Investigation of tomato ringspot virus (ToRSV) by Real-Time TaqMan RT-PCR in Hakkari province, Turkey

Nevin AKDURA*, Murat ŞEVİKb

*aHakkari University, Faculty of Education, Department of Mathematics and Science Education, 30000, Hakkari, TURKEY
bHatay Mustafa Kemal University, Faculty of Veterinary, Department of Virology, 31135, Hatay, TURKEY

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Corresponding Author: Nevin AKDURA, E-mail: nevinakdura@hakkari.edu.tr, Tel: +90 (438) 212 12 11
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AUTHORS ORCID ID:
(Nevin AKDURA: 0000-0001-6162-0500), (Murat ŞEVİK: 0000-0002-9604-3341)

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Investigation of tomato ringspot virus (ToRSV) by Real-Time TaqMan RT-PCR in Hakkari province, Turkey

Nevin AKDURA\textsuperscript{a}, Murat ŞEVİK\textsuperscript{b}

\textsuperscript{a}Hakkari University, Faculty of Education, Department of Mathematics and Science Education, 30000, Hakkari, TURKEY

\textsuperscript{b}Hatay Mustafa Kemal University, Faculty of Veterinary, Department of Virology,, 31135, Hatay, TURKEY

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Abstract
Tomato ringspot virus (ToRSV) belongs to the Nepovirus genus in the family Secoviridae. It has a wide host range and is listed as a quarantine virus in Turkey. In this study, 80 leaf samples were collected from tomato, pepper, cucumber and grapevine cultivation sites located in three different parts of Hakkari province: Şemdinli, Çukurca and Center districts. Real-time TaqMan RT-PCR (Reverse Transcription-Polymerase Chain Reaction) method was used for the detection of the virus. Amplification was carried out in reaction mix including TaqMan probe, QuantiNova Probe RT-PCR kit (Qiagen, Germany) using primers based on 3'-UTR (untranslated region) of virus, which amplified a 182 bp product. ToRSV was detected in 13 of 80 samples and threshold cycle (CT) values ranged between 23.9–37.4. 16.25% of the samples collected from the districts were found to be infected with ToRSV whereas no ToRSV was detected in the samples collected from the Center. In Çukurca district, the virus was detected on pepper and cucumber samples and it was present on tomato, pepper, cucumber and grapevine samples in Şemdinli district. This study provides, to our knowledge, the first report of molecular detection of ToRSV by real-time TaqMan RT-PCR in Turkey.

Keywords: Tomato ringspot virus, Real-time TaqMan RT-PCR, Hakkari
Hakkari İlinde Domates halkalı leke virüsü (Tomato ringspot virus; ToRSV)’nün Real-Time TaqMan RT-PCR Yöntemi ile Araştırılması

Özet

Domates halkalı leke virüsü (Tomato ringspot virus; ToRSV) Secoviridae familyası, Nepovirus cinsine aittir. Geniş bir konukçu aralığına sahip bu etmen Türkiye’de iç karantinaya tabi hastalık ve zararlılar listesinde yer almaktadır. Bu çalışmada, sebze üretiminin ve üzüm yetiştiriciliğinin yapıldığı Hakkari’nin Şemdinli, Çukurca ve Merkez ilçelerinde survey çalışmaları yürütülerek domates, biber, hıyar ve asmadan 80 yaprak örneği toplanmıştır. Örneklerden viral RNA, RNeasy Plant Mini Kiti (Qiagen, Almanya) kullanılarak izole edilmiştir. RNA izolasyonu yapılan örneklerde, ToRSV’nin varlığı real-time TaqMan RT-PCR (gerçek zamanlı TaqMan ters transkripsiyon- polimeraz zincir reaksiyonu) yöntemi kullanılarak araştırılmıştır. Amplifikasyon, ToRSV’nin 3’-'UTR (translasyon olmayan bölge) bölgesinde 182 bç’lik bölgeyi hedef alan primerler ve Taqman probu içeren reaksiyon karışımı ile gerçekleştirilmiştir. Real-time Taqman RT-PCR çalışmaları sonucunda 80 örnekten 13’ü ToRSV ile enfekteli tespit edilmiştir. Pozitif tespit edilen örneklerin eşik döngü değerlerinin (CT=Cycle Threshold) 23.9-37.4 arasında değiştiği gözlenmiştir. Hakkari ilçelerinden toplanan örneklerin %16.25’i enfekteli bulunurken merkez ilçede ToRSV tespit edilmemiştir. Çukurca da biber ile hıyar örneklerinde ve Şemdinli’de domates, biber, hıyar ile asmada bu etmenin varlığı saptanmıştır. Bu çalışma, bildiğimiz kadarıyla, Türkiye'de real-time TaqMan RT-PCR ile ToRSV’nin moleküler tespiti için ilk rapوردur.

