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RT-PCR AND QRT-PCR DETECTION AND CHARACTERIZATION OF THE ISOLATE OF TOMATO SPOTTED WILT VIRUS IN EGGPLANTS

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Abstract

The study was planned after the ring spot symptoms of Tomato spotted wilt virus (TSWV), which is characterized in pepper plants, and also seen in eggplant plants grown in Antalya. In this context, a total of 101 eggplant samples were collected, of which 32 from Alanya, 33 from Serik, and 36 from Kumluca. The symptoms observed in the leaves and fruits of the eggplant plant were associated with the typical ring spot symptom of TSWV in tomato and pepper fruits, and the presence of the virus in the eggplant samples was determined by RT-PCR method. Coat protein-specific primers are incapable of detecting any mutations in the TSWV genome. In this study, a primer pair was designed that enables the identification of TSWV-RB isolate in eggplants infected with TSWV and covers the C118Y mutation region in the genome of the virus. Sequence analysis of the RT-PCR product was performed with primers that amplify the mutation region and because of contig studies with Non-resistance isolates (NRB) that did not break resistance, it was revealed that eggplant plants were infected with TSWV-RB isolate, contained nucleotide change in the C118Y mutation region, and that TSWV-RB isolate was also the host. The primers that identified the TSWV-RB isolate and developed in this study give successful results in qRT-PCR studies and Melt Curve analyzes. Thanks to these primers, it has been possible to detect easily and quickly that the infection in many different hosts such as tomatoes, peppers, eggplants infected with TSWV is caused by the isolate that breaks the resistance.

Keywords: Eggplant, TSWV, Resistance-breaking, RT-PCR, qRT-PCR

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INTRODUCTION

Eggplant (*Solanum melongena*) is a vegetable belonging to the Solanum genus of the Solanaceae family. Eggplant is one of the five most important vegetable crops (tomato, pepper, potato, tobacco, eggplant) in Asia and the Mediterranean (Fidan and Sarikaya 2020). Turkey produced 835 422 tonnes of eggplants in 2020. Antalya, the city where eggplant plants are collected with the virus, which is the subject of this study, and is located in the Mediterranean region, is the province with the highest eggplant production (TUIK 2020). Solanaceae plants are exposed to many pathogens including threatening fungi, bacteria, and viruses. Plant viruses are among the major disease-inducing agents affecting Solanaceae crops as they are transmitted by vegetative reproduction, by contact between infected and healthy plants, and by different vectors, including insects. Eggplant is naturally infected by several viruses that cause disease outbreaks and represent increased agronomic threats (Abou Kubaa et al. 2021).

Tomato spotted wilt orthotospovirus is one of the most common viral diseases in tomatoes. TSWV is a member of the Orthotospovirus genus, Bunyaviridae family (Adams, 2017) and reduces tomato production, especially in greenhouses (Todd et al.,1995). TSWV was first found in 1915 in Australia (Brittlebank, 1919). TSWV virus is transmitted by thrips (Thysanoptera, Thripidae), the most common vector being *Frankliniella occidentalis* in the permanent and circulatory form (Mandal et al., 2001).

Tomato spotted wilt virus infecting eggplant have been reported in different parts of the world such as Italy (Betti 1992); Spain (Soler et al. 2007), Turkey (Kamberoglu 2009), (Fidan and Sarikaya 2020); Syria (Halabi et al. 2014), United States (Badillo-Vargas et al. 2015).

Studies on isolates that break resistance to TSWV continue from past to present. TSWV isolates were obtained from a wide variety of infected host plants (Latham et al. 1998). In 2002, typical symptoms of TSWV were seen in tomato plants that carry the Sw5 gene growing in a field in Barcelona, Spain (Aramburu and Marti 2003). In 2004, the TSWV field isolate was investigated for the ability of the resistance gene to overcome Sw5 by mechanically inoculating F1 hybrid tomato varieties carrying Sw5 (Ciuffo et al. 2005).

Some pepper cultivars shown to be resistant to tomato spotted wilt virus (TSWV) with symptoms commonly associated with TSWV infection were tested and inoculated into the resistant plant Capsicum chinense with infected sap. They published the first report of TSWV strains, which broke the resistance provided by the Tsw gene with the disease of this resistant plant, in Spain (Roggero et al. 2002). Comparison of the nucleotide and amino acid sequences showed that tomato resistance provided by the Sw5 gene could be inoculated with the C to Y amino acid substitution or T120N at position 118 (C118Y) in the TSWV movement protein NSm. Phylogenetic analysis revealed that the C118Y substitution occurred independently three times in isolates studied by convergent evolution, whereas the T120N substitution was a unique occurrence. Analysis of the rates of non-synonymous and synonym changes in individual codons showed that the substituent C118Y was chosen as positive (Lopez et al. 2011). In the summer of 2012, typical symptoms of Tomato spotted wilt disease were detected in tomato plants of different resistant varieties (carrying the Sw5 gene) in different areas in southern Italy. The virus isolate obtained from symptomatic plants that carrying the Sw5 gene was mechanically inoculated by *Frankliniella occidentalis* into several different tomato hybrids. Inoculated tomato plants showed systemic infection occurred several days before local lesions appeared in inoculated leaves because of the typical hypersensitivity reaction caused by the Sw5 gene (Crescenzi et al 2013).

