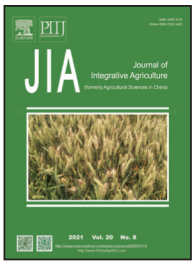
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RESEARCH ARTICLE

Robust molecular detection of the new Tomato brown rugose fruit virus in infected tomato and pepper plants from Turkey

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Abstract

Tomato brown rugose fruit virus (ToBRFV) causes severe fruit loss in tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) plants. It is an emerging *Tobamovirus* that is spreading globally. The major challenge is to develop a reliable method for the detection of the virus, and to better characterize the symptoms it causes. The aims of this study, therefore, were to characterize the symptom development on tomato and pepper plants, and to establish a reliable detection method for the virus. Following infection of the tomato and pepper plants with ToBRFV, the leaves turned chlorotic, mosaic or mottled, while the fruit became rugose, necrotic and marbled, and showed discoloration with yellow or brown spots. Transmission electron microscopy (TEM) revealed single rod-like virus particles characteristic of the *Tobamoviruses*. Classical reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR) with specific primers and probes confirmed that the virus is ToBRFV. We found that the resistance genes from tomato, *Tm-2²*, and pepper, *L1*, *L2*, *L3* and *L4*, did not confer resistance to ToBRFV. Here, we present a PCR-based method as a diagnostic test for detecting ToBRFV in infected seeds. This method will help to prevent further spread of the virus in commercial seeds.

Keywords: tomato, pepper, *Tobamovirus*, ToBRFV, resistance

1. Introduction

The world's most widely consumed vegetables are tomato (*Solanum lycopersicum*) and capsicum peppers (*Capsicum annuum*). Tomato is an important food source with several beneficial properties including antiaging, anticarcinogenic, nutritional value and taste (Agarwal and Rao 2000; Story *et al*. 2010). The annual global market value of tomato is roughly 510 billion USA dollars (FAO 2019). Pepper is a globally-important spice commodity. The pepper is also an excellent source of micronutrients and antioxidants such as vitamins C and E and carotenoids, and its consumption is critically important in preventing or reducing chronic and age-related diseases (Palevitch and Craker 2012). The production and consumption of pepper have increased to 36 million tons fresh weight in recent years (Tripodi and Kumar 2019). Both tomato and pepper are grown either in the field or in greenhouses. The increasing incidence and severity of virus infections have caused significant

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losses in yield and quality. Several viruses affect both commercially-grown tomato and pepper, including Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), Tomato spotted wilt virus (TSWV), Tomato yellow leaf curl virus (TYLCV), Tomato chlorosis virus (ToCV) and many others (Hanssen *et al*. 2010).

The TMV and ToMV cause diseases in several species of *Solanaceae* and are not only the oldest known viruses but also have great potential for reducing production of these crops (Smith and Dombrovsky 2019). An important means for controlling viral diseases is through disease resistant genes found in the *Solanaceae* plants which reduce viral multiplication. However, the resistance can be broken through if the virus mutates in such a way that it is no longer recognized by the host immune receptors, failing to trigger host defense mechanisms (Xu *et al*. 2017). Tomato brown rugose fruit virus (ToBRFV), which belongs to *Tobamoviruses*, is an emerging virus on commercial crops of tomato and pepper, and is spreading worldwide (Salem *et al*. 2016). Tomato and pepper show foliar symptoms including chlorosis, mosaic, and mottling, with occasional leaf narrowing, and necrotic spots on the peduncle, calyces and petioles (Cambrón-Crisantos *et al*. 2018; EPPO 2019; Fidan *et al*. 2019). Typical fruit symptoms on commercial cultivars develop as yellow areas, roughness — often described as rugose — and necrotic and brown areas (Luria *et al*. 2017; Cambrón-Crisantos *et al*. 2018; Fidan *et al*. 2019). The ToBRFV causes a reduction in the yield of plants and destroys the aesthetic structure of both tomato and pepper fruits (Luria *et al*. 2017).

The ToBRFV, TMV and ToMV all belongs to the genus *Tobamovirus*. The *Tobamoviruses* are single strand (ss) RNA viruses, and form one of the six genera of the *Virgaviridae* family of positive-strand viruses without a DNA stage (Adams *et al*. 2009). The *Tobamovirus* particle has a typical rod-shaped morphology, a 3´-t-RNA-like structure without a poly(A) tail, and a genome of 6.2 to 6.4 kb encoding four open reading frames (ORFs). The ORF1 and ORF2 are separated by a stop codon and encode non-structural proteins. The ORF3 codifies the movement protein of 30 kDa, while ORF4 encodes the coat protein (CP) of 17.5 kDa (Luria *et al*. 2017).

Three genes, *Tm-1*, *Tm-2* and its allele *Tm-2²*, control resistance against TMV. However, only *Tm-2* and *Tm-2²* confer resistance in tomatoes because different domains in the movement protein (MP) and different protein structural requirements are necessary for each resistance (Meshi *et al*. 1989). The *Tm-2²* resistance interferes with viral cell-to-cell movement in plants; for example, Tomato mosaic virus (ToMV) strain ToMV-22 needs to suppress two amino acid (aa) exchanges in the carboxy-terminal region of the viral 30-kDa MP to overcome *Tm-2²* resistance (De Ronde *et al*. 2014). The ToMV's MP domains, where respective mutations are necessary to overcome either *Tm-2* or *Tm-2²* resistance, interact or are close together. Therefore, two positionally differing sets of mutations are able to affect the two highly similar interactions between the two R proteins and MP or the complex involving MP. Close proximity or interaction of the two domains could explain how virulent ToBRFV is able to overcome both the *Tm-2* and *Tm-2²* resistance (Lanfermeijer *et al*. 2005).