Anahtar Kelimeler: Domates halkalı leke virüsü, Real-time TaqMan RT-PCR, Hakkari

1. Introduction

ToRSV (tomato ringspot virus, genus Nepovirus, subgroup C, family Secoviridae) is a bipartite single-stranded, positive sense RNA virus (Sanfaçon et al 2006; 2009). ToRSV primarily infects perennial plants such as tomato (Lycopersicon esculentum Mill.), tobacco (Nicotiana tabacum), grapevine (Vitis vinifera), blueberry (Vaccinium corymbosum), strawberry (Fragaria vesca), geranium (Pelargonium domesticum),
raspberry (*Rubus idaeus*), blackberry (*Rubus fruticosus, Rubus sp*), walnut (*Juglans regia*) and ornamental plants and causing diseases that results in great economic losses. Experimental host diversity of ToRSV is also very high and about 35 families are susceptible to this virus (Samuitiene et al 2003; OEPP/EPPO 2005; Fuchs et al 2010; Sneideris et al 2012; Tzanetakis & Martin 2013; Zindovic et al 2014). The most typical symptom of ToRSV infection in plants is the presence of annular spots on the leaves. It also has other conspicuous symptoms in fruit trees and grapevines. In the grapevines, the virus manifests itself especially with necrotic pitting, spongy phloem tissue, fall of fruit, the rosette formation of leaves, ring spots on the leaves and general decrease in yield (OEPP/EPPO 2013). In infected plants, the effect of the virus can be seen as, pale yellow and pale green spots on the leaves that develop along the major side veins or the main vein of the leaves and causing systemic chlorotic or necrotic ring stains and deformation as well as inhibition of the fruit growth. In certain cases the virus does not show any visible symptoms, being usually characterized by a decrease in the yield. ToRSV is transmitted by natural ways, such as seeds, transplantation, pollen, vegetative organs and different species of *Xiphinema* (Bitterlin et al 1987; Pinkerton et al 2008).

The objective of this research is to determine the presence of ToRSV by real-time TaqMan RT-PCR method in tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*) and grapevine (*Vitis vinifera*) samples were collected from three different districts of Hakkari province.

2. Material and Methods

2.1. Field surveys and sample collection

In early autumn of 2014 and summer of 2015, 80 leaf samples of tomato, pepper, cucumber and grapevine plants were collected from Çukurca, Şemdinli and Center districts of Hakkari province (Durankaya, Kırmık, Üzümçü, Çimenli, Geçitli villages in the Center; Narlı, Geçimli, Kayalı villages in Çukurca; Bağlar, Şapatan, Güzelkonak, Yukarıyokuş, Balova villages in Şemdinli district). The samples were collected from various plant species based on the presence of suspicious viral symptoms at the time of sampling, such as necrosis, chlorosis, mosaic, and ring stains and transported to the laboratory in cool conditions and stored at 4 °C until tested.
2.2. Preparation of primers and total nucleic acid extraction

The primer pair and probe to amplify the 182-bp region in 3’-UTR of ToRSV RNA1 genome sequencing were synthesized and used at the real-time TaqMan RT-PCR (Table 1). RNA extraction from leaf samples of tomato, pepper, cucumber and grapevine were conducted by using the RNeasy Plant Mini Kit from Qiagen GmbH (Hilden, Germany) as specified in the manufacturer's protocol.