As a continuation of resistance breaking studies, similar studies were conducted by Margaria et al. (2015) in Italy, by Batuman et al. (2017) in California, and by Fidan and Sarı (2018) in Turkey. In contrast to the determination of resistance-breaking isolates in tomatoes, this study was conducted for the detection and symptoms of these isolates in eggplant plants. Two different PCR-based allelic distinction techniques, namely Custom TaqMan™ SNP Genotyping and high-resolution melting (HRM) assays, were developed and compared for their ability to individuate between avirulent resistance-breaking and non-resistance breaking biotypes. In our study, instead of using the Sw5 resistance-breaking isolates of TSWV using

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HRM method (di Rienzo et al. 2018), a very specific primer was developed for the genome of the resistance-breaking isolate and was used successfully.

MATERIALS AND METHODS

Survey and plant collecting

Virus-like symptoms were determined in eggplants grown in Antalya province. Samples were collected from plants showing symptoms such as necrosis, shortening between nodes, necrotic spots, and wilt. (Figure 1). During the surveys, 32 samples from Alanya, 33 from Serik and 36 from Kumluca were collected and kept in appropriate laboratory conditions.



Figure 1. Eggplant leaves and fruits showing TSWV symptom

Nucleic acid extraction and RT-PCR assays

Samples were taken from the leaves and fruits of eggplant plants infected with TSWV and nucleic acid was obtained using an RNA purification kit (ThermoFisher Scientific). Infection in eggplants was investigated using TSWVF: ATCAGTCGAAATGGTCGGCA and TSWVR: AATTGCCTTGCAACCAATTC primers (Mumford et al. 1996) used to detect the presence of TSWV. RT-PCR was performed for TSWV using 1-Step RT-PCR ReddyMix Kit (Thermo Scientific) under the following conditions: 50°C for 15 min and 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 45 s and 72°C for 30 s, and a final extension at 72°C for 7 min. RT-PCR products were checked by running on 1,5 % agarose gel.

Primer Design Studies of The Resitance-Breaking Isolate

As a result of molecular studies, samples confirmed positive for TSWV infection in an agarose gel were sent for sequence analysis. The sequencing results were analyzed in BioEdit programs; Clear peaks were obtained by removing impurities at the beginning and end of the sequences. A single sequence was determined by overlapping the sequences analyzed directionally. Primers covering the mutation region of resistance-breaking isolate compared to the old isolate have been developed (Figure 2). Considering the sequences of resistance-breaking isolates that show high similarity in the NCBI database of our eggplant isolates, the common primer regions were created using the Primer3 program. While this primer pair gives a certain band in resistance-breaking isolates, but it does not give band in non-resistance-

breaking isolates

>MH367503.1:101-1009 Tomato spotted wilt orthotospovirus isolate TSWVAntRB segment M, complete sequence
ATGTTGACTCTTTTCGGTAACAAGAGGCCTTCTAAGTCTGCCGGAAAGGATGAAGGTCCTTTAGTTTCACTTGCTAAACATAATGGCAATGTTGAAGTCTCAAAACCATGGTCTTCTTCTGATGAAAAG
CTTGCTTTAACCAAAGCCATGGACGCATCCAAAGGAAAGATACTGTTGAACACTGAGGGAACATCTTCCTTTGGAACCTATGAATCTGATTCTATCACAGAATCAGAGGGTTATGATCTTTCTGCTAGA
ATGATAGTAGATACAAACCATCATATCTCAAACTGGAAAAATGATCTTTTTGTTGGCAACGGAAAGCAAAATGCTAATAAGGTTATCAGGATCTATCCAACTTGGGACAGCAGAAAACAATACATGATG
ATTTCCAGGATTGTGATATGGGTATGCCCCACTATACCAAACCCTACAGGGAAACTTGTGGTTGCTTTAGTTGATCCCAACATGCCATCTGAAAAGCAAGTCATCCTGAAGGGTCAAGGGACAATAACT
GATCCTATCTGCTTTGTTTTTTATCTGAACTGGTCTATTCCGAAGATGAACAACACCCCAGAAAACTGTTGCCAGCTGCATTTGATGTGCAACCAAGAATACAAGAAAGGGGTTTCTTTTGGTAGTGTC
ATGTATTCTTGGACAAAAGAGTTTTGCGATTCACCCAGAGCTGATAAAGACAAAAGTTGTATGGTTATACCTCTAAACAGGGCCATTAGAGCTAGGTCTCAAGCATTCATTGAAGCCTGCAAGCTGATA
ATTCCTAAAGGAAACAGCGAGAAGCAGATTAAAAAACAGCTTAAAGAATTGAGCTCAAGTCTTGAGAGATCAGTTGAAGAAGAAGAGGAAGGGATTTCTGACAGTGTTGCTCAGTTATCCTTTGATGAA
ATATAG