As reported in the European and Mediterranean Plant Protection Organization (EPPO) Bulletin (2019), ToBRFV has been transmitted on contaminated tools, hands and clothing, direct plant to plant contact, and through propagation materials such cuttings and grafts. *Tobamoviruses* may be transmitted in infected seeds, and may remain in soil contaminated by an infected plant for years. Seed transmissions of plant viruses have great epidemiological significance and cause disease outbreaks worldwide (Dombrovsky and Smith 2017). The ToBRFV has been reported so far in Israel, Brazil, Germany, Turkey, and the United Kingdom (Salem *et al*. 2016; Cambrón-Crisantos *et al*. 2018; Fidan *et al*. 2019; Menzel *et al*. 2019; Skelton *et al*. 2019).

Pepper plants have four *L* genes that provide resistance to *Tobamovirus* species, where *L1* controls resistance to P0 pathotype viruses such as ToMV; *L2* confers resistance to P0 and P1 pathotype viruses such as Paprika mild mottle virus (PaMMV); *L3* controls resistance to P0, P1 and P1-2 pathotypes of Pepper mild mottle virus (PMMoV); and *L4* controls resistance to P0, P1, P1-2, and P1-2-3 pathotypes of PMMoV (Tomita *et al*. 2011). The newly identified ToBRFV has appeared on commercial plants recently and whether any of these resistance genes are effective against this virus remains to be determined.

The goal of our research was to confirm whether the newly emerging disease of tomato and pepper in Turkey was indeed caused by ToBRFV. For this, we first characterized the symptoms on infected plants. We subsequently isolated a virus from the infected materials and determined that the nucleotide sequences of the viruses from tomato and pepper corresponded to strains of ToBRFV. We used these sequences to develop a RT-PCR based method that can be used to identify the presence of the virus in seed lots, as a quarantine measure, and in commercial practice in order to avoid its inadvertent local spread. This new method will be able to help scientists, seed companies, and quarantine organizations test for contamination with ToBRFV.

2. Materials and methods

2.1. ToBRFV isolates

More than 100 samples from tomato and pepper plants showing symptoms of viral infection were collected from

commercial greenhouses cultivating tomato and pepper crops in Antalya, the West Mediterranean Region of Turkey. Foliar symptoms included chlorosis, mosaic with dark green bulges and narrowing. Fruit symptoms consisted of deformed and irregular maturation, and necrotic spots on tomato fruits and around the calyces, with occasional rugose symptoms (Fidan *et al*. 2019). These samples were used to isolate the virus for molecular analysis and for TEM analyses. For RNA purification, 100 mg fresh or frozen leaves or fruits were used from tomato and pepper plants. All molecular analyses were conducted within 24 h after samples were brought to the laboratory at the Faculty of Agriculture, Akdeniz University, Antalya, Turkey.

2.2. Virus purification and TEM

Virions were isolated from 100 g of symptomatic tomato and pepper plants. The leaves were homogenized in 200 mL of 0.2 mol L^{-1} potassium phosphate, pH 7.6, containing 3% (v/v) urea and 200 µL of thioglycolic acid for 4 min in a mixer. After centrifugation at 10 000×g for 15 min, the supernatant was collected. A 10 µL aliquot of this supernatant and 10 µL of 1% phosphotungstic acid (Fluka analytical) were placed together on copper grids of 300 mesh (Electron Microscopy Sciences®, the United Kingdom) covered with a Formvar/Carbon® membrane. The droplets were mixed using a pipette, left for 30 s, and the excess liquid was removed with a piece of filter paper. Grids were observed with a Zeiss Leo 906 E TEM (Germany) operated at 120 kV and photographed. Scaling used a standard of known size and the dimensions of viral particles were calculated.

2.3. Host range analyses

Various test plants for inoculation were raised in sterilized soil in a growth chamber with a photoperiod of 16 h of light and a target air temperature set at 28°C/20°C day/night. They included at least four plants each of 3-week-old tomato and pepper of different varieties, *Chenopodium* spp., *Nicotiana* spp., eggplant and black nightshade plants (Appendix A). In the host range analyses, the same isolate was used for inoculation of all the hosts. The inoculum was prepared from the collected symptomatic fruit and leaf samples which were individually homogenized in 0.01 mol L^{-1} phosphate buffer (0.8 mol L^{-1} KH_2PO_4, 0.1 mol L^{-1} Na_2HPO_4, pH 7.0). A sponge was dipped into the inoculum and rubbed across healthy, immature leaves of the test plants. This process created micro-abrasions that served as entry points for virus infection. After inoculation, test plants were returned to the growth chamber. The inoculated plants were examined daily and the symptoms, which appeared 15–25 days post inoculation (dpi), were recorded. The experimental controls were healthy, non-inoculated tomato and pepper plants grown in a separate growth chamber under the same conditions.