2.3. The real-time TaqMan RT-PCR

The 3’-UTR sequence of RNA1 genome of ToRSV was amplified by real-time TaqMan RT-PCR method. Positive control was used to obtain high accuracy and optimization in real-time TaqMan RT-PCR. The plant sample (the original host: Pelargonium sp) obtained from Leibniz-Institut DSMZ German Collection of Microorganisms and Cell Cultures (Germany) was used as a positive control. The total RT-PCR reaction mix was prepared by using QuantiFast Probe PCR (Qiagen, Germany) and it consisted of 1 µl primer-1 (0.3 µM), 1 µl primer-2 (0.3 µM), 1 µl prob (0.5 µM), 10 µl 2xProbe RT-PCR Mix, 0.2 µl QuantiFast RT Mix, 4.4 µl MgCl2 (5.5 µM) and 1.4 µl RNase-free water. 5 µl of RNA isolated from leaf samples was added to the mix, amounting a total of 20 µl. For amplification, reverse transcription cDNA (complementary DNA) was synthesized at 50°C for 10 min, initial denaturation was conducted at 95 °C for 5 min and amplification step were performed in a total of 40 cycles at 95 °C for 15 min, and at 60 °C for 45 sec. In the real time TaqMan RT-PCR studies, Rotor Gene Q Series Software (version 2.3.1) and Rotor-Gene Q (Qiagen, Germany) Real Time appliance were used.

3. Results and Discussion

3.1. Field observation

Field surveys were conducted in Çukurca, Şemdinli and Center districts of Hakkari province during the 2014-2015 growing season. It was observed during the surveys that vegetable farming is generally done without the use of pesticide in these areas and so that plants are susceptible to viral and other risks (bacteria, fungi etc.). The samples were collected in accordance with the common symptoms that are known to be caused by ToRSV on tomato, pepper, cucumber and grapevine (Figure 1). The plants that did not display
known symptoms were also sampled for control. Eighty samples were collected from 13 villages in mentioned areas (Table 2).

3.2. Molecular detection

Total nucleic acids were isolated from 80 samples and tested for the presence of ToRSV along with positive controls and negative controls. A fragment of 182 bp was detected in 3'-UTR of ToRSV RNA1 genome sequencing in the infected leaves of tomato, pepper, cucumber and grapevine. Tang et al (2014) recommended the use of primers and probes in the detection process, but the QuantiFast Probe PCR kit from Qiagen (Qiagen, Germany) was preferred in this study. A nuclease-free water sample was used as a negative control in the tests. CT value of the positive control was 15.6. After determining the appropriate program for real-time TaqMan RT-PCR with the positive control, the procedure was applied to the other samples. After the tests for optimization, a total of 80 samples were evaluated by real-time TaqMan RT-PCR. The real-time TaqMan RT-PCR tests conclusively proved the presence of ToRSV in the province. Real-time TaqMan RT-PCR analysis of 80 samples collected in the field surveys revealed that 13 samples were infected with ToRSV. According to the real-time TaqMan RT-PCR results, CT value of ToRSV infected samples ranged between 23.9-37.4. Samples with CT value greater than 38 were ignored. The CT values of some tested samples were ranged from 23.88 to 37.41 (Table 3). The infection rate of ToRSV in tested samples was found as 16.25%.

The data obtained showed that ToRSV incidence was the highest in Şemdinli district (28.20%) and the lowest in Çukurca district (10%). ToRSV was detected in the tested tomato, pepper, cucumber and grapevine samples. None of the samples collected from Center were found to be infected with the ToRSV (Table 4). The results showed that although ToRSV can be found in various cultivation sites in Hakkari province, the virus is not wide spread in Hakkari in general.

