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# Sensitive detection of *Tomato ringspot virus* by real-time TaqMan RT-PCR targeting the highly conserved 3′-UTR region

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A real-time TaqMan RT-PCR assay was developed for the rapid and sensitive detection of *Tomato ringspot virus* (ToRSV), an important plant virus which infects a wide range of fruit and ornamental crops. Primers and a probe were designed based on the highly conserved 3′-untranslated region (UTR) sequences of ToRSV, to amplify a 182 bp fragment of this region of RNA-1 and RNA-2. The assay was demonstrated to reliably amplify all ToRSV isolates tested. The detection limit was estimated to be about 12 copies of the ToRSV target region. No amplification was observed from the RNA of other nepoviruses or healthy host species. A comparison with a published conventional RT-PCR and a SYBR-based qRT-PCR indicated that both of the published assays lacked reliability and sensitivity, as neither were able to amplify all ToRSV isolates tested, and both were approximately 1000 times less sensitive than the novel TaqMan real-time assay. This TaqMan real-time assay was tested using four different reagent kits and was shown to be robust and stable, with no significant differences in sensitivity between kits. It is expected that the implementation of this TaqMan real-time RT-PCR assay will facilitate efficient phytosanitary certification of nursery stock requiring testing for ToRSV by regulatory agencies, and will also have wider uses for the general detection of ToRSV in a range of hosts.



## 1. Introduction

*Tomato ringspot virus* (ToRSV) is a distinctive member of the genus *Nepovirus* (subgroup C) in the family *Secoviridae*. The virus has a bipartite single-stranded positive-sense RNA genome encapsidated in icosahedral particles (Sanfaçon et al., 2006, 2009). RNA1 is 8214 bp in length and contains a single long open reading frame (ORF) of 6591 bp, while RNA2 is 7273 bp in length and also contains a single long ORF of 5646 bp (Rott et al., 1991a, 1995). ToRSV RNA1 and RNA2 have a 3′untranslated region (3′-UTR) of 1533 bp that are identical (Rott et al., 1991b), and are distinctly characteristic of the *Nepovirus* subgroup C (Borja et al., 1995; Lehto et al., 2004; Rott et al., 1991a).

ToRSV infects a wide range of economically important crop species such as apple (*Malus domestica*), grapevine (*Vitis vinifera*), stonefruit (*Prunus* spp.), raspberry (*Rubus idaeus*) and causes serious disease problems in North America. The most serious diseases associated with ToRSV are Yellow bud mosaic in peach, Brownline disease in prune, Union necrosis and Decline in apple, Ringspot and Decline in red raspberry and Decline in grapevine (Stace-Smith, 1984). ToRSV has also been isolated from trees (e.g. American ash), berryfruits (e.g. gooseberry, strawberry, blueberry, blackcurrant), ornamentals (e.g. pelargonium, hydrangea, gladiolus, narcissus, lily, daphne, butterfly bush) and perennial weed species (Kim and Choi, 1990; Hibben et al., 1988; Hughes and Scott, 2003; OEPP/EPPO, 2005; Stace-Smith, 1984).

ToRSV is transmitted in nature by a nematode, *Xiphinema americanum sensu lato*, which is a species complex and yet to be defined (Brown et al., 1994; OEPP/EPPO, 2009). ToRSV is seed-transmitted in several host plants and can be spread from pollen to seed or by pollen to the pollinated plant (OEPP/EPPO, 2005). Outside USA and Canada, ToRSV has been reported in China, Chile, Japan, Korea, Jordan, Egypt, Iran, Brazil, Australia, New Zealand and many European countries (CABI, 2013; Pearson et al., 2006). However, ToRSV remains a quarantine pest in Europe, as well as in Australia and New Zealand. Phytosanitary certification often requires that such nursery stock be tested and found free from ToRSV by regulatory agencies. A cost-effective diagnostic tool to detect ToRSV reliably in bulked samples is also required by the industries. As such, sensitive and fast detection methods are essential for the virus detection in quarantine or, in the event of an incursion.

Serological (e.g. Enzyme-linked immunosorbent assay, ELISA) and molecular methods have been used widely for the detection of ToRSV. Like many other viruses, however, the specificity and

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sensitivity of detection for ToRSV using serological methods are not reliable (Bitterlin and Gonsalves, 1988; Griesbach, 1995; Stewart et al., 2007). In 1995, Griesbach developed a reverse transcription-polymerase chain reaction (RT-PCR) based protocol, which has greater specificity and sensitivity than ELISA, for the detection of ToRSV (Griesbach, 1995). More recently, a SYBR Green (Stewart et al., 2007) and a TaqMan (Osman et al., 2008) real-time RT-PCR assay have also been published for the detection of ToRSV. However, both these assays are designed to detect ToRSV strains specifically from grapevine and therefore, strains of ToRSV from other hosts may not be amplified.

In this study, a one-step TaqMan real-time RT-PCR assay targeting the highly conserved 3′-UTR sequences of ToRSV was developed. The specificity, sensitivity and reliability of this assay are validated and compared to extant methods of ToRSV detection.