2.4. Effect of *R*-genes on infection by ToBRFV

The Turkish isolate of ToBRFV was mechanically inoculated into test plants of tomato varieties harboring the resistance gene *Tm-2²*, and test plants of pepper varieties containing the *L1*, *L2*, *L3* and *L4* resistance genes. The test was performed in triplicate on batches of at least four plants per variety. Controls were tomato and pepper plants lacking the corresponding resistance gene, and mock inoculations were conducted using only the phosphate buffer (pH 7.0).

2.5. Whole genome sequencing of ToBRFV

Leaf samples that contained infectious virus were presumed to be ToBRFV because of the symptoms observed. Total nucleic acids were extracted from infected leaves using CTAB extraction method (Doyle and Doyle, 1990). The nucleic acids were amplified with 12 primer sets (Table 1) reported by Luria *et al*. (2017). The amplified products were run on 1.5% agarose gels and then amplified fragments were cut from the gels and purified using GeneJet Gel Extraction Kit (Thermo Fisher Scientific, USA). The sequences of the amplified and gel-purified PCR products were obtained from Ficus Company (Ankara, Turkey). Additionally, to obtain sequences from the 5´-ends, the FirstChoice® RLM-RACE Kit (Thermo Fisher Scientific, USA) was used according to manufacturer's instructions.

The obtained sequences were aligned after removing minor errors using the BioEdit (version 7.2) and Chromas (version 2.6) programs. This generated a single sequence, overlapping and bidirectional, with the forward and complementary sequences corresponding to ToBRFV for the samples from both tomato and pepper. The entire sequences were deposited as tomato (ToBRFV-Ant-Tom: MT107885) and pepper (ToBRFV-Ant-Pep: MT118666) in the the GenBank Database at the National Center for Biotechnology Information (NCBI).

2.6. PCR analysis for molecular detection

Total RNA was extracted with GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific, USA) from infected tomato and pepper plants. The extracted RNA samples were assayed using specific primer sets (Table 2) for TMV, ToMV, Pepper mild mottle virus (PMMoV) and Cucumber green mottle mosaic virus (CGMMV) in classic and reverse-transcription PCR (RT-PCR) analyses. Meanwhile, additional ELISA test analyses were also

conducted with the four *Tobamovirus* species (TMV, ToMV, PMMoV and CGMMV) and a *Potexvirus*, Pepino mosaic virus (PepMV). All ELISA kits (Agdia Inc., USA) were used according to manufacturer's instructions.

ToBRFV cDNAs were obtained with a high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. For molecular detection of ToBRFV, two specific primer sets were designed from the genome sequences of ToBRFV-Ant-Tom and ToBRFV-Ant-Pep (GenBank numbers MT107885 and MT118666). Because the RNA-dependent RNA polymerase region shows the most significant differences between the *Tobamovirus*es, two primer sets of ToBRFV1 forward (bp 620–639) and ToBRFV1 reverse (bp 1 073–1 092) and ToBRFV2 forward (bp 741–760) and ToBRFV2 reverse (bp 1 074–1 093) were designed to amplify a region of the RNA-dependent RNA polymerase ORF. The primer sets of ToBRFV1 and ToBRFV2 were used for RT-PCR amplification of 472 and 351 bp fragments, respectively. The RT-PCR amplification was performed in a total volume of 50 μL containing 2 μL template RNA, 200 nmol L^{-1} of each of the primers, 1 μL Verso enzyme mix, 25 μL 2× 1-Step PCR ReddyMix (Thermo Fisher Scientific, USA), and 20 μL nuclease-free water. The RT-PCR program executed the reverse transcription of RNA at 50°C for 30 min, and performed the PCR step at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, 56°C for TMV and for ToMV or 52°C for PMMoV for 30 s, 59°C for ToBRFV, and 72°C for 1 min, followed by a final 72°C extension step for 5 min. The RT-PCR amplified products were purified on a 1.5% agarose gel and then visualized.

2.7. Primers and probes designed for ToBRFV

In order to develop a qPCR assay, primers and probes were designed based on the sequences of the ToBRFV-Ant-Tom (MT107885) and ToBRFV-Ant-Pep (MT118666) genes published in NCBI GenBank library using Chromas (Technelsium DNA Sequencing Software, Australia) and CodonCode Corporation (Florida) Software. All primers

Table 1 Forward and complementary primers used for whole genome sequencing of Tomato brown rugose fruit virus (ToBRFV)

Primer number	Orientation	Name (nucleotide position, bp)	Sequence (5′→3′)
1	Forward	F-TobGEN (3 666)	ATGGTACGAACGGCGGCAG
2	Complementary	R-TobGEN (4 718)	CAATCCTTGATGTGTTTAGCAC
3	Forward	F-1 (1)	GTGTATTTTTTACAACATATACCAAC
4	Complementary	R-1572 (1 572)	CTAATGCGTCTCCCGACACT
5	Forward	F-1534 (1 534)	AGATTTCCCTGGCTTTTGGA
6	Complementary	R-3733 (3 733)	ATCATCGCCACCAAATTTTC
7	Forward	F-4587 (4 587)	CCAACCTTATGTGGAATTTCG
8	Complementary	R-6392 (6 392)	GAACCCCCGGTAGGGGCCCA
9	Complementary	R-Ex-480 (480)	TTATGTCGCGGACATCAAGA
10	Complementary	R-In-408 (408)	GTGAACATATGCCCGTCCTT
11	Forward	F-Ex-5931 (5 931)	ACAATGCGGTACTAGATCCTCT
12	Forward	F-In-6041 (6 041)	GACGCAACGGTGGCTATAAG