ToRSV is a virus with a very wide host range. The damage caused on plants by this virus has encouraged us to work on it. ToRSV spreading from North America to other parts of the world, is also reported from Netherlands, Chile, Australia, Iran (Samuitiene et al 2003; Moini 2010; Sokhansanj et al 2012; Rivera et al 2016; Roberts et al 2018).
Presence of ToRSV can be determined by biological indexing, serological and molecular methods. Mechanical inoculation to herbaceous plants is also applied and is known to be simple and reliable. Biological indexing, on the other hand, is a time-consuming method and it requires considerable experience, meaning that only a limited number of plants can be tested by use of this method. ELISA (Enzyme Linked Immunosorbent Assay) and DAS-ELISA (Double-Antibody Sandwich Enzyme Linked Immunosorbent Assay) can be used for serological diagnosis of ToRSV. Moini (2010) detected ToRSV by ELISA method in the leaf samples collected from apples in the north-east of Iran. The genome of most plant viruses consists of RNA. Detection of the RNA sequence by PCR requires some changes. Prior to the application of PCR, RNA must have a reverse copy called cDNA. The RT-PCR is a very sensitive method and there may be inhibition problems in the samples. It should also be noted that the use of this method requires experienced researchers (OEPP/EPPO 2005). Detection of ToRSV by RT-PCR has been developed for multiple strains of ToRSV in both herbaceous and woody plants (Griesbach 1995). Msikita (2007) compared ELISA with RT-PCR methods for ToRSV detection and preferred RT-PCR with the identified appropriate primary sequence. Digiaro et al (2007) studied the development of degenerate and specific primers for differential and simultaneous RT-PCR detection between subgroups A, B and C of grape infecting nepoviruses. They were designed specifically for RNA-1 3'-UTR for grapevine and provided a source for studies on the determination of this factor in grapevine with obtained positive results. A real-time RT-PCR test has been developed for rapid and sensitive detection of ToRSV. Stewart et al (2007) tested samples for ToRSV primarily by ELISA. Real-time RT-PCR detection of ToRSV was performed in host tissues and a comparison was made between real-time PCR and ELISA. It was concluded that the results obtained by real-time PCR were more sensitive than ELISA. It was also seen that the samples that did not show positive results by ELISA were positive when tested in much lower amounts by real-time RT-PCR. Osman et al (2008) compared low-density sequences using real-time TaqMan PCR and RT-PCR in the detection of grapevine viruses and examined the reliability of the results for ToRSV. This was the first report on the use of low-density sequences in the detection of plant viruses. Tang et al (2014) detected the presence of ToRSV on grapevine by targeting RNA-1 3'-UTR region by real-time Taqman RT-PCR. In terms of specificity,
sensitivity and reliability in the detection of ToRSV, real-time TaqMan RT-PCR and other real-time RT-PCR methods were compared. The real-time TaqMan RT-PCR used in that study was designed for the highly conserved region of ToRSV 3′-UTR. The TaqMan real-time RT-PCR test showed that the method can be widely used in the overall detection of ToRSV over a wide range of hosts and it also served as a resource for the method used in our research.

