculate the amplification efficiency according to the equation $E = 10^{(-1/slope)} - 1$ (Osman et al., 2015). *In silico* Primer-BLAST assessment for analytical specificity of newly designed primers and probe were carried out against ToMMV sequences available on NCBI. Non-target virus samples such as Tomato Brown Rugose Fruit Virus (ToBRFV) (DSMZ PV-1236), Tobacco mosaic virus (TMV) (DSMZ PV-0055), Tomato mosaic virus (ToMV) (DSMZ PV-0135), Pepper mild mottle virus (PMMoV) (DSMZ PV-0093), Cucumber green mottle mosaic virus (CGMMV) (DSMZ PV-0159) were also tested for assay exclusivity. For assay repeatability and reproducibility, three different samples diluted up to the established LOD (10^{-5}) were amplified three times and by two different operators.

Results

Taqman probe and primers design

In order to design the TaqMan probe and primers for specific detection of ToMMV strains; different sequences in the open reading frame 3 (ORF3) region (Movement protein) of ToMMV strains were aligned by following accession numbers (KF477193.1), (KR824951.1), (KT810183.1), (KU594507.2), (MG171192.1), (MH381817.1), (MN654021.1), (MW582804.1). Figure 1 shows the alignment of the sequences, TaqMan probe and primers binding sites.

Assay validation

Analytical sensitivity was performed by generating a standard curve of seven 10-fold (from 10^{-1} to 10^{-7}) serial dilutions of ToMMV DSMZ PV-1267 total RNA extract. The limit of detection was recorded at 10^{-7} dilutions with an average Cq

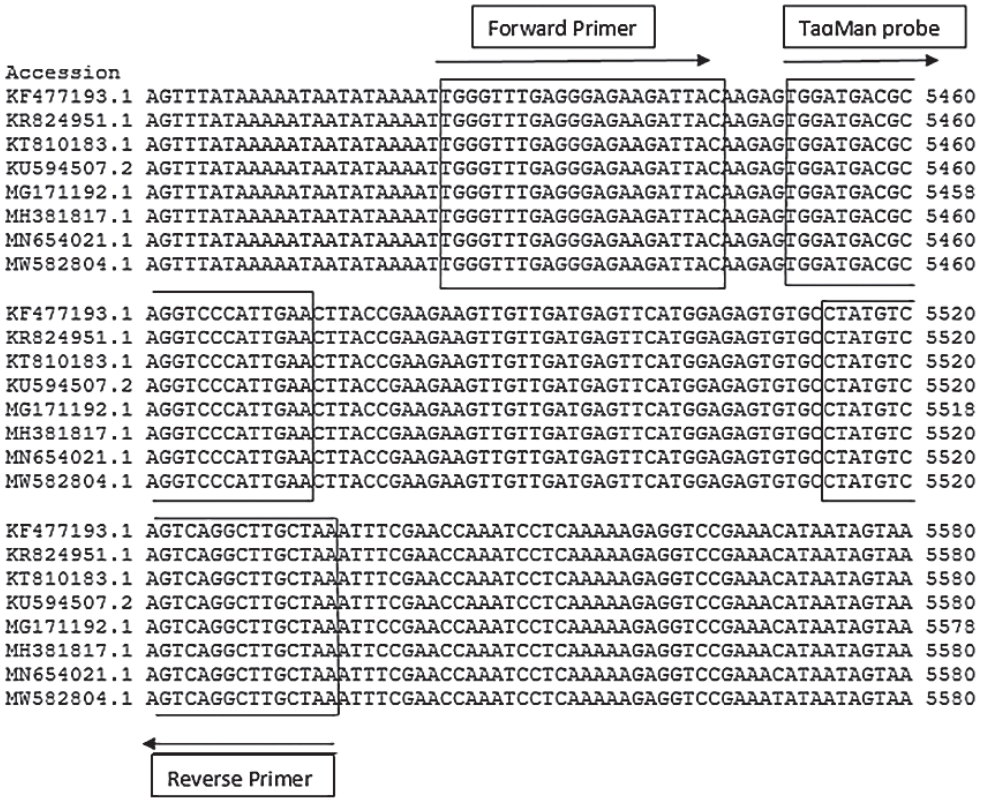

Fig. 1. Nucleotide alignment of partial genomic sequences (180nt) in the open reading frame 3 (ORF3) region (Movement protein) of ToMMV strains: MX5 (KF477193.1), TiLhaLJ (KR824951.1), NY-13 (KT810183.1), VLC-1 (KU594507.2), Hainan (MG171192.1), HN (MH381817.1), 19-02305 (MN654021.1), DSMZ PV-1267(MW582804.1). Alignment was performed by Geneious Prime software. Primers and probe for amplifying 120nt amplicon designed in this study are indicated in boxes