Table 2 List of specific primers sets used for retrieving the *Tobamovirus* and the designed primers and probes for Tomato brown rugose fruit virus (ToBRFV)[1)]

Host and *Tobamovirus* species	Primer name and sequences	Fragment size (reference)
Tomato–General *Tobomovirus* primer	R-4718 5′-CAATCCTTGATGTGTTTAGCAC-3′ F-3666 5′-ATGGTACGAACGGCGGCAG-3′	1 052 bp (Luria *et al.* 2017)
Tomato–TMV	TMV F 5′-GCACATCAGCCGATGCAGC-3′ TMV R 5′-ACCGTTTTCGAACCGAGACT-3′	880 bp (Fidan and Barut 2019)
Tomato–ToMV	ToMV F 5′-CGAGAGGGGCAACAAACAT-3′ ToMV R 5′-ACCTGTCTCCATCTCTTTGG-3′	318 bp (Fidan and Barut 2019)
Pepper–PMMoV	P12/3 F 5′-ACAGCGTTTGGATCTTAGTAT-3′ P12/3A R 5′-GTGCGGTCTTAATAACCTCA-3′	836 bp (Fidan and Barut 2019)
ToBRFV1	ToBRFV1 F 5′-CTTCCAAACGTGTACGCACC-3′ ToBRFV1 R 5′-ATGCATCTTCCATTGCGCTG-3′	475 bp In this study
ToBRFV2	ToBRFV2 F 5′-CATGTTTGTTACGCCGCCTT-3′ ToBRFV2 R 5′-CATGCATCTTCCATTGCGCT-3′	351 bp In this study
ToBRFV	ToBRFV-F1 5′-AAGTTGTCCCAAATTTCCAAAA-3′ TaqMan probe 5′-FAM-ATACGCTGAAACGCCAGACGAAG-TAMRA-3′ ToBRFV-R1 5′-CAGGTATATCGTATATACTGTGCA-3′	158 bp In this study

[1)] TMV, Tobacco mosaic virus; ToMV, Tomato mosaic virus; PMMoV, Pepper mild mottle virus.

and probes were designed to target the conserved RNA-dependent RNA polymerase ORF region within a 158 bp (544–570 and 679–702 positions) fragment (Table 2). The TaqMan probe was labeled with 6-carboxy-fluorescein (FAM, excitation wavelength 494 nm, emission wavelength 521 nm) reporter dye at 5′-end and 6-carboxytetramethylrhodamine (TAMRA) fluorescent quencher at the 3′-end. The details of designed primers and TaqMan probe are listed in Table 2.

2.8. One-step qPCR assays

One-step qRT-PCR assay was performed with a final volume of 25 µL using the Verso 1-step RT-PCR Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Each reaction was carried out using 2 µL of total RNA extracted from collected samples as the template on the Bio-Rad RealTime PCR Detection System (Bio-Rad, Germany). During the amplification process, the fluorescence intensity of the reporter dye (FAM) and a quencher dye (TAMRA) were recorded. These data allowed the calculation of the normalized reporter signal, which is linked to the amount of product amplified. The threshold cycle (Ct values, number of amplification cycles for the fluorescence to reach the threshold) refers to the number of amplification cycles required for a significant increase in the reporter's fluorescence. The data were analyzed with the Bio-Rad RealTime PCR Detection System Program.

Forward and reverse primers were subjected to a 3×3 optimization matrix using a concentration of 100, 200, and 400 nmol μL^{-1} for each concentration of primer under the 200 nmol μL^{-1} probe concentration. Subsequently, the concentration of the TaqMan probe was optimized. ssRNA transcripts synthesized *in vitro* were used as templates. The most suitable program and parameter were reverse transcription of the RNA at 50°C for 5 min. The optimum denaturation time was 10 s at 95°C. Annealing-extension time was 45 s at 60°C. A standard curve was generated using ten-fold serial dilutions ranging from 10^9 to 10^2 copies of ssRNA transcripts or 100 ng to 1 pg of total RNA extracted from tomato leaf with ToBRFV-Ant-Tom infected isolate. Ct values were measured in three duplicates and plotted against the known copy numbers of the standard samples. These standard curves were used to estimate the reaction efficiency and the quantification of viral target in the unknown samples. The reaction efficiency under our experimental conditions is expressed as % and calculated with the formula $E=(10^{-1}/\text{slope}-1)\times100\%$.

2.9. Detection of ToBRFV from tomato and pepper seeds

Maximum care was taken to avoid any contamination during the testing of tomato and pepper seed samples. A minimum sample size was 3 000 seeds, which was equally divided into 12 sub-samples where each sub-sample contained 250 seeds. Individually, each sub-sample was placed in a special extraction bag (#480100, Bioreba, Switzerland) and 5 mL of 0.1 mol L^{-1} phosphate buffer (Na_2HPO_4/KH_2PO_4 pH 7.2) was added for immersion and then the extraction bag was placed into a +4°C refrigerator overnight. The soaked seeds in the bag were properly ground and a 500 µL aliquot was sampled into a sterile Eppendorf tube. The aliquot was used to extract RNA using the GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions. RNA extractions were performed on ice and they were analyzed using classical RT-PCR and RT-qPCR as described above.