4. Conclusions

The presence of ToRSV in Turkey has been reported in tomato, pepper, cucumber (Fidan 1995; Arlı- Sökmen & Şevik 2006), stone fruit (Azeri & Çiçek 1997), blackberry (Sertkaya 2010) and strawberry (Yeşilçöllü et al 2011). The methods used in these studies were ELISA and RT-PCR. In the studies where the primary method was real-time RT-PCR, grapevine was preferred as a host for detection of ToRSV. In this study, we detected the presence of ToRSV in different hosts. Samples with CT values ≤38 were accepted infected with ToRSV. CT value of the positive control was found to be 15.6, but CT value of the other samples that were considered positive was higher. CT values increased as the density of the virus decreased in the samples. This may have stemmed from the evaluation of different hosts. In the survey, ToRSV was detected in tomato, pepper, cucumber and grapevine in Şemdinli district and in pepper and cucumber in Çukurca of Hakkari province. 16.25% of the total samples were found to be infected with ToRSV and it is good to note that areas infected with ToRSV are uncommon. ToRSV-infected plants are concentrated mostly in Şemdinli. It is noteworthy that the uncommon use of pesticides and the use of local seeds in the fields observed are widespread. ToRSV can be transmitted by mechanically, nematode vectors, seeds and pollen in some plants, therefore it will be appropriate to comply with the internal quarantine rules. Although there have been a number of attempts to identify the presence of ToRSV in Turkey, this study is the first report of molecular detection of ToRSV in different hosts by real-time TaqMan RT-PCR.

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**Figures**
Figure 1- Symptomatic plants collected in the field survey from Hakkari province: a) tomato b) grapevine c) pepper d) cucumber

Tables

Table 1- Primer and probe information used in real-time TaqMan RT-PCR analyses

| Primer/Probe         | Sequence                             | Target gene and position | Reference       |
|----------------------|--------------------------------------|--------------------------|-----------------|
| ToRSV-UTR (Forward primer) | F 5'-GAATGTTCCAGCCACTT-3'           | 3'–UTR 7686-7704 bp of RNA1 |                 |
| ToRSV-UTR (Reverse primer) | R 5'-AGTCTCACTTAACATACCAC-3'       | 3'–UTR 7847-7867 bp of RNA1 | Tang et al., 2014 |
| ToRSV-UTR (Probe)    | P FAM-5'-AGGATCGC-TACTCCTCGTCAAC-3'-BHQ-1 | prob –7746–7768 bp      |                 |

Table 2- List of plant samples collected from Hakkari province for real-time TaqMan RT-PCR analyses

| No | Host   | Location  | No | Host   | Location  |
|----|--------|-----------|----|--------|-----------|
| 1  | Tomato | Çukurca-Narlı | 41 | Cucumber | Şemdinli-Balova |
| 2  | Tomato | Çukurca-Narlı | 42 | Grapevine | Şemdinli-Bağlar |
| 3  | Tomato | Çukurca- Narlı | 43 | Grapevine | Şemdinli-Bağlar |
Table 3 - CT (cycle threshold) values obtained from real-time TaqMan RT-PCR analyses of different plant samples collected from Hakkari province

| The collected field | Host       | No of infected ToRSV the sample | CT value |
|---------------------|------------|---------------------------------|----------|
| Çukurca-Geçimli     | Pepper     | 12                              | 33.49    |
| Çukurca-Kayalı      | Cucumber   | 20                              | 35.99    |
| Şemdinli-Balova     | Tomato     | 21                              | 32.66    |
| Şemdinli-Balova     | Tomato     | 22                              | 34.09    |
| Province          | District | Tomato | Pepper | Cucumber | Grapevine | Avarage infection rate (%) |
|-------------------|----------|--------|--------|----------|-----------|----------------------------|
| Şemdinli-Güzelkonak |          |        |        |          |           |                            |
| Şemdinli-Güzelkonak |          |        |        |          |           |                            |
| Şemdinli-Yukarıyokuş |        |        |        |          |           |                            |
| Şemdinli-Yukarıyokuş |        |        |        |          |           |                            |
| Şemdinli-Yukarıyokuş |        |        |        |          |           |                            |
| Şemdinli-Balova     |          |        |        |          |           |                            |
| Şemdinli-Balova     |          |        |        |          |           |                            |
| Şemdinli-Balova     |          |        |        |          |           |                            |
| Şemdinli-Şapatan    |          |        |        |          |           |                            |
| Şemdinli-Şapatan    |          |        |        |          |           |                            |

Table 4- ToRSV infection rate in tomato, pepper, cucumber and grapevine samples collected from Hakkari province