Figure 2. Mutation site and primer design on the Nsm protein of TSWV genome (primers with yellow, mutation site with red)

When these primers are used with the Luna kit (One-Step RT-qPCR Kit, NEB) they also give accurate results with Realtime PCR because they contain SYBR green dye. When the Melt curve method is used, it distinguishes the resistance breaking and non-breaking isolates with the peaks it gives.

Sequencing and bioinformatic analysis of mutation region

After confirming TSWV infection by RT-PCR method of nucleic acids obtained from eggplant plants showing severe TSWV symptoms; First, eggplant plant and a tomato sample known to be positive for RB-isolate infection were sent for sequence analysis. The sequence results were searched in the NCBI database and compared with the isolates with high similarity. In this comparison study, both resistance breaking and non-resistance breaking isolates were included in order to determine the differences. These isolates were examined in the Bioedit program, and the mutation site has been characterized.

RESULTS and DISCUSSION

RT-PCR of TSWV infection and RB-isolates results

The results of the RT-PCR test performed for eggplants showing TSWV symptoms in the field were examined. Eggplants are infected with TSWV, as can be seen from the gel picture (Fig. 3) of the samples carried out at 60 volts in % 1.5 agarose gel electrophoresis. Since the primers used in this infection test are specific to the coating protein of the virus, that is, they are not in mid-segment where the mutation occurs, both resistance-breaking and non-resistance breaking isolates have been successfully used for infection detection because they have a common coating protein. After 276 bp confirmation of TSWV infection, RT-PCR was performed again with the primers developed by this study based on the sequences of resistance-breaking isolates. Band sizes of 339 bp including amplification of primers and mutation site are shown in the agarose gel image (Fig. 3).

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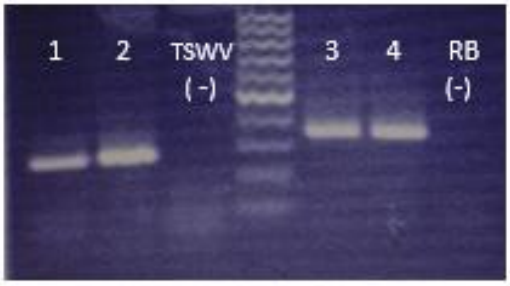


Figure 3. RT-PCR results of TSWV coat protein primer of infected eggplants and current study primer. Respectively 1: TSWV infected eggplant, 2: positive control, TSWV negative control; 100 bp DNA ladder, 3: RB primers with eggplant, 4: RB primers with positive control, RB negative control

Sequencing and bioinformatic analysis results

In this analysis, a comparison is made by selecting resistance breaking and non-resistance breaking isolates. As it well known that TGT or CGT nucleotides make up the cysteine amino acid (Lopez et al. 2011). In the C118Y mutation reported by the TGT sequence that encodes cysteine transforms into TAT sequence and encodes tyrosine amino acid. Based on this information, we checked the sequence of the eggplant isolates. In this analysis, we have found that our eggplant isolate (Eggplant B) has the same mutation region as MH367503.1 (Turkey), HM015519.1, MK715443.1, MG457158.1 isolates, which is different from isolates containing the TGT sequence such as FR692606.1 and AY744486.1 as shown in Figure 4.

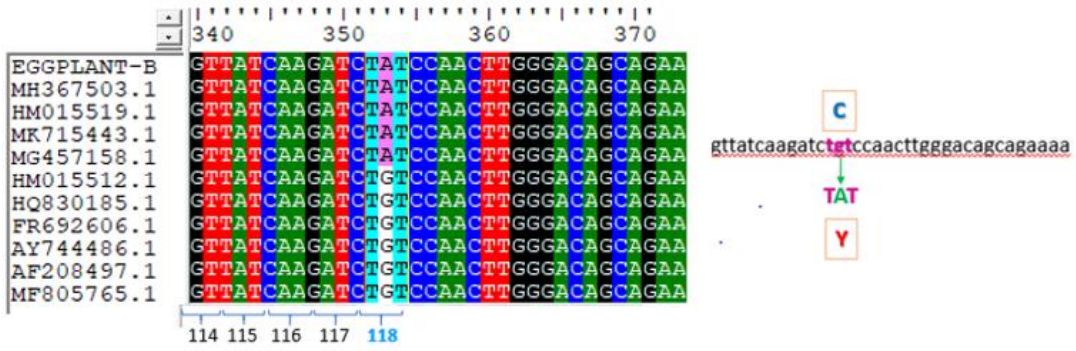


Figure 4. Comparison of RB and NRB isolates and determination of mutation site

Following the sequencing studies, the mutation region was studied in detail and primers covering this region were designed (Table 1).