3. Results

3.1. Observations on the virus particles

The results obtained by TEM microscopy of extracts of infected tissues revealed a rod-shaped virus particle with an average size of (250±50) nm length and (18±5) nm width (Fig. 1). Naturally infected and artificially infected tomato and pepper samples either prepared fresh (Fig. 1-C) or stored at –20°C for 2 days (Fig. 1-A and B) were separately examined by TEM. The resolution of the virus particles was noticeably better in the frozen samples (Fig. 1-A and B). The presence of single particles and clusters of particles is typical of the *Tobamovirus* genus.

3.2. A new *Tobamovirus* is ToBRFV

In 2019, a new and severe disease appeared on commercial crops of tomato and pepper in greenhouses in Antalya Province of Turkey. The diseased tomato plants showed severe virus-like symptoms with mosaic of the leaves with mottling, rugosity and occasional narrowing (Fig. 2-A, B, and C). Necrotic spots were observed on the peduncle, calyces and petioles (Fig. 2-D) and the fruit was rough, or rugose, with chlorotic and necrotic patches (Fig. 2-E). Simultaneously, pepper plants developed necrotic lesions, yellowing (Fig. 2-F and G), a dried apoptotic, hypersensitive response like dried spots on the leaves (Fig. 2-H) and necrotic, irregular spots (Fig. 2-I) and discoloring on fruit (Fig. 2-J) which reduced yield. Because the typical disease symptoms developed very quickly in the greenhouses, the causal agent was speculated to be a *Tobamovirus* rather than an *Orthotospovirus*. Meanwhile, all symptomless tomato and pepper plants were monitored by RT-PCR and the results showed that all samples were positive.

Samples were also analyzed by ELISA with monoclonal

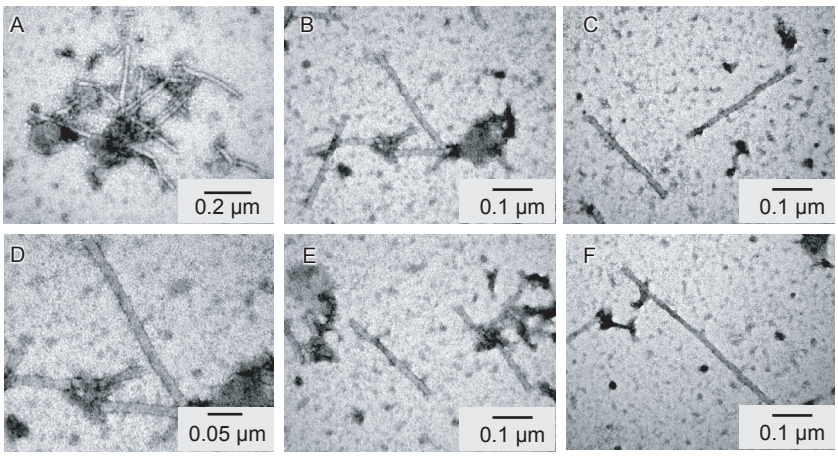


Fig. 1 Morphological characterization of viral particles under transmission electron microscope (TEM). A–C, tomato. D–F, pepper plants. A and D, electron micrograph illustrations of viral particles. B and E, particles of ToBRFV stored at –20°C. C and F, particles of Tomato brown rugose fruits virus (ToBRFV) freshly prepared before microscopy. Bar=0.1 μm.

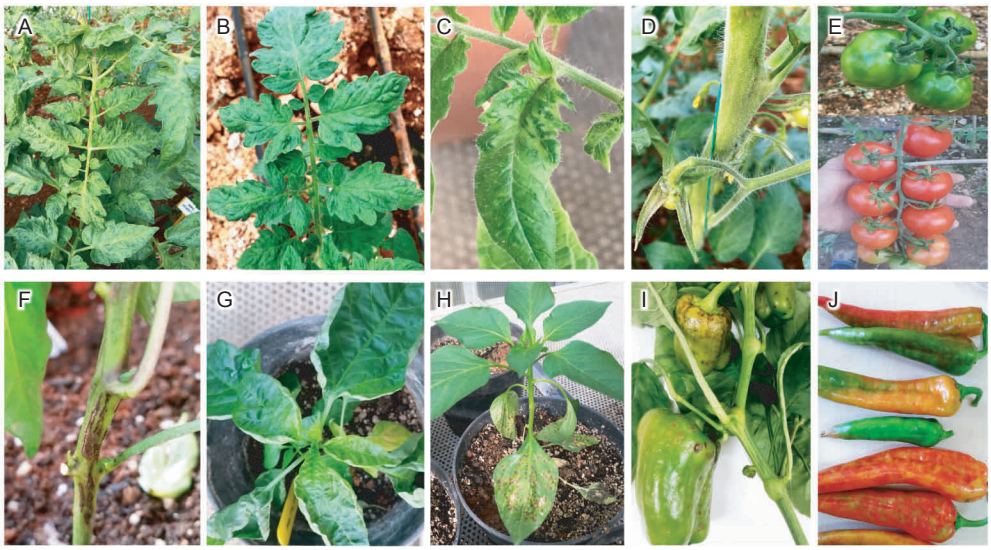


Fig. 2 Naturally and artificially infected tomato and pepper plants with Tomato brown rugose fruits virus (ToBRFV). A–C, typical mosaic rugose pattern on leaves of tomato plants. D, necrotic, dried peduncles and calyces on tomato plants leading to fruit abscission. E, typical fruit symptoms, marbling, discoloration on tomato fruits. F, necrotic lesions on stem of pepper plant. Yellowing on leaves (G) and typical spots (H) on pepper leaves due to *L3* and *L4* resistance genes and developing a hypersensitive response. I–J, typical pot symptoms of discoloration, marbling and yellowing on fruits leading to a firm structure like charcoaled kebab on bell and capia pepper pots, respectively.

antibodies for TMV, ToMV, Pepper mild mottle virus (PMMoV) and Cucumber green mottle mosaic virus (CGMMV). Both leaf and fruit samples of tomato and pepper plants reacted strongly with the TMV antibody (data not shown). TMV on tomato and pepper is common in Turkey and it reacts with the TMV antibody. To further investigate the causal agent of the disease, the nucleic acids were extracted from infected tissues in preparations containing the virus particles, and RT-PCR was conducted with primer sets for general *Tobamovirus* (R-4718/F3666), TMV (TMVF/TMVR), ToMV (ToMVF/ToMVR) and PMMoV (P12/3F/P12/3AR). The RT-PCR results revealed a 1 052-bp specific band (Fig. 3-A) generated with the general *Tobamovirus* primer pair; however, no bands were obtained with the specific primer pairs for TMV, ToMV or PMMoV (Fig. 3-A). Hence, we further searched for the ToBRFV virus from *Tobamovirus* genus using its sequence data in the NCBI. In classical PCR tests, two primer sets, ToBRFV1F/ToBRFV1R and ToBRFV2F/ToBRFV2R, were used in the RT-PCR analysis of extracts of infected plants. The ToBRFV1 and ToBRFV2

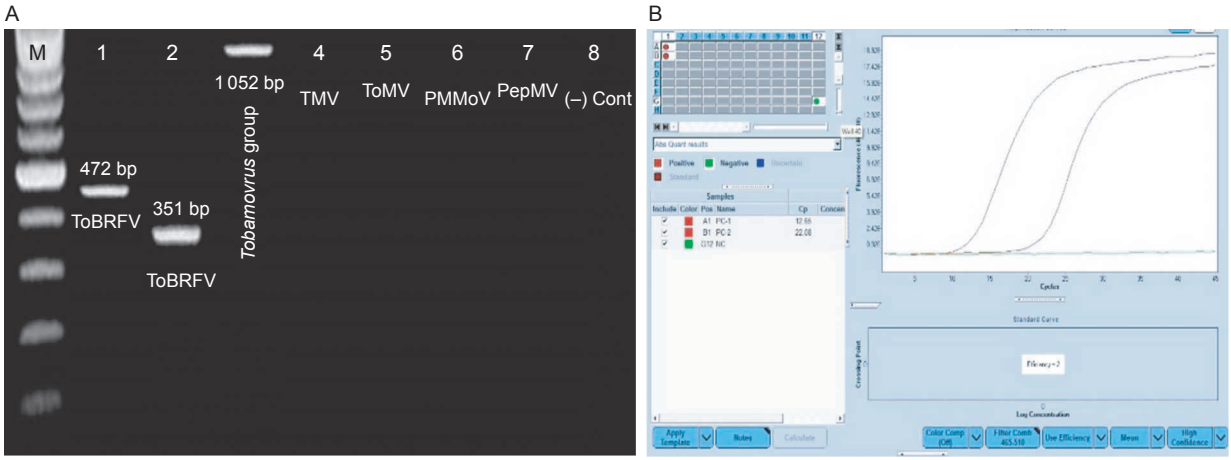


Fig. 3 A, amplification detection by using two Tomato brown rugose fruit virus (ToBRFV), *Tobamovirus* group, Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), Pepper mild mottle virus (PMMoV), and Pepino mosaic virus (PepMV) primer sets and a negative control. B, comparison of samples showing high Ct values in qPCR with electrophoretic gel. Amplification curves obtained with the primer pair ToBRFV-F1/ToBRFV-R1 and probe (bp 544–702) of the samples using qPCR with TaqMan probe.

specific primer sets produced 472 and 351 bp amplicons, respectively (Fig. 3-A), from both the tomato and pepper plant samples showing symptoms of the infection, but not from samples infected with other *Tobamoviruses* (Fig. 3-A). We conclude that primer sets ToBRFV1F/ToBRFV1R and ToBRFV2F/ToBRFV2R amplify only ToBRFV but not other *Tobamoviruses*. Importantly, these results indicate that the causal agent of the newly identified disease on tomato and pepper in the Antalya greenhouses is caused by ToBRFV.

3.3. Primers ToBRFV1 and ToBRFV2 and TaqMan probe are specific for ToBRFV

To determine the specificity of the qPCR assay with ToBRFV1 and ToBRFV2 and the TaqMan probe, samples from tomato and pepper plants were analyzed. As shown in Fig. 3-B, RNA samples from tomato and pepper plants either naturally or artificially infected with ToBRFV gave positive signals with Ct values that ranged from 12.65 to 22.08. The negative control plant (G12) did not produce any signal in the same assay (Fig. 3-B).

3.4. Genome organization

The whole genome of ToBRFV was assembled using 12 sequencing primers and was named as ToBRFV-Ant-Tom (6 386 bp) from tomato and ToBRFV-Ant-Pep (6 373 bp) from pepper plants. These sequences were submitted to the NCBI database as ToBRFV-Ant-Tom and ToBRFV-Ant-Pep, with GenBank accession numbers MT107885 and MT118666, respectively. Their whole genome sequences were compared with other ToBRFV sequences (Appendix B) in the NCBI database and a cladogram was constructed (Fig. 4) which displays related clades with other ToBRFV isolates (Appendix B). BLAST analysis of the sequence of the ToBRFV-Ant-Tom revealed 99.97% sequence identity (Fig. 4, pink area) to isolates of ToBRFV from Israel (KX619418) and State of Palestine (MN013188). BLAST analyses with the ToBRFV-Ant-Pep sequence showed 99.91% sequence identity (Fig. 4, green area) to isolates of ToBRFV from Germany (MK133093) and Mexico (MK319944). This indicates different geographic origins of ToBRFV-Ant-Tom and ToBRFV-Ant-Pep, respectively (Fig. 4).

3.5. Tomato *Tm-2²* does not control resistance of commercial crops to ToBRFV

Artificial pathogenicity tests with ToBRFV showed that tomato plants with the *Tm-2²* resistant gene suppressed symptoms of the disease on seedlings and mature plants until the fruit appeared, which developed disease symptoms including light brown to black discoloration, yellow spotted rugose fruits, chlorosis, mosaic and mottling (Fig. 2-E). Together, these symptoms make the fruit unmarketable. This finding is consistent with the grower's observations that the new disease has caused large product losses on tomato varieties with or without the *Tm-2²* gene. The losses were due in part to the unaesthetic appearance of the affected tomato fruits, leading to the loss of commercial value (Fig. 2). We noted the tomato plants containing *Tm-2²* gene inoculated with ToBRFV did not show symptoms if the temperatures rose to 35–40°C for 10 days; however, altered temperatures led to severe symptoms on fruit, narrowing of leaves and rugose symptoms on leaves (Fig. 2). Hence, the reaction of the tomato plants to ToBRFV could not be reliably

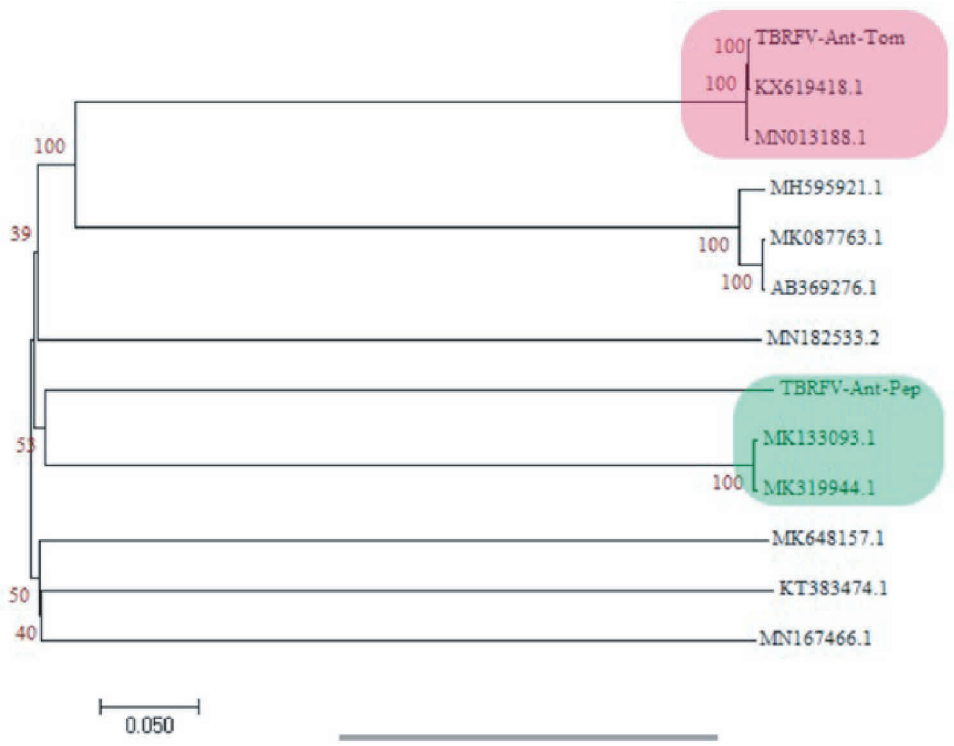


Fig. 4 Phylogenetic analysis of Turkish ToBRFV-Ant-Tom (pink area) and ToBRFV-Ant-Pep (green area) isolates with the aligned genomic nucleic acid sequences from other ToBRFV genome sequences. The sequences included in the analysis are represented by the acronym of the virus and GenBank access numbers. The nodal values support the evolutionary relationship of the analyzed sequences (Appendices B and C).

assessed until 90 dpi of the seedlings. Neither eggplant nor black nightshade plants showed any symptoms following ToBRFV inoculations.

3.6. Pepper *L* genes do not confer resistance to ToBRFV at above 30°C

Pepper plants without any *L* resistance genes are highly susceptible to ToBRFV (Fig. 2). Each individual *L1* or *L2* gene did not confer resistance and the plants displayed yellow to brown rugose, wrinkled, necrotic spots on the fruit (Fig. 2-I and J), similar to the reaction of pepper plants lacking any *L* gene. However, both *L3* and *L4* resistance genes conferred resistance to ToBRFV, and a hypersensitive response (HR) was induced (Fig. 2-H) but no other symptoms appeared on inoculated leaves or upper uninoculated leaves (Fig. 2-H). When pepper plants containing both *L3* and *L4* were inoculated with ToBRFV and incubated at 32°C or above, severe necrotic, rugose, and discolored disease symptoms appeared on the stems (Fig. 2-F), leaves (Fig. 2-G) and fruit (Fig. 2-I and J). This suggested that the *L3* and *L4* resistance mechanisms are inactive at higher temperatures and ToBRFV repetitive inoculation dependent resulting in susceptibility. Bell peppers were found to be more susceptible to ToBRFV than other peppers (Fig. 2-I).

4. Discussion

We report here that a recent and continuing severe disease of commercial tomato and pepper crops growing in Antalya, Turkey was caused by strains of ToBRFV. We determined the nucleotide sequence of the new strains of ToBRFV infecting tomato (ToBRFV-Tom) and pepper (ToBRFV-Pep), and noted their similarities to previously characterized strains of the virus from Israel and from Germany, respectively. We used the nucleotide sequences of the two strains to develop a qRT-PCR assay for the detection of the virus. This assay could be used to identify the virus in samples, as part of a programme to exclude the virus from the commercial crops, fulfilling a goal of previous studies (Luria *et al*. 2017; Cambrón-Crisantos *et al*. 2018). The RT-PCR assay could be used, for example, to identify the source of infection for the commercial crop. It is known, and confirmed in the present study, that ToBRFV is transmitted mechanically. The RT-PCR could be used to examine the role of mechanical transmission of the virus in commercial settings. ToBRFV is also seed-transmitted and is subject to quarantine of infested seeds. The seed export could be tested for ToBRFV as an addition to existing quarantine controls for the virus. It is

significant that our RT-PCR assay detected ToBRFV in a batch of 250 seeds containing a single infected seed, for a detection threshold of 1:249 (1 infected seed/249 healthy seeds). This level would more than meet the requirement for a quarantine test on imported seed as 12 batches of 250 seeds each (3 000 seeds in total) was previously suggested for other *Tobamoviruses* (Dombrovsky and Smith 2017).

The ToBRFV particles may remain viable in infested crop debris, stakes and pots (Luria *et al*. 2017). ToBRFV would survive in crops debris for many years, so rotation to non-host crops such as bean is recommended following a disease epidemic. Greenhouse surfaces, pots, and stakes should be properly disinfected before being used for crop production. Additionally, tobacco products used by workers could contain viable virus particles and therefore serve as a source of inoculum when tomato and pepper plants are being handled. All workers should therefore wash their hands, change their clothes and disinfect tools regularly. Only disease-free seed and transplants should be used, and destroying an infected tomato or pepper crop promptly will help to eradicate the viral disease.

There are specific interactions between *L* genes and *Tobamovirus* spp. where studies have identified viral coat proteins (CP) as Avr effectors for *L* gene-mediated resistance (Gilardi *et al*. 2004; Matsumoto *et al*. 2008). Comparisons of amino acid sequences revealed that CP of ToMV, PaMMV, and PMMoV are distantly related to each other, but those from the P1-2, P1-2-3, and P1-2-3-4 pathotypes of PMMoV strains differ in only one or two amino acid residues (Hamada *et al*. 2002; Genda *et al*. 2007; Antignus *et al*. 2008; Tomita *et al*. 2011).

We confirm here that the dominant resistance genes *Tm-2*^{2} and *L4* do not confer resistance to ToBRFV. This finding points to an urgent need to discover novel sources of resistance to guard tomato and pepper cultivars against ToBRFV. Several seed companies have begun to use molecular techniques such the CRISPR-Cas9 based gene-editing methodology as an alternative for producing non-transgenic tomato and pepper plants that are resistant to the ToBRFV (Liu *et al*. 2020).

The rod-shaped virus particles of ToBRFV could be distinguished from the round virus particles of Tomato spotted wilt virus (TSWV) by observation under electron microscopy (data not included). However, in pepper both ToBRFV and TSWV cause many similar macroscopic characteristics including yellowing, necrosis, discoloration but when the pods are opened the seeds of TSWV-infected plants are white, whereas in ToBRFV infected plants the seeds are discolored brown.

Antalya is in the heart of the West Mediterranean Region of Turkey, and is important for production of winter tomato and pepper. It also represents the main point for the export and import of plant material. This situation may increase the risk of introducing new pathogens into a key region for commercial crop production in Turkey. The outbreak of ToBRFV represents a threat due to its multiple transmission methods. Our work revealed that ToBRFV-Ant-Tom has 99.97% identity to the Israeli ToBRFV-II isolate, and that ToBRFV-Ant-Pep has 99.91% identity to a South Korean TMV isolate. In addition, the 99.95% identity reported here between ToBRFV-Ant-Tom and ToBRFV-Ant-Pep points to a common origin of these two isolates. While the current situation persists, with an absence of tomato and peppers varieties resistant to these two viruses, there is an imminent danger of these viruses spreading to the other tomato- and pepper-growing regions of Turkey. The diagnostic test we report here may represent one of the few tools available for early diagnosis and implementation of preventive measures in crop management, reducing the further spread of ToBRFV within Turkey, and its export to other countries

5. Conclusion

The study revealed a new virus which causes epidemics on tomato and pepper plants in Turkey. The virus is identified as Tomato brown rugose fruit virus and its molecular detection systems have been developed. Currently, neither the *Tm-2*^{2} gene in tomato nor the *L* genes in pepper confer resistance against this virus.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendices associated with this paper are available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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