## 2. Materials and methods

### 2.1. Virus isolates and RNA extraction

ToRSV isolates were obtained from a range of commercial and academic sources and healthy plant samples, known to be hosts of ToRSV, were obtained from Plant Health and Environment Laboratory (PHEL) reference collection (Table 1). While the isolates that were purchased from commercial suppliers were likely maintained in herbaceous species, the other infected samples were naturally infected woody hosts such as apple, grapevine and peach. Total RNA was extracted from all samples using an InviMag Plant Kit and Kingfisher ml extraction system (Invitek GmbH; Berlin, Germany). An internal control PCR was done for all RNA extracts using *nad5*-s and *nad5*-as primers (Menzel et al., 2002), to ensure the PCR competency of the RNA extracts (data not shown). For the ToRSV PCRs, each PCR product (182 bp) was directly sequenced by EcoGene™, Auckland, to confirm the identity of the amplicon.

An additional panel of 18 ToRSV isolates, kindly provided by Dr Zongrang Liu from USDA – ARS (Appalachian Fruit Research Station, Kearneysville, USA) was also used to evaluate the specificity of the ToRSV TaqMan real-time RT-PCR assay developed in this study. This panel was provided as plasmid DNA containing a cDNA insert of 1.3 kb of 3′-UTR fragment of 18 ToRSV isolates derived from apple, apricot, blueberry, grapevine, peach, raspberry, redcurrant and unknown hosts. Details of these ToRSV isolates are described in Li et al. (2011).

### 2.2. Primer design

The primers and TaqMan probe for real-time RT-PCR amplification of ToRSV were designed based on an alignment of 23 3′-UTR sequences of RNA1 and RNA2 available in NCBI. The melting temperature, self-dimer and heterodimer properties of the primers and probe were examined *in silico* using Oligo Analyzer (IDT Technologies, Coralville, IA, USA). Names and sequences of the primers and probe are as follows. The nucleotide positions of the primers and probe shown in brackets refer to the area of the genome of RNA1 of ToRSV (NCBI Accession No. L19655).

ToRSV-UTRf: 5′-GAATGGTTCCCAGCCACTT-3′ (forward primer, 7686-7704); ToRSV-UTRr: 5′-AGTCTCAACTTAACATACCAC-3′ (reverse primer, 7847-7867); ToRSV-UTRp: FAM-5′-AGGATCGC-TACTCCTCCGTCAAC-3′-BHQ-1 (probe, 7746–7768).

### 2.3. Reaction optimization

The $MgSO_4$ concentration (3.0 vs. 5.5 mM), primer concentration (300 vs. 500 nM), probe concentration (150 vs. 250 nM), reverse transcription time (15 vs. 30 min), and annealing/extension temperature (58 vs. 60 vs. 62 °C) were compared using an Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Life Technologies, Carlsbad, CA, USA). The final optimized reaction of a 20 μl volume contained: 10 μl qRT-PCR master mix, 0.5 μl SuperScript III and Platinum *Taq* enzyme mix, 300 nM forward and reverse primers, 150 nM FAM-labeled probe, additional $MgSO_4$ to a final concentration of 5.5 mM, 0.4 μg/μl bovine serum albumin (BSA) and 2 μl total RNA extract. Thermocycling conditions were reverse transcription at 50 °C for 15 min, initial denaturation at 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 45 s. All qRT-PCRs were run on a CFX96 real-time thermocycler (Bio-Rad, Hercules, CA, USA).

### 2.4. Specificity and sensitivity

Specificity was evaluated against RNA extracts from 10 isolates of ToRSV, and against RNA from four virus species from the nepovirus subgroups A, B and C, and from six healthy host species (Table 1). An additional panel of 18 ToRSV isolates, provided as plasmid with an insert of 1.3 kb 3′-UTR region, was used to further evaluate the specificity of the ToRSV real-time TaqMan RT-PCR assay. All samples were tested in triplicate wells, and sample threshold, baseline values and reaction efficiency values were calculated automatically by the CFX96 manager software (Bio-Rad). Samples with a threshold cycle (Ct) value greater than 38 were ignored, as well the samples with an inter-replicate standard deviation greater than 0.5 cycles. The sensitivity and detection limit of the assay was explored by generating a standard curve using a plasmid with a known copy number of the ToRSV insert. The plasmid with an approximate $10^7$ copies/μl was 10-fold serially diluted ($1 \times 10^7$–$1 \times 10^1$) in RNA extract from healthy *Vitis vinifera* to account for the effects of inhibitors from the plant tissue (the presence of inhibitors was determined by measurements of the optical density ratio of $A_{260/230}$ and $A_{260/280}$ for diluted and undiluted RNA extracts, using a Nanodrop spectrophotometer – data not shown). The plasmid copy number was calculated using the formula: copies/μl = (concentration in ng $\times$ $6.023 \times 10^{23}$)/(genome length $\times$ $1 \times 10^9$ $\times$ 650). Total RNA extracted from the ToRSV isolate (FPS Davis, CA, USA) was serially diluted by a factor