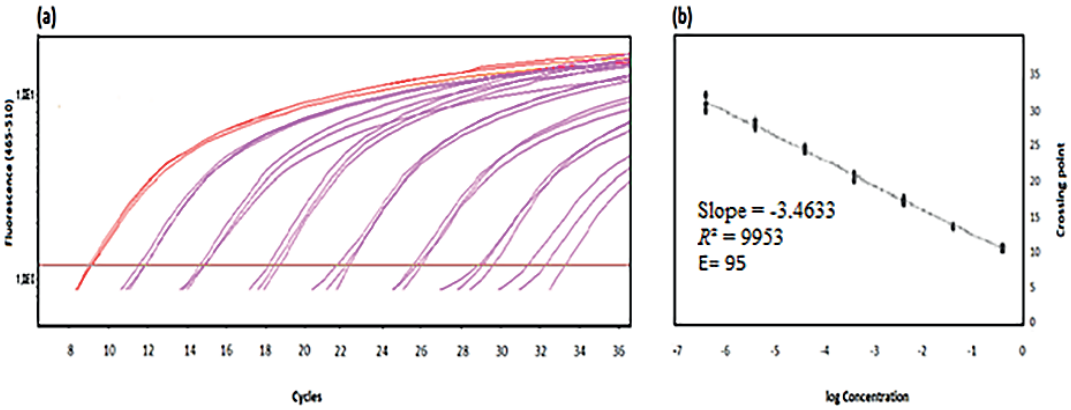

Figure 2. Analytical sensitivity of the Real-time one-step RT-PCR assay.
a) Amplification curves obtained by testing tenfold serial dilutions of quantitated RNA standards obtained from ToMMV DSMZ PV-1267. The x-axis shows the number of PCR cycles. The y-axis shows the fluorescence values on a logarithmic scale. b) Standard curve generated from the above data. The x-axis indicates the logarithm of the RNA concentration and the y-axis shows the CP values

value of 32.026 ± 0.76 (Figure 2). The efficiency of the PCR test was E= 95 with slope of -3.4633 and R^2> 0.99 (Figure 2).

For analytical specificity, *in sillico* assessment by BLAST nucleotide analysis of the newly designed primers and a TaqMan probe showed a 100% match to the other ToMMV isolates retrieved from National Centre for Biotechnology Information (NCBI). For assay exclusivity the following non-target *Tobamovirus* strains were used: Tomato Brown Rugose Fruit Virus (ToBRFV) Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), Pepper mild mottle virus (PMMoV), Cucumber green mottle mosaic virus (CGMMV). No positive result was acquired from tested samples. Repeatability and reproducibility were also 100%.

Discussion

ToMMV infection in plants can be detected using electron microscopy, serological and RT-PCR assays. ToMMV single-stranded RNA (+ssRNA) sense genome contains four ORFs which encode two replication proteins, movement protein and a coat protein which show significant nucleotide similarity with ToMV genome (Sui et al., 2017) resulting in serological cross-reactivity between these two viruses (Li et al., 2021).

RT-PCR based studies were designed to identify tobamoviruses. In order to detect and differentiate TMV and ToMV immunocapture RT-PCR assays were designed based on the conserved regions of movement protein and coat protein genes (Jacobi et al., 1998; Letschert et al., 2002).

Generic RT-PCR assays using degenerate primers were developed based on RNA depended RNA polymerase (RdRp) conserved regions to detect tobamoviruses. However, they were not applicable to detect all tobamovirus groups (Gibbs et al., 1998). A generic RT-PCR assay was also developed to detect nine tobamoviruses including ToMMV (Li et al., 2018). Another study also reported a multiplex RT-PCR method to detect and differentiate TMV and ToMV in tomato and pepper seeds (Kumar et al., 2011).

A spot nested RT-PCR-RFLP for tobamovirus detection was developed utilizing dI-containing and homologous dG-containing primers using RdRp domains of tobamoviruses (Dovas et al., 2004). In a study two detection methods were developed for specific detection of ToMMV, a hybridization method with digoxigenin (DIG)-labelled RNA probes and a one-step RT-PCR assay which primers designed using conserved regions of movement protein gene (Ambrós et al., 2017).

The high specificity and inclusivity together with the absence of cross-reactions with the host genome of several plant species make this real-time PCR test particularly suitable for a specific, quick and reliable detection of ToMMV.

Conclusion

This study is the validated real-time RT-PCR detection method for ToMMV according to the parameters required by the new diagnostic EPPO standard PM7/98. In addition, these primers and probe which are designed using conserved MP gene region match all of the ToMMV isolates. This real-time RT-PCR protocol can be successfully applied for specific detection of ToMMV in pepper and tomato seeds at Quarantine Laboratories of the Turkish Ministry of Agriculture and Forestry.

Data Availability

All data generated or analysed during this study are included in this published article.

Conflicts of interest

The authors declare no conflict of interest.

Authors' contributions

All authors contributed equally.

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