Table 1. Spesific primers' sequences of RB isolates

Primer name	Sequence	Annealing temp.	Literature
TSWVRB-F:	CTGTTGAACACTGAGGGAACA	53°C	In this study
TSWVRB-R	TGTCCCTTGACCCTTCAGGA		

Primers were made slightly from the beginning and end of that region to cover the mutation area. When the forward and reverse primers are matched to the target site during in vitro annealing; Since Taq polymerase enzyme will read and complete the gene region between the two primers according to the genome sequence, the mutation region was amplified.

Morphological examination of eggplant fruits infected with TSWV RB-isolate and healthy eggplant plants showed significant differences in length. The size of the virus-infected fruit is rather short, the healthy fruit is rather hard compared to the fruit. Eggplant leaves infected with TSWV-RB isolate were examined at every stage. Although the leaf symptoms are mild at the leaflet and fresh leaf stage, an increase in symptoms was observed as the plant grows. When the TSWV-RB isolate was compared with the typical symptom in tomato, the nested ring symptom was found to be similar. This symptom was clearly observed in both eggplant fruits and leaves.

In this study, we brought together molecular applications such as Melting curve assays and classical PCR studies at a common point. We developed a primer specific to the target mutation site. In this way, the detection of resistance-breaking isolates depending on the preference of the employee and laboratory conditions was possible with classical RT-PCR methods and Realtime-based analyzes.

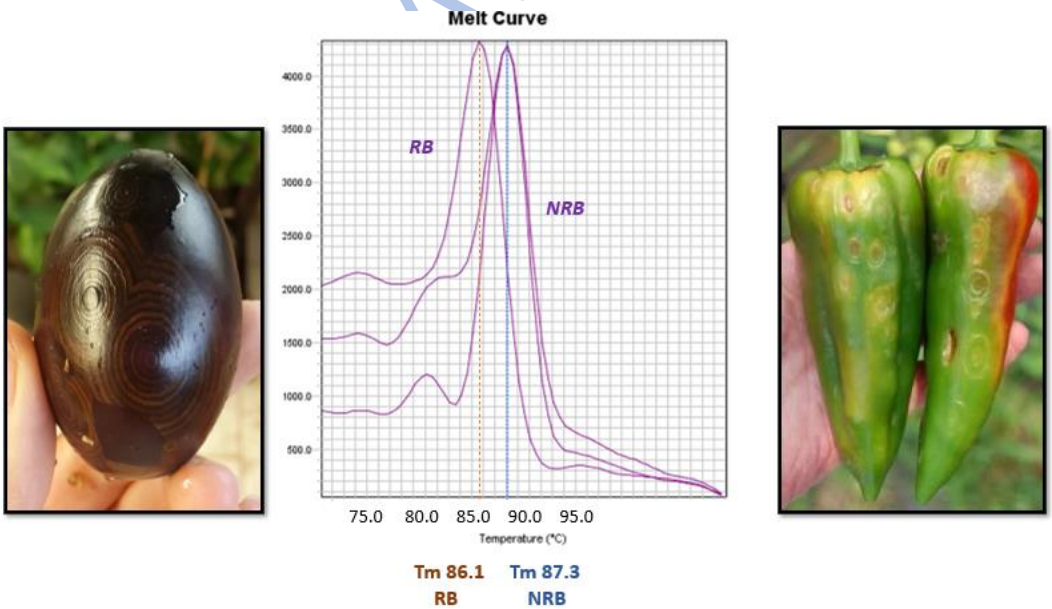


Figure 5. Melt curve analysis of RB and NRB isolates

Our primers pair is made as specific as possible so that results with the precision of Melt Curve analysis can be obtained The Non-resistance breaking isolate was denatured at 87.3 °C, while the RB isolate was at 86.1 °C (Figure 5). Thanks to our primer, it is possible to detect all types

of hosts infected by TSWV Because the primers are not associated with the Sw5 gene in tomato, but directly with the Nsm region of the virus particle. In this way, the fact that the primer can work without the host separation has increased the importance of this study.

CONCLUSION

Our primers pair have been made as specific as possible so that the accurate Melt Curve analysis results can be obtained (Figure 5). Thanks to our primer, it is possible to detect all types of hosts infected by TSWV because the primers are not associated with the Sw5 gene in tomato, but directly with the NSm region of the virus particle. In this way, the fact that the primer can work without the host separation has increased the importance of this study.

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Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

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