Comparative Analysis of Host Range, Ability to Infect Tomato Cultivars with *Tm-2²* Gene, and Real-Time Reverse Transcription PCR Detection of Tomato Brown Rugose Fruit Virus

Bidisha Chanda, Andrea Gilliard, Namrata Jaiswal, and Kai-Shu Ling[†]

U.S. Vegetable Laboratory, U.S. Department of Agriculture–Agricultural Research Service, Charleston, SC 29414

Abstract

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetables in the world. However, tomato is also susceptible to many viral diseases. Several tobamoviruses, including tomato mosaic virus (ToMV), tomato mottle mosaic virus (ToMMV), and tomato brown rugose fruit virus (ToBRFV), are highly contagious pathogens that could result in significant economic losses if not controlled effectively. Tobamoviruses have been managed relatively well with broad adaptation of tomato cultivars with resistance genes. However, emergence of ToBRFV was shown to break down resistance conferred by the common resistance genes, resulting in serious outbreaks in many countries in Asia, Europe, and North America. The objective of this study was to conduct a comparative analysis of biological properties, including host range and disease resistance of ToMV, ToMMV, and ToBRFV. Results showed that despite many similarities in the host range, there were some unique host plant responses for each of the three

viruses. In a comparative evaluation of disease resistance using the same tomato cultivars with or without *Tm-2²* gene, there was a striking difference in responses from tomato plants with *Tm-2²* gene inoculated with ToBRFV, ToMV, or ToMMV. Whereas these test plants were resistant to ToMV or ToMMV infection, all test plants were susceptible to ToBRFV. Further, for ToBRFV detection, a sensitive and reliable multiplex real-time reverse transcription (RT)-PCR assay using Taq-Man probe with an internal 18S rRNA control was also developed. With simple modifications to RNA extraction and seed soaking, real-time RT-PCR could consistently detect the virus in single infested seed in varied levels of contamination, suggesting its usefulness for seed health assay.

Keywords: fruit, real-time RT-PCR, seed health test, *Solanum lycopersicum*, tobamovirus, ToBRFV, tomato

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops grown worldwide, with the United States as the third largest producer after China and India. Tomato production in the United States is estimated at 12.6 million tonnes annually (<http://www.fao.org/faostat/en/#data/QC>). Tomato is especially susceptible to viral disease infections, with hundreds of viruses in 40 genera recognized infecting solanaceous plants (Hančinský et al. 2020). Viral diseases are difficult to manage; thus, planting disease-resistant cultivars is currently the most effective and economical means of viral disease management.

Several species of the genus *Tobamovirus* (family Virgaviridae), such as tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), tomato mottle mosaic virus (ToMMV), and tomato brown rugose fruit virus (ToBRFV), have been recognized to infect tomato crops. Depending on the genotype and environmental conditions, symptoms expressed on the infected tomato plants by these tobamoviruses are similar, including mosaic, mottling, necrosis, leaf distortion, stunted plants, and smaller sized fruits. In the case of ToBRFV, leaf necrotic lesions and fruits with brown rugose may develop on certain tomato cultivars (Fig. 1). Structurally, tobamoviruses are rod-shaped, positive sense single-stranded RNA viruses. They typically contain four open reading frames, encoding two replication-associated proteins (126 and

183 kDa, with the second protein expressed through leaky expression of the UAG stop codon), a movement protein (30 kDa), and a coat protein (17.5 kDa) (Ishibashi and Ishikawa 2016). RNA viruses evolve rapidly because of their high rate of mutation during replication from error-prone RNA-dependent RNA polymerase and their short genome length. These mutations may be lethal, neutral, or beneficial to the viral fitness. However, during coevolutionary race between plants and viruses, the natural selection will exert pressure on this mutant virus population to increase biological fitness (Hall 1980). This process may lead to speciation through mutations and horizontal/lateral gene transfer with other related viruses. These new virus species may be introduced to different parts of the world through increasing global trade activities involving seeds, seedlings, planting materials, or fruits, resulting in new disease epidemics.

A new species of tobamovirus, ToBRFV, originally identified in the Middle East, Jordan (Salem et al. 2016), and Israel (Luria et al. 2017), has been found to cause outbreaks in greenhouse tomatoes around the world. Countries reporting the presence of ToBRFV are China (Yan et al. 2019) and Palestine (Alkowni et al. 2019) in Asia; Egypt in Africa (Amer and Mahmoud 2020); Germany (Menzel et al. 2019); Greece (Beris et al. 2020), Italy (Panno et al. 2019a), The Netherlands (van de Vossenberg et al. 2020), Spain (Alfaro-Fernandez et al. 2020), Turkey (Fidan et al. 2019), and the United Kingdom (Skelton et al. 2019) in Europe; and Canada (Davidson 2019), Mexico (Cambrón-Crisantos et al. 2018; Camacho-Beltran et al. 2019), and the United States (Ling et al. 2019; Chanda et al. 2020) in North America. To curb the virus spread, the United States issued a federal order on ToBRFV in November 2019 to inspect for the presence of ToBRFV on tomato and pepper produce imported from selected countries as well as on tomato and pepper seeds and planting materials worldwide (U.S. Department of Agriculture 2019). Tobamoviruses are considered seedborne viruses and can transmit to seedlings from contaminated seeds (Dombrovsky and Smith 2017). However, its true seed transmission through embryo in contaminated seeds is still in question (Ellis et al. 2020). As a tobamovirus, ToBRFV is a seedborne and mechanically transmissible virus, deeming contaminated seeds a potential pathway for virus spread, thereby necessitating the development of a sensitive, reliable seed health

[†]Corresponding author: K.-S. Ling; kai.ling@usda.gov

Funding: This work was supported in part by U.S. Department of Agriculture–Agricultural Research Service, National Plant Disease Recovery System Program grant 6080-22000-028 (to K.-S. Ling).

*The e-Xtra logo stands for “electronic extra” and indicates there is supplementary material published online.

The author(s) declare no conflict of interest.

Accepted for publication 27 May 2021.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2021.

test to achieve effective management of this emerging virus. In greenhouse tomato production, it is necessary to introduce pollinators, particularly bumblebees, to improve crop production through pollination. However, in a number of cases, pollinators, bumblebees, and honey bees are shown to aid the spread of tobamoviruses, including ToBRFV (Levitzky et al. 2019; Okada et al. 2000; Roberts et al. 2018).

The current global ToBRFV outbreaks are reported largely on tomato and pepper crops grown under greenhouse or other protected cultivation conditions. As it quickly spreads around the world, it is important to uncover ToBRFV's potential impact on other crops as well as on some weed hosts. Previous studies have reported a range of host plants for ToBRFV using isolates from Jordan, Israel, and China (Luria et al. 2017; Salem et al. 2016; Yan et al. 2020). These studies, however, were not comprehensive and could benefit from additional analysis using different isolates of ToBRFV. In addition, with the existence of several closely related tobamoviruses infecting tomato, information on their host range through a direct comparative analysis among ToMV, ToMMV, and ToBRFV would be valuable, not only for improving current knowledge but also for ensuring practical disease management. Through many years of breeding, breeders have been successful in introducing the dominant resistance R genes *Tm-1*, *Tm-2*, and *Tm-2²* in cultivated tomato (Pelham 1966; Lanfermeijer et al. 2003; Ohmori et al. 1996; Young et al. 1988). The *Tm-1* gene is

derived from *Solanum habrochaites*, whereas the *Tm-2* and *Tm-2²* genes are derived from *Solanum peruvianum* and are allelic. *Tm-2²* confers durable resistance to diverse ToMV strains (Shi et al. 2011). In a previous study, *Tm-2²* conferred resistance to ToMMV in some tomato cultivars (e.g., Endeavour) but only partial resistance in other cultivars (e.g., Brioso) (Sui et al. 2017), which was also the case when tested against Brazilian isolates of ToMMV (Nagai et al. 2019). The *Tm-2* and *Tm-2²* genes are dominant resistance genes and encode the CC-NB-LRR class of resistance proteins (Lanfermeijer et al. 2003). According to the gene-for-gene model, for every dominant resistant gene in the host plant there is a matching dominant avirulent gene in the pathogen (Flor 1942). The viral movement protein is the avirulent factor that interacts with the tomato *Tm-2* and *Tm-2²* resistance proteins upon perception (Calder and Palukaitis 1992; Weber et al. 1993). For nearly half a century, tobamoviruses were managed by using commercial tomato cultivars containing the *Tm-2* and/or *Tm-2²* resistance genes. The emerging ToBRFV demonstrated its ability to break the durable *Tm-2* and *Tm-2²* gene resistance, which raises serious concerns among tomato growers (Luria et al. 2017; Maayan et al. 2018). In the present study, we were interested in using a U.S. isolate of ToBRFV to assess the effect of resistance in tomato cultivars with or without the *Tm-2²* gene. More importantly, we conducted a direct comparative analysis on disease resistance to three tobamoviruses: ToBRFV, ToMV, and ToMMV. Tomato cultivars with and without *Tm-2²* gene were evaluated for resistance breaking, including the same three cultivars (Brioso, Endeavour, and Idolini) that were used in our previous study for ToMMV (Sui et al. 2017).

The goal of the present study is to provide additional information for this emerging virus by (i) determining its host range on different plant species while making a comparative evaluation with two related viruses: ToMV and ToMMV; (ii) evaluating resistance-breaking ability of ToBRFV using selected commercial tomato cultivars containing the *Tm-2²* gene in a direct comparison with two other tobamoviruses: ToMV and ToMMV; and (iii) developing a real-time reverse transcription (RT)-PCR method to achieve sensitive, reliable ToBRFV detection that is useful for virus diagnosis in plant, bees, and seed samples.

Materials and Methods

Virus isolates and generation of a pure culture of ToBRFV.

The ToBRFV-US isolate CA18-01 was collected from tomato in a greenhouse in southern California in August 2018 (Ling et al. 2019); ToMMV isolate SC13-05 was collected from a greenhouse in South Carolina in 2013; and ToMV isolate V13-07 was collected from tomatoes in California (Sui et al. 2017). The individual virus isolates were maintained on 'Moneymaker' tomato plants in an insect-proof bugdorm (BioQuip Products, Compton, CA) within a containment greenhouse with temperature maintained at 25 to 30°C at the U.S. Vegetable Laboratory, Charleston, SC. The initial greenhouse-collected sample of isolate CA18-01 contained a mix of two viruses: ToBRFV and pepino mosaic virus (PepMV) (Ling et al. 2019). To conduct accurate host range evaluation for ToBRFV, it was necessary to obtain a pure culture of ToBRFV, devoid of PepMV. This was achieved by two passages of local lesion isolations after mechanical inoculation on *Nicotiana tabacum* var. *samsun*, which is a host plant for ToBRFV but a nonhost for PepMV (Gómez et al. 2009). The first passage was by inoculating two healthy *N. tabacum* var. *samsun* plants with an inoculum prepared from the field-collected sample. A local lesion that developed on the inoculated leaf was dissected 2 weeks postinoculation and used to prepare a new inoculum for the second passage inoculation on a healthy plant of *N. tabacum* var. *samsun*. After two passages, symptomatic systemic leaves of inoculated plants were harvested and confirmed through RT-PCR tests to be positive for ToBRFV and negative for PepMV. This pure isolate of ToBRFV (CA18-01) was used for subsequent experiments.

For seed health assays, experimental seeds were extracted from fruits harvested from ToBRFV-infected tomato 'Moneymaker' plants in a containment greenhouse. Similarly, control healthy seeds were obtained from fruits of the uninoculated 'Moneymaker' plants. Seeds

Fig. 1. Typical symptoms of tomato brown rugose fruit virus infection on tomato and pepper. **A**, Typical brown rugose fruit with necrotic patches on tomato cultivar Progression with *Tm-2²*. **B**, A truss of tomatoes with blotchy and brown necrotic patches and necrotic spots on calyxes and peduncle. **C**, A close-up look at a tomato with typical brown necrotic patches. **D**, Abscission and drying of flowers from infected tomato plants. **E**, Leaf symptoms showing mosaic, mottling, and narrow leaves on tomato 'Moneymaker' without *Tm-2²* resistance gene. **F**, Necrotic lesions and leaf deformation developed on a tomato cultivar Endeavour with the *Tm-2²* resistance gene. **G**, Necrotic spots developed on young leaves at 5 days postinoculation on pepper (*Capsicum annuum* 'Feferona', PI 379126) to ToBRFV infection. **H**, The initial necrotic leaves drop off from the infected pepper cultivar Feferona plant, and newly developed leaves appeared to recover from infection at 10 days postinoculation. **I**, Typical disease symptoms on pepper, including mosaic, mottling, and necrotic spots, reappeared on plants 4 weeks postinoculation. **J**, The infected pepper cultivar Feferona plants could still produce fruits, although there were some necrotic spots on fruits and pedicels.

were manually extracted from ripened fruits, air-dried, and used for optimizing seed health tests.

Host range analysis. For host range studies, a total of 31 plant species from seven families was evaluated. Seedlings at the two- to three-leaf stage were lightly dusted with Carborundum (320-grit; Thermo Fisher Scientific, Gaithersburg, MD) and gently inoculated with a cotton swab soaked in an inoculum of ToBRFV CA18-01 prepared (1:5 wt/vol) in a 1× phosphate-buffered saline solution, pH 7.0 (140 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, and 0.8 mM Na₂SO₃). The host range experiment was conducted twice in replicated experiments, each with three biological replicates per species. For mock inoculation, at least one plant per species was set aside and inoculated in the same method with inoculation buffer as the healthy control. The test plants were observed for symptoms daily during the first week postinoculation on inoculated leaves and then weekly for systemic infection. Symptoms on emerging leaves could be visible at 7 days postinoculation on some susceptible plants. The final reading was made at 4 weeks postinoculation. To confirm ToBRFV infection, leaf tissue was collected from systemically infected leaves and tested for the presence of the target virus through RT-PCR using virus-specific primers (Ling et al. 2019).

Evaluating resistance-breaking ability to the *Tm-2²* gene by tobamoviruses. To evaluate the resistance-breaking ability by ToBRFV against the *Tm-2²* gene (Luria et al. 2017), we conducted replicate experiments through direct comparison of plant responses to three tobamoviruses (ToMV, ToMMV, and ToBRFV) using seeds of the same three commercial tomato cultivars containing *Tm-2²* (Brioso, Endeavour, and Idolini, F1 hybrids developed by Rijk Zwaan, The Netherlands) as those used for ToMMV evaluation (Sui et al. 2017). Along with a susceptible control cultivar Moneymaker, we used the same experimental design (Sui et al. 2017) as the mechanical inoculation method described above in the host range study. The evaluation was conducted in three independent experiments. In the first experiment, 21 to 28 plants from each of three tomato cultivars (Endeavour, Brioso, and Idolini) and 20 plants from the control 'Moneymaker' were used for ToBRFV evaluation. Between 8 and 10 plants were inoculated with ToMV or ToMMV for each tomato cultivar, along with six plants for control 'Moneymaker'. Two additional experiments were conducted, with each experiment using five plants per cultivar (Endeavour, Brioso, or Idolini) as well as the control 'Moneymaker' for inoculation with three tobamoviruses (ToBRFV, ToMV, and ToMMV). For mock inoculation with inoculation buffer, three plants per cultivar were used for each experiment. Symptom observation was conducted weekly post-inoculation and concluded at 4 weeks postinoculation. To verify virus infection, leaf tissue samples from each of the test plants were collected and used for serological analysis (enzyme-linked immunosorbent assay [ELISA]) as described below. Plants with disease symptoms that were confirmed to be infected by the target virus as tested in ELISA were considered susceptible. Statistical analysis using two-way ANOVA followed by Dunnett's multiple comparisons ($\alpha = 0.05$) test of mean percent infection was used to analyze the level of significance among ToBRFV, ToMV, and ToMMV on their infection on four tomato cultivars in three replicated experiments.

In addition, to evaluate resistance phenotype in other commercial tomato cultivars against ToBRFV, a total of 23 cultivars with or without *Tm-2²* gene was evaluated using mechanical inoculation method as described above in the host range analysis. The virus infection was recorded 4 weeks postinoculation by symptom observation and confirmation test using serological method as described below.

Serological test using ELISA. Because ToBRFV-specific antibody was not available to us and with known serological cross-reactivities among tomato-infecting tobamoviruses, a commercial ELISA kit for TMV was used to detect ToBRFV following the manufacturer's instructions (Agdia, Elkhart, IN). The absorbance values at OD_{405nm} were measured using a SpectraMax ELISA microplate reader (Molecular Devices, San Jose, CA). An absorbance value that was at least twice that of the healthy control was considered positive.

Total RNA extraction from seed, leaf, and bumblebee tissues. Total RNA preparations from greenhouse-collected plant leaf and fruit tissues or bumblebees (used by growers to enhance pollination) were

extracted using TRIzol reagent following manufacturer's instructions (Thermo Fisher Scientific). However, for seed samples, the initial step in sample processing was modified to include soaking of crushed seed tissues in 1× ELISA general extraction buffer (GEB) to allow for release of virus particles to the tissue extraction buffer before RNA extraction. The 1× buffer was prepared from 10× concentrate of GEB from a commercial source (BioReba, Reinach, Switzerland). For seed extract preparation, one tomato seed was initially processed in 1 ml of 1× GEB buffer, 100 seeds in 2 ml of GEB, 200 seeds in 3 ml of GEB, 400 seeds in 5 ml of GEB, and 800 seeds in 7 ml of GEB, which was just enough to cover the seeds and generate a sufficient amount of processed seed slurry needed for downstream RNA extraction. After placing the desired number of seeds in a plastic tissue extraction bag (BioReba), seeds were laid flat and carefully crushed with a mallet without puncturing the plastic bag. Lightly crushed seeds were soaked in a desired amount of 1× GEB and incubated on ice for 60 min. The soaked seed tissue was homogenized for 10 s at top speed using a Homex homogenizer (BioReba). An aliquot of aqueous phase of the seed extract (100 µl) was transferred to a 1.5-ml Eppendorf tube containing 500 µl of TRIzol Reagent and vortexed rigorously, and then the mixture was allowed to settle for 5 min at room temperature. After centrifugation for 10 min at 12,000 × g, 500 µl of supernatant was transferred to a new tube with 200 µl of chloroform. The mixture was vortexed vigorously for 15 s and then allowed to settle for 15 min at room temperature. The process was followed by centrifugation for 15 min at 12,000 × g in a refrigerated microcentrifuge, after which 300 µl of supernatant was transferred to a new tube, mixed with 500 µl of ice-cold isopropanol, and allowed to settle for 10 min. After another centrifugation for 10 min at 12,000 × g and removal of the supernatant, the resulting pellet was rinsed twice with 75% ethanol (250 µl each) and air dried at room temperature in a laminar flow hood. Purified total RNA was prepared by resuspending the pellet in 100 µl of RNase-free water, quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific), and used for virus detection in RT-PCR reactions or stored at -80°C for future use.

Conventional RT-PCR. For ToBRFV detection, conventional RT-PCR was conducted using primers and protocols described by Ling et al. (2019), and for detection of ToMV and ToMMV, conventional RT-PCR was performed as described previously (Sui et al. 2017). The PCR products were analyzed by gel electrophoresis in a 1.5% agarose gel and SYBR Safe DNA Gel Stain (Thermo Fisher Scientific).

Developing multiplex RT-quantitative PCR useful for seed health assay. To develop a real-time RT quantitative PCR (RT-qPCR) assay for sensitive and species-specific detection of ToBRFV, it is important to identify a sequence region in the virus genome that is conserved among ToBRFV isolates but sufficiently divergent from other closely related tobamoviruses (e.g., TMV, ToMV, ToMMV, and cucumber green mottle mosaic virus [CGMMV]) for primers and TaqMan probe. From a multiple sequence alignment, a 92-nt conserved sequence region in the movement protein gene was identified; consequently, primers and probe were selected manually to consider significant sequence divergence, particularly at the 3' terminal sequence of a primer if possible, to other related tobamoviruses (Supplementary Fig. S1). In addition, these primer and probe sequences (ToBRFV-F1, 5'-GCCCATGGAACATCAGAAGAA-3'; ToBRFV-R1, 5'-TTCCGGTCTTCGAACGAAAT-3'; ToBRFV-P1, FAM-AGTCCCGATGTCTGTAAGGCTTGC-TAMRA) were verified to share no significant sequence identity to possible off-target species by *in silico* analysis in GenBank using the BLASTn program (NCBI). To develop a multiplex RT-qPCR, an endogenous gene of 18S rRNA was selected to serve as an internal control (Osman et al. 2007) using 18S rRNA 449_F: 5'-GTGACGGAGAATTAGGGTTCGA-3', 18S rRNA 498_R: 5'-CTGCCTTCC TTGGATGTGGTA-3', and 18S rRNA 475_P: CY5-CCGGAGAGGGAGCCTGAGAAACGG-BHQ-2. Real-time RT-qPCR was performed using a One Step PrimeScript RT-PCR kit following the manufacturer's instructions (Takara Bio USA, Mountain View, CA). A master reaction mixture was prepared according to the number of samples to be tested, with the following compositions for each 10-µl aliquot: 5 µl of 2× buffer, 0.25 µl of the Ex Taq HS mix, 0.25 µl of the RTase mix, 0.25 µl of forward primer ToBRFV-F1 (10 µM), 0.25 µl of reverse primer ToBRFV-R1 (10 µM), 0.25 µl of TaqMan probe

ToBRFV-P1 (10 µM), 0.25 µl of forward primer 18S rRNA 449_F (10 µM), 0.25 µl of reverse primer 18S rRNA 498_R (10 µM), 0.25 µl of TaqMan probe 18S rRNA 475_P (10 µM), 0.2 µl of diluted Ref Dye (ROX), 0.5 µl of RNA preparation as the template, and the appropriate amount of RNase-free water to a total of 10 µl. For multiple reactions, the RNA preparation was added to individual tubes as a last step after placing each aliquot of master mixture into a PCR tube with optical cap. Real-time RT-PCR reaction was carried out in an MX3000P Realtime PCR machine (Stratagene, San Diego, CA) with the following thermocycling program: 50°C for 30 min of RT, followed by denaturation at 95°C for 2 min, and then 40 cycles of 95°C for 10 s and 55°C for 30 s.

Results

Comparative host range of ToBRFV with that of ToMV and ToMMV. The ToBRFV-US isolate CA18-01 (GenBank accession no. MT002973), collected in August 2018 from an infected tomato plant in a southern California greenhouse, was originally described in the first report of ToBRFV in the United States (Ling et al. 2019). Tomato brown rugose fruit disease symptoms varied greatly on infected tomatoes, from mild mosaic, mottling, leaf deformation, and necrotic spots to brown rugose fruits, depending on the tomato cultivars and greenhouse conditions (Fig. 1A to F). ToBRFV-infected pepper plants

showed similar symptoms (mosaic and mottling but without rugose on fruit) as those in tomato (Fig. 1G to J).

For host range evaluation, a total of 31 plant species belonging to seven families was tested. The resulting host range of ToBRFV US isolate (Table 1) was then compared with those of ToMV and ToMMV, obtained in a previous study (Sui et al. 2017). Overall, the host ranges among three tomato-infecting tobamoviruses were similar (Table 2), with many host species in common. However, some unique host species were also observed for each of the viruses. Several plant species belonging to four families (Amaranthaceae, Apocynaceae, Asteraceae, and Solanaceae) were found to be hosts of ToBRFV. On the other hand, test plants from three other families (Brassicaceae, Cucurbitaceae, and Verbenaceae) were nonhosts for ToBRFV. Many (11 of 12 tested) plant species in the family Solanaceae showed a high degree of susceptibility to ToBRFV infection. In addition to tomato and pepper (Fig. 1), garden petunia, black nightshade, husk tomato, cutleaf groundcherry, jimsonweed, and tobacco were also susceptible to ToBRFV infection (Fig. 2). One exception in the family Solanaceae is eggplant (*Solanum melongena*), a nonhost for ToMV and ToMMV, which is also a nonhost for ToBRFV (Fig. 2; Tables 1 and 2).

Plant species in the family Amaranthaceae were also susceptible to ToBRFV infection. While globe amaranth (*Gomphrena globosa*) developed systemic infection, four *Chenopodium* species produced

Table 1. Evaluation of the experimental host range of the tomato brown rugose fruit virus isolate CA18-01

Plant family, species, and cultivar	Common name	ToBRFV		
		Local symptoms ^a	Systemic symptoms ^a	RT-PCR ^b
Amaranthaceae				
<i>Chenopodium album</i> , Ames 21063	Lamb's quarters	CLL, NRS	C, DL	+
<i>Chenopodium berlandieri</i> var. <i>bushianum</i> , PI 608030	Lamb's quarters	CLL, NRS	–	–
<i>Chenopodium giganteum</i> , NSL 86650	Three spinach	NRS	–	–
<i>Chenopodium quinoa</i> ^c	Quinoa	CLL, NRS	M, DL	+
<i>Gomphrena globosa</i> , PI 606070	Globe amaranth	–	M	+
Apocynaceae				
<i>Catharanthus roseus</i> , PI 608581	Rosy periwinkle	–	–	+
Asteraceae				
<i>Emilia sonchifolia</i>	Lilac tasselflower	NLL	VN	+
<i>Glebionis coronaria</i> , PI 564828	Crown daisy	–	M, LM	+
<i>Lactuca sativa</i> , W6 29844	Lettuce	–	–	–
Brassicaceae				
<i>Brassica rapa</i> , PI 370742	Field mustard	–	–	–
Cucurbitaceae				
<i>Citrullus lanatus</i> 'Charleston Gray'	Watermelon	–	–	–
<i>Cucumis melon</i> , F1 hybrid	Cantaloupe	–	–	–
<i>Cucumis metulifer</i> , PI 202681	Horned melon	–	–	–
<i>Cucurbita moschata</i>	Butternut squash	–	–	–
Solanaceae				
<i>Nicotiana glutinosa</i> ^c	Tobacco	N	PD	+
<i>Nicotiana occidentalis</i> subsp. <i>hesperis</i> , PI 555687 ^c	Native tobacco	NRS	M, N, CP	+
<i>Nicotiana benthamiana</i> , PI 555478 ^c	Benthi	N	PD	+
<i>Datura stramonium</i> ^c	Jimsonweed	N	PD	+
<i>Nicotiana debneyi</i> , PI 555497	Debney's tobacco	–	M	+
<i>Nicotiana rustica</i> , PI 555555	Aztec tobacco	CLL, NRS	M, DL, S	+
<i>Nicotiana tabacum</i> 'Samsun', PI 512288 ^c	Tobacco	–	M, N, CP	+
<i>Nicotiana tabacum</i> 'Xanthi nc'	Tobacco	NLL	PD	+
<i>Petunia</i> × <i>hybrida</i> , G 28643 ^c	Garden petunia	–	M	+
<i>Physalis angulata</i> , PI 305457	Cutleaf groundcherry	N	NL	+
<i>Physalis pubescens</i> , PI 644008	Husk tomato	N	NL	+
<i>Solanum lycopersicum</i> 'Moneymaker' ^c	Tomato	–	M, D	+
<i>Solanum lycopersicum</i> 'Rutgers'	Tomato	–	M, D	+
<i>Solanum melongena</i> ^c	Eggplant	–	–	–
<i>Solanum nigrum</i> , PI 381290 ^c	Black nightshade	CLL, NRS	MM	+
<i>Capsicum annuum</i> 'Feferona', PI 379126 ^c	Pepper	N	C, YM, NRS	+
Verbenaceae				
<i>Verbena officinalis</i> var. <i>halei</i> , PI 608581	Common verbena	–	–	–

^a Symptoms observed on inoculated (local symptoms) and noninoculated leaves (systemic symptoms) are indicated in their respective columns. CLL = chlorotic local lesion; NRS = necrotic ringspot; C = chlorosis; DL: deformed leaf; – = no symptom; LM: leaf mottling; M = mosaic; MM: mild mosaic; NLL = necrotic local lesion; VN = vein necrosis; N = necrosis; PD = plant death; CP = chlorotic plant; NL = necrotic lesion; S = stunting; YM = yellow mosaic.

^b Indicate presence of the virus in upper noninoculated leaves. All systemic infections were confirmed by species-specific reverse transcription PCR for tomato brown rugose fruit virus (ToBRFV). + = positive results; – = negative results.

^c These plant species have already been shown to be hosts for ToBRFV when using an Israeli isolate of ToBRFV (Luria et al. 2017).

chlorotic local lesions, with two species producing local infection only and the two other species escalated to systemic infection (Fig. 2; Tables 1 and 2). Rose periwinkle, a popular ornamental plant found in gardens and homes across the world, a nonhost for ToMMV, was an asymptomatic carrier for ToBRFV. Lilac tasselflower, a common weed in tropical regions around the world, was also a nonhost for ToMV and ToMMV but a symptomatic host for ToBRFV. Debney's tobacco (*Nicotiana debneyi*) was a local lesion host for ToMV and ToMMV, but it was a host for ToBRFV and developed systemic mosaic symptoms on leaves (Fig. 2). Conversely, common verbena (*Verbena officinalis* var. *halei*), an asymptomatic carrier for both ToMV and ToMMV, tested negative for ToBRFV.

ToBRFV infects tomato cultivars with *Tm-2²* gene that are resistant to ToMV and ToMMV. To evaluate virulence effect of ToBRFV on tomato plants with or without the *Tm-2²* resistance gene, three independent replicated experiments were conducted using four representative tomato cultivars for their resistance properties against infection by three tobamoviruses (ToMV, ToMMV, and ToBRFV) (Supplementary Table S1). The cultivar Endeavour containing *Tm-2²*, resistant to infection by ToMV (5.9% infection rate) and ToMMV (16.7% infection rate), was 100% susceptible to ToBRFV infection (Fig. 3; Supplementary Table S1). Inoculated plants of cultivar Endeavour developed necrotic local lesions on inoculated leaves at approximately 1 week postinoculation. By 4 weeks postinoculation, test plants from the susceptible cultivar Moneymaker developed severe mosaic, mottling, and distorted leaf symptoms. Inoculated plants

from cultivar Endeavour also developed similar mosaic symptoms in addition to some necrotic lesions on systemic leaves by 4 weeks postinoculation. The cultivar Brioso (also containing *Tm-2²*) was resistant to ToMV (5% infection rate) and ToMMV (10% infection rate) but was 100% susceptible to ToBRFV infection (Fig. 3; Supplementary Table S1). The cultivar Idolini was more susceptible to infection by ToMV (35% infection rate) and ToMMV (25% infection rate) than that of cultivars Endeavour and Brioso, which was also 100% infection by ToBRFV (Fig. 3; Supplementary Table S1). Symptoms (mild mosaic and mottling) caused by ToBRFV on cultivars Brioso and Idolini were more subtle than that on cultivar Endeavour, which also developed necrotic lesions on systemic leaves (Fig. 3). Upon determination of susceptibility of tomato cultivars with the *Tm-2²* gene to ToBRFV CA18-01, we tested other commercial tomato cultivars (with or without *Tm-2²*) for ToBRFV resistance. In a total of 23 tomato cultivars tested, regardless of being with or without *Tm-2²*, all test plants were susceptible and tested positive for ToBRFV (Supplementary Table S2).

Developing an RT-qPCR assay for species-specific detection of ToBRFV. Because of the cross-serological reactivity of tomato-infecting tobamoviruses, the current serological test could not achieve species-specific identification. In our previous study, a species-specific conventional RT-PCR was developed for ToBRFV detection (Ling et al. 2019). In this study, we attempted to develop a real-time RT-qPCR that could be scalable to allow for high throughput sample processing, quantification, and timely analysis.

Table 2. Comparison of the experimental host range of the tomato brown rugose fruit virus with that of the tomato mottle mosaic virus and the tomato mosaic virus

Plant family, species, and cultivar (accession number)	Common name	ToBRFV ^a	ToMMV ^a	ToMV ^a
Amaranthaceae				
<i>Chenopodium album</i> , Ames 21063	Lamb's quarters	+	— ^b	+
<i>Chenopodium berlandieri</i> var. <i>bushianum</i> , PI 608030	Lamb's quarters	— ^b	— ^b	+
<i>Chenopodium giganteum</i> , NSL 86650	Three spinach	— ^b	— ^b	+
<i>Chenopodium quinoa</i>	Quinoa	+	+	+
<i>Gomphrena globosa</i> , PI 606070	Globe amaranth	+	+	+
Apocynaceae				
<i>Catharanthus roseus</i> , PI 608581	Rosy periwinkle	+	—	NA
Asteraceae				
<i>Emilia sonchifolia</i>	Lilac tasselflower	+	—	—
<i>Glebionis coronaria</i> , PI 564828	Crown daisy	+	+	NA
<i>Lactuca sativa</i> , W6 29844	Lettuce	—	—	—
Brassicaceae				
<i>Brassica rapa</i> , PI 370742	Field mustard	—	—	—
Cucurbitaceae				
<i>Citrullus lanatus</i> 'Charleston Gray'	Watermelon	—	—	NA
<i>Cucumis melon</i> , F1 hybrid	Cantaloupe	—	—	—
<i>Cucumis metulifer</i> , PI 202681	Horned melon	—	—	—
<i>Cucurbita moschata</i>	Butternut squash	—	—	NA
Solanaceae				
<i>Nicotiana glutinosa</i>	Tobacco	+	NA	NA
<i>Nicotiana occidentalis</i> subsp. <i>hesperis</i> , PI 555687	Native tobacco	+	NA	NA
<i>Nicotiana benthamiana</i> , PI 555478	Benthi	+	+	NA
<i>Datura stramonium</i>	Jimsonweed	+	+	—
<i>Nicotiana debneyi</i> , PI 555497	Debney's tobacco	+	— ^b	— ^b
<i>Nicotiana rustica</i> , PI 555555	Aztec tobacco	+	+	+
<i>Nicotiana tabacum</i> 'Samsun', PI 512288	Tobacco	+	+	+
<i>Nicotiana tabacum</i> 'Xanthi nc'	Tobacco	+	+	— ^b
<i>Petunia × hybrida</i> , G 28643	Garden petunia	+	+	NA
<i>Physalis angulata</i> , PI 305457	Cutleaf groundcherry	+	+	+
<i>Physalis pubescens</i> , PI 644008	Husk tomato	+	+	+
<i>Solanum lycopersicum</i> 'Moneymaker'	Tomato	+	+	+
<i>Solanum lycopersicum</i> 'Rutgers'	Tomato	+	NA	NA
<i>Solanum melongena</i>	Eggplant	—	— ^b	— ^b
<i>Solanum nigrum</i> , PI 381290	Black nightshade	+	+	+
<i>Capsicum annum</i> 'Feferona', PI 379126	Pepper	+	NA	NA
Verbenaceae				
<i>Verbena officinalis</i> var. <i>halei</i> , PI 608581	Common verbena	—	+ ^c	+ ^c

^a Information on the host range for the tomato brown rugose fruit virus (ToBRFV) is derived from Table 1 in the present study. The host ranges for tomato mottle mosaic virus (ToMMV) and tomato mosaic virus (ToMV) were derived from our previous publication (Sui et al. 2017). + = host; — = nonhost; NA = not available.

^b Systemic infection was not detected on these test plants, but necrotic or chlorotic local lesions were observed on inoculated leaves.

^c Asymptomatic host and latent infection based on PCR detection of the specific virus.

To develop sensitive and species-specific detection of ToBRFV, a 92-nucleotide conserved sequence located in the movement protein gene was identified through multiple genome sequence alignment of known sequences of ToBRFV isolates (Supplementary Fig. S1). Both forward and reverse primers and TaqMan probe were designed based on their conserved sequence for ToBRFV with significant divergence from other related tobamoviruses. Upon optimization of the newly developed RT-qPCR, species-specific detection of ToBRFV was demonstrated in which only those samples containing ToBRFV were positive, and the

TaqMan assay did not detect other tobamoviruses (TMV, ToMV, ToMMV, and CGMMV) or other common viruses infecting greenhouse tomatoes, such as PepMV (Supplementary Table S3A). To evaluate sensitivity of the newly developed RT-qPCR protocol, purified plant RNA from ToBRFV-infected tomato tissue was prepared through serial 10-fold dilutions. The RT-qPCR could detect ToBRFV up to 1:10⁶ dilutions of total plant RNA (Supplementary Table S3B).

The newly developed RT-qPCR was evaluated for effective detection of ToBRFV in greenhouse-collected tomato leaf or fruit tissues and

Fig. 2. Phenotypic responses of various plant species upon mechanical inoculation by ToBRFV CA18-01 in host range study. 1, *Chenopodium album*. 2, *Chenopodium berlandieri*. 3, *Chenopodium giganteum*. 4, *Chenopodium quinoa*. 5, *Gomphrena globosa*. 6, *Catharanthus roseus*. 7, *Emilia sonchifolia*. 8, *Glebionis coronaria*. 9, *Nicotiana glutinosa*. 10, *Nicotiana occidentalis*. 11, *Nicotiana benthamiana*. 12, *Datura stramonium*. 13, *Nicotiana debneyi*. 14, *Nicotiana rustica*. 15, *Nicotiana tabacum* 'Samsun'. 16, *Nicotiana tabacum* 'Xanthi nc'. 17, *Petunia* × *hybrida*. 18, *Physalis angulata*. 19, *Physalis pubescens*. 20, *Solanum lycopersicum* 'Moneymaker'. 21, *Solanum lycopersicum* 'Rutgers'. 22, *Solanum nigrum*. 23, *Capsicum annum* 'PI379126'. 24, *Solanum melongena*. 25, *Verbena officinalis* var. *halei*.

bumblebees and validated with the previously developed conventional RT-PCR (Ling et al. 2019). In seven representative tomato tissue and three bumblebee samples tested, results generated from RT-qPCR and conventional RT-PCR were in total agreement (Table 3).

Optimizing RT-qPCR for use in seed health assay. To assess the suitability of RT-qPCR for seed health assay, we observed positive reactions not only in samples containing 5 to 10 ToBRFV-contaminated seeds but also in those with only one seed, resulting in mean Ct values of 20.56 for 1-seed samples, 16.07 for 5-seed samples, and 14.68 for 10-seed subsamples. With optimized conditions for total RNA extraction from a large number of seeds, amount of high-quality total RNA extracted from different treatments of seeds was in an average concentration of 3.675 ng/μl for individual infected seed samples, 3.933 ng/μl for 100-seed samples, 5.367 ng/μl for 200-seed samples, 5.167 ng/μl for 400-seed samples, and 5.933 ng/μl for 800-seed samples. In addition, the average RNA concentration was 3.2 ng/μl for those uninfected tomato seed samples and 4.4 ng/μl for ToBRFV-positive controls. After we found that the RT-qPCR was able to

consistently detect ToBRFV from single contaminated seeds, we evaluated its effectiveness as a seed health test by spiking batches of 100, 200, 400, and 800 healthy seeds each with a single contaminated seed. RT-qPCR was able to detect the target virus in all samples spiked with one individual ToBRFV-contaminated seed, whether it was mixed with 100, 200, 400, or 800 uninfected tomato seeds (Table 4), with mean Ct values ranging from 25.69 to 18.54 comparable with the mean Ct value of 19.36 obtained from four single contaminated seed samples tested (Table 4). In multiplex RT-qPCR tests, the internal control of 18S rRNA with its TaqMan probe labeled in CY5 generated 16.57 for single infected seed samples, and 11.37 to 12.55 for samples with spiked one contaminated seed in 100 to 800 seeds (Table 4). When the seed extracts were tested using an ELISA kit prepared with TMV antibody, a range of mean optical density absorbance readings from 0.720 to 0.930 were achieved in all subsamples of spiking one contaminated seed in 100 to 800 uninfected seeds, validating the effectiveness of this newly developed multiplex RT-qPCR as a useful method for seed health assay of ToBRFV on tomato.

Fig. 3. Resistance breaking in tomato cultivars with *Tm-2* resistance gene upon infection by tomato brown rugose fruit virus (ToBRFV). The left panel contains photos of typical symptoms on tomato plants infected by ToBRFV on 'E' cultivar Endeavour (*Tm-2*) with necrotic lesions; on 'B' cultivar Brioso (*Tm-2*) with mosaic and mottling; on 'I' cultivar Idolini with mild mosaic; and on cultivar Moneymaker with severe mosaic and mottling. The graph in the right panel shows mean and standard deviation of percentage test plants on tomato cultivars infected by three tobamoviruses, ToBRFV, tomato mottle mosaic virus (ToMMV), and tomato mosaic virus (ToMV), in three replicated experiments. Statistical analysis was conducted using two-way analysis of variance followed by Dunnett's multiple comparisons ($\alpha = 0.05$) test using mean percent infection to analyze infection rate and test their dependency on the resistance *Tm-2* gene among four tomato cultivars. The "ns" (not significant) has an adjusted *P* value > 0.5. Significant treatments are designated as "****" with an adjusted *P* value ≤ 0.0001. The y axis represents the mean percent infection, and the x axis represents the tomato cultivars. The infection status of individual test plant was determined by symptom observation with a confirmation test using an enzyme-linked immunosorbent assay to tobacco mosaic virus, which has a broad serological reactivity to other tobamoviruses (ToMV, ToMMV, and ToBRFV) used in this study.

Table 3. Validation of reverse transcription quantitative PCR detection of tomato brown rugose fruit virus (ToBRFV) with conventional reverse transcription PCR

Sample	Source material	PepMV RT-qPCR (Ct) ^a	ToBRFV RT-qPCR (Ct)	ToBRFV RT-PCR ^b
V18-157	Tomato green fruits	22.79 (+)	6.76 (+)	(+)
V18-158	Tomato leaves	10.84 (+)	8.26 (+)	(+)
V18-159	Tomato fruits	14.30 (+)	12.04 (+)	(+)
V18-160	Tomato leaves	11.00 (+)	7.15 (+)	(+)
V18-161	Tomato leaf and fruit	22.18 (+)	6.35 (+)	(+)
V18-162	Tomato leaves	10.43 (+)	6.26 (+)	(+)
V18-163	Tomato mature fruits	28.56 (+)	16.38 (+)	(+)
V19-85	Bumblebees	NA	25.71 (+)	(+)
V19-86	Bumblebees	NA	26.62 (+)	(+)
V19-87	Bumblebees	NA	25.73 (+)	(+)
ToBRFV control	Tomato leaves	15.55 (+)	6.93 (+)	(+)
Healthy control	Tomato leaves	32.38 (–)	32.05 (–)	(–)
Nontemplate control	Water	32.94 (–)	34.40 (–)	(–)

^a Greenhouse collected tomato samples were ubiquitously infected with pepino mosaic virus (PepMV). Reverse transcription quantitative PCR (RT-qPCR) test for PepMV was conducted following the method described in Ling et al. (2007). Ct value <30 was considered positive. Ct values between 30 and 35 were on borderline; thus, the Ct value from the internal healthy control was used as cutoff for background reaction. NA = not available or not tested.

^b The conventional RT-PCR for ToBRFV was conducted following the procedure described by Ling et al. (2019). (+) = positive; (–) = negative.

Discussion

Although the current reported cases of ToBRFV infection are limited to tomato and pepper crops under protected cultivation, a comprehensive evaluation of potential host range would help growers and governmental regulators to understand the potential risk to economically important crops. Consequently, appropriate management strategies are necessary, including crop rotation with nonhost crops or control of those host plants considered as weeds. However, there are still conflicting conclusions on eggplant and potato as host plants for ToBRFV. Although previous reports (Luria et al. 2017; Panno et al. 2019b) as well as the present study did not support eggplant as a host, a report from China considered it a host with latent infection (Yan et al. 2020). Eggplant's broad immunity to tobamoviruses is important because it may be a rootstock candidate for tomato grafting (Black et al. 2003). This property is important, especially if the virus is confirmed to spread through the root system or irrigation water in a hydroponic production system. Similarly, Yan et al. (2020) reported potato as a host with latent infection, which was not considered to be a host for ToBRFV by Luria et al. (2017). Therefore, it is necessary to conduct additional analysis on host range using a different isolate of ToBRFV. Our host range results using a U.S. isolate of ToBRFV agree with those plant species that were tested earlier using an Israeli isolate of ToBRFV (Luria et al. 2017). However, our study also expanded the knowledge on host range for ToBRFV, from plant species mainly in Amaranthaceae and Solanaceae (Luria et al. 2017; Panno et al. 2019b; Salem et al. 2016; Yan et al. 2020) to other species in Apocynaceae and Asteraceae (Table 1). Some of these species are important ornamental (*Catharanthus roseus*) or weed plant (*Emilia sonchifolia*). *Glebionis coronaria*, native to the Mediterranean region and commonly cultivated in East Asia as a leaf vegetable, is considered an invasive weed species in California.

We also made a comparative evaluation of host ranges for three tomato-infecting tobamoviruses, including ToBRFV, ToMV, and ToMMV (Table 2). Although these three tobamoviruses share many common host species, we identified some unique host plants for each individual virus. Rosy periwinkle (*C. roseus*) is a host for ToBRFV but not for ToMMV. Lilac tasselflower (*E. sonchifolia*) and Debney's tobacco (*N. debneyi*) were preferred hosts for ToBRFV but not for ToMMV and ToMV. Jimsonweed (*D. stramonium*) is a host for ToBRFV and ToMMV but not ToMV. Tobacco (*N. tabacum* cultivar Xanthi nc) is infected by ToBRFV and ToMMV but not by ToMV. We also found that common verbena (*V. officinalis*), a nonhost for ToBRFV, is a host for ToMMV and ToMV (Table 2).

ToBRFV has been shown to infect tobamovirus-resistant cultivars containing the *Tm-2²* resistance gene (Luria et al. 2017). In the present study, we observed the lack of resistance in tomato cultivars with *Tm-2²* gene to ToBRFV. We made a direct comparison of several tomato cultivars with or without the *Tm-2²* gene, evaluating their responses to infection by ToMV, ToMMV, and ToBRFV. As evaluated from three independent replicated experiments, the *Tm-2²*-harboring tomato

cultivars Endeavour, Brioso, and Idolini were largely resistant to ToMV and ToMMV but had 100% susceptibility to ToBRFV infection. We are still puzzled by some small proportions of test plants from the commercial tomato cultivars (Endeavour and Brioso with the *Tm-2²* gene) that were infected by ToMV and ToMMV, which were observed in repeated experiments in the present study (Fig. 3). One explanation is that because tomatoes are open pollinated, some degree of cross-pollination with pollen from non-*Tm-2²* genotype(s) might have introduced susceptibility in the F1 hybrid population. There is a need to better characterize the genetic background of these tomato cultivars because a similar effect was also observed in our previous study (Sui et al. 2017). It is also important to understand the mechanism of resistance of the *Tm-2²* gene to ToMV and ToMMV and its complete failure to ToBRFV infection. Lanfermeijer et al. (2003) demonstrated that recognition of the movement protein of ToMV by the tomato *Tm-2²* gene product results in ToMV resistance. Comparative sequence analysis revealed some variations of amino acid sequences in the movement protein of ToBRFV to other tobamoviruses (Maayan et al. 2018). However, we are still unsure what might have caused the resistance breakdown in tomato cultivars with *Tm-2²* by ToBRFV, which requires further investigation. As more ToBRFV outbreaks are reported in different countries around the world, the need to find a new source of genetic resistance against ToBRFV becomes even more urgent. Extensive efforts to screen tomato germplasm for resistance to ToBRFV are currently underway in our laboratory as well as others, with some promising results (Zinger et al. 2021).

With a cross-serological reactivity among tomato-infecting tobamoviruses, it is important to develop a sensitive and species-specific detection for ToBRFV, particularly useful for seed health testing. In the present study, we developed a multiplex RT-qPCR with an internal control 18S rRNA to achieve a sensitive and reliable assay of ToBRFV in spiked seed samples useful for up to 1:800 seeds. The inclusion of an endogenous gene (18S rRNA) labeled with a different fluorescent dye (CY5) as an internal control would allow us to assess the quality of total RNA extracted from the seed samples as well as the conditions suitable for RT-qPCR reactions. During the course of this manuscript preparation, another real-time RT-PCR detection was published for ToBRFV (Panno et al. 2019b). However, they did not demonstrate its usefulness for seed health test. On the other hand, several other primer and probe combinations have been applied in RT-qPCR for seed health assays by seed companies (ISHI-Veg 2020). However, these techniques need rigorous validation. In the present study, we developed an RT-qPCR not only to demonstrate a sensitive species-specific detection of the target virus in plant tissue or on bumblebees but also to offer a reliable detection of ToBRFV from one individual contaminated seed mixed with up to 800 uninfected seeds. Reliability of the seed health test was made possible with improved viral RNA extraction from initial soaking of crushed seed extract in the general ELISA tissue extraction buffer to release virus particles into the buffer. The total RNA purified from seed extract after soaking in the extraction buffer

Table 4. Evaluation on sensitivity of tomato brown rugose fruit virus (ToBRFV) detection using multiplex reverse transcription quantitative PCR in comparison with enzyme-linked immunosorbent assay for seed health testing

Sample	n ^a	ELISA ^b	Multiplex RT-qPCR ^c		Result
		Mean OD _{405nm} ± SD	Mean Ct ± SD for ToBRFV (FAM)	Mean Ct ± SD for internal 18S rRNA (CY5)	
Single infected seed	4	1.552 ± 0.319	19.36 ± 3.14	16.57 ± 1.40	+
Spiked one infected seed in 100 noninfected seeds	3	0.930 ± 0.029	25.69 ± 1.49	12.55 ± 0.95	+
Spiked one infected seed in 200 noninfected seeds	3	0.784 ± 0.052	25.79 ± 0.08	11.37 ± 0.91	+
Spiked one infected seed in 400 noninfected seeds	3	0.720 ± 0.100	28.56 ± 2.38	11.99 ± 0.29	+
Spiked one infected seed in 800 noninfected seeds	3	0.831 ± 0.089	28.54 ± 1.22	12.03 ± 0.86	+
Noninfected seed control (100–800 seeds)	4	−0.101 ± 0.005	37.17 ± 0.81	13.34 ± 1.06	−
ToBRFV–leaf tissue control	1	1.273	7.63 ± 0.26	19.52 ± 0.36	+
Healthy tomato leaf tissue control	1	0	35.02 ± 0.31	9.38 ± 0.29	−

^a n = number of biological replications.

^b Enzyme-linked immunosorbent assay (ELISA) was conducted using tomato mosaic virus antibody from Agdia. SD, standard deviation.

^c The background cutoff Ct value was set at 35 for seed health testing of the tomato brown rugose fruit virus (ToBRFV) in this experiment for positive (+) or negative (−) for ToBRFV. An internal control with 18S rRNA was developed in multiplex reverse transcription quantitative PCR (RT-qPCR), which would allow objective assessment on the quality of RNA extraction and the reaction conditions that are suitable for RT-qPCR.

for 30 min resulted in quite consistent Ct readings across three biological and three experiment replications in RT-qPCR (Table 4).

Intensive greenhouse production system, frequent touching of plants during a production season, and the lack of resistance gene all contribute to ToBRFV outbreaks. Comparative analysis of common and unique plant species as host plants for the three tomato-infecting tobamoviruses (ToMV, ToMMV and ToBRFV) provides us general guidance in disease management through crop rotation and weed control. Finally, a multiplex RT-qPCR assay with internal control was developed to achieve sensitive and reliable species-specific detection of ToBRFV in contaminated plant and seed samples, which could be useful for plant and seed healthy testing. Because sequenced ToBRFV isolates share high-nucleotide sequence identity (99.6 to 99.9%), such low genetic diversity suggests a short evolutionary history because of the emergence of this virus, which may have been in circulation around the world through global trade of seed and produce (Klap et al. 2020). The RT-qPCR assay is expected to do well in global seed health testing and government regulatory programs.

Acknowledgments

The authors thank Sharon Andreason and Omotola Olaniyi for critical review and comments on an earlier version of the manuscript.

Literature Cited

- Alfaro-Fernandez, A., Castillo, P., Sanahuja, E., Rodriguez-Salido, M. C., and Font, M. I. 2020. First report of tomato brown rugose fruit virus in tomato in Spain. *Plant Dis.* 105:515.
- Alkowni, R., Alabdallah, O., and Fadda, Z. 2019. Molecular identification of tomato brown rugose fruit virus in tomato in Palestine. *J. Plant Pathol.* 101: 719-723.
- Amer, M. A., and Mahmoud, S. Y. 2020. First report of tomato brown rugose fruit virus on tomato in Egypt. *New Dis. Rep.* 41:24.
- Beris, D., Malandraki, I., Kektisidou, O., Theologidis, I., Vassilakos, N., and Varveri, C. 2020. First report of tomato brown rugose fruit virus infecting tomato in Greece. *Plant Dis.* 104:2035.
- Black, L. L., Wu, D. L., Wang, J. F., Kalb, T., Abbass, D., and Chen, J. H. 2003. Grafting tomatoes for production in the hot-wet season. AVRDC International Cooperators Guide, AVRDC Publication #03-551, Tainan, Taiwan.
- Calder, V. L., and Palukaitis, P. 1992. Nucleotide sequence analysis of the movement genes of resistance breaking strains of tomato mosaic virus. *J. Gen. Virol.* 73:165-168.
- Camacho-Beltrán, E., Perez-Villarreal, A., Rodríguez-Negrete, E. A., Cenicerio-Ojeda, E., Leyva-López, N. E., and Mendez-Lozano, J. 2019. Occurrence of tomato brown rugose fruit virus infecting tomato crops in Mexico. *Plant Dis.* 103:1440.
- Cambrón-Crisantos, J. M., Rodríguez-Mendoza, J., Valencia-Luna, J. B., Alcasio-Rangel, S., García-Ávila, C. J., López-Buenfil, J. A., and Ochoa-Martínez, D. L. 2018. First report of tomato brown rugose fruit virus (ToBRFV) in Michoacan, Mexico. *Rev. Mex. Fitopatol.* 37: 185-192.
- Chanda, B., Rivera, Y., Nunziata, S. O., Galvez, M. E., Gilliard, A. C., and Ling, K.-S. 2020. Complete genome sequence of a tomato brown rugose fruit virus isolated in the United States. *Microbiol. Resour. Announc.* 9: e00630-e20.
- Davidson, K. 2019. Tomato brown rugose fruit virus identified in Ontario. <http://thegrower.org/news/tomato-brown-rugose-fruit-virus-identified-ontario>
- Dombrovsky, A., and Smith, E. 2017. Seed transmission of tobamoviruses: aspects of global disease distribution. In: *Advances in Seed Biology*. J. C. Jimenez-Lopez, ed. InTechOpen Limited, London. <https://www.intechopen.com/chapters/56506>
- Ellis, M. D., Hoak, J. M., Ellis, B. W., Brown, J. A., Sit, T. L., Wilkinson, C. A., Reed, T. D., and Welbaum, G. E. 2020. Quantitative real-time PCR analysis of individual flue-cured tobacco seeds and seedlings reveals seed transmission of tobacco mosaic virus. *Phytopathology* 110:194-205.
- Fidan, H., Sarikaya, P., and Calis, O. 2019. First report of tomato brown rugose fruit virus on tomato in Turkey. *New Dis. Rep.* 39:18.
- Flor, H. H. 1942. Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* 32:653-669.
- Gómez, P., Sempere, R. N., Elena, S. F., and Aranda, M. A. 2009. Mixed infections of pepino mosaic virus strains modulate the evolutionary dynamics of this emergent virus. *J. Virol.* 83:12378-12387.
- Hall, T. J. 1980. Resistance at the *Tm-2* locus in the tomato to tomato mosaic virus. *Euphytica* 29:189-197.
- Hančinský, R., Mihálik, D., Mrkvová, M., Candresse, T., and Glasa, M. 2020. Plant viruses infecting *Solanaceae* family members in the cultivated and wild environments: a review. *Plants* 9:667.
- ISHI-Veg. 2020. Detection of infectious tomato brown rugose fruit virus (ToBRFV) in tomato and pepper seed. https://www.worldseed.org/wp-content/uploads/2020/03/Tomato-ToBRFV_2020.03.pdf
- Ishibashi, K., and Ishikawa, M. 2016. Replication of tobamovirus RNA. *Annu. Rev. Phytopathol.* 54:55-78.
- Klap, C., Luria, N., Smith, E., Bakelman, E., Belausov, E., Laskar, O., Lachman, O., Gal-on, A., and Dombrovsky, A. 2020. The potential risk of plant-virus disease initiation by infected tomatoes. *Plants* 9:623.
- Lafermeijer, F. C., Dijkhuis, J., Sturre, M. J., Haan, P., and Hille, J. 2003. Cloning and characterization of the durable tomato mosaic virus resistance gene *Tm-2²* from *Lycopersicon esculentum*. *Plant Mol. Biol.* 52: 1039-1051.
- Levitzy, N., Smith, E., Lachman, O., Luria, N., Mizrahi, Y., Bakelman, H., Sela, N., Laskar, O., Milrot, E., and Dombrovsky, A. 2019. The bumblebee *Bombus terrestris* carries a primary inoculum of tomato brown rugose fruit virus contributing to disease spread in tomatoes. *PLoS One* 14:e0210871.
- Ling, K.-S., Tian, T., Gurung, S., Salati, R., and Gilliard, A. C. 2019. First report of tomato brown rugose fruit virus infecting greenhouse tomato in the U.S. *Plant Dis.* 103:1439.
- Ling, K.-S., Wechter, W. P., and Jordan, R. 2007. Development of a one-step immunocapture real-time TaqManTM RT-PCR assay for the broad-spectrum detection of pepino mosaic virus. *J. Virol. Methods* 144:65-72.
- Luria, N., Smith, E., Reingold, V., Bekelman, I., Lapidot, M., Levin, I., Elad, N., Tam, Y., Sela, N., Abu-Ras, A., Ezra, N., Haberman, A., Yitzhak, L., Lachman, O., and Dombrovsky, A. 2017. A new Israeli tobamovirus isolate infects tomato plants harboring *Tm-2²* resistance genes. *PLoS One* 12: e0170429.
- Maayan, Y., Pandaranayaka, E., Srivastava, D. A., Lapidot, M., Levin, I., Dombrovsky, A., and Harel, A. 2018. Using genomic analysis to identify tomato *Tm-2* resistance breaking mutations and their underlined evolutionary path in a new and emerging tobamovirus. *Arch. Virol.* 163: 1863-1875.
- Menzel, W., Knierim, D., Winter, S., Hamacher, J., and Heupel, M. 2019. First report of tomato brown rugose fruit virus infecting tomato in Germany. *New Dis. Rep.* 39:1.
- Nagai, A., Duarte, L. M. L., Chaves, A. L. R., Peres, L. E. P., and dos Santos, D. Y. A. C. 2019. Tomato mottle mosaic virus in Brazil and its relationship with *Tm-2²* gene. *Eur. J. Plant Pathol.* 155:353-359.
- Ohmori, T., Murata, M., and Motoyoshi, F. 1996. Molecular characterization of RAPD and SCAR markers linked to the *Tm-1* locus in tomato. *Theor. Appl. Genet.* 92:151-156.
- Okada, K., Kusakari, S., Kawatani, M., Negoro, J., Ohk, S. T., and Osak, T. 2000. Tobacco mosaic virus is transmissible from tomato to tomato by pollinating bumblebees. *J. Gen. Plant Pathol.* 66:71-74.
- Osman, F., Leutenegger, C., Golino, D., and Rowhani, A. 2007. Real-time RT-PCR (TaqMan) assays for the detection of grapevine leafroll associated viruses 1-5 and 9. *J. Virol. Methods* 141:22-29.
- Panno, S., Caruso, A. G., and Davino, V. 2019a. First report of tomato brown rugose fruit virus on tomato crops in Italy. *Plant Dis.* 103:1443.
- Panno, S., Ruiz-Ruis, S., Caruso, A. G., Alfaro-Fernandez, A., Font San Ambrosio, M. I., and Davino, S. 2019b. Real-time reverse transcription polymerase chain reaction development for rapid detection of tomato brown rugose fruit virus and comparison with other techniques. *PeerJ* 7: e7928.
- Pelham, J. 1966. Resistance in tomato to Tobacco mosaic virus. *Euphytica* 15: 258-267.
- Roberts, J. M. K., Ireland, K. B., Tay, W. T., and Paini, D. 2018. Honey bee-assisted surveillance for early plant virus detection. *Ann. Appl. Biol.* 173:285-293.
- Salem, N., Mansour, A., Ciuffo, M., Falk, B. W., and Turina, M. 2016. A new tobamovirus infecting tomato crops in Jordan. *Arch. Virol.* 161: 503-506.
- Shi, A., Vierling, R., Grazzini, R., Chen, P., Caton, H., and Panthee, D. 2011. Molecular markers for *Tm-2* alleles of tomato mosaic virus resistance in tomato. *Am. J. Plant Sci.* 2:180-189.
- Skelton, A., Buxton-Kirk, A., Ward, R., Harju, V., Frew, L., Fowkes, A., Long, M., Negus, A., Forde, S., Adams, I. P., Pufal, H., McGreig, S., Weekes, R., and Fox, A. 2019. First report of tomato brown rugose fruit virus in tomato in the United Kingdom. *New Dis. Rep.* 40:12.
- Sui, X., Zheng, Y., Li, R., Padmanabhan, C., Tian, T., Groth-Helms, D., Keinath, A. P., Fei, Z., Wu, Z., and Ling, K.-S. 2017. Molecular and biological characterization of tomato mottle mosaic virus and development of RT-PCR detection. *Plant Dis.* 101:704-711.
- U.S. Department of Agriculture. 2019. Tomato Brown Rugose Fruit Virus. <https://www.aphis.usda.gov/aphis/ourfocus/planthealth/import-information/federal-import-orders/tobrfv/tomato-brown-rugose-fruit-virus>
- van de Vossen, B. T. L. H., Visser, M., Bruinsma, M., Koenraadt, H. M. S., Westenberg, M., and Botermans, M. 2020. Real-time tracking of tomato brown rugose fruit virus (ToBRFV) outbreaks in the Netherlands using Nextstrain. *PLoS One* 15:e0234671.
- Weber, H., Schultze, S., and Pfützner, A. J. 1993. Two amino acid substitutions in the tomato mosaic virus 30-kilodalton movement protein confer the ability to overcome the *Tm-2²* resistance gene in the tomato. *J. Virol.* 67: 6432-6438.

- Yan, Z., Ma, H., Han, S., Geng, C., Tian, Y., and Li, X. 2019. First report of tomato brown rugose fruit virus infecting tomato in China. *Plant Dis.* 103: 2973.
- Yan, Z.-Y., Zhao, M.-S., Ma, H.-Y., Liu, L.-Z., Yang, G.-L., Geng, C., Tian, Y.-P., and Li, X.-D. 2020. Biological and molecular characterization of tomato brown rugose fruit virus and development of quadruplex RT-PCR detection. *J. Integr. Agric.* 19:2-10.
- Young, N. D., Zamir, D., Ganai, M. W., and Tanksley, S. D. 1988. Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the tm-2a gene in tomato. *Genetics* 120:579-585.
- Zinger, A., Lapidot, M., Harel, A., Doron-Faigenboim, A., Gelbart, D., and Levin, I. 2021. Identification and mapping of tomato genome loci controlling tolerance and resistance to tomato brown rugose fruit virus. *Plants* 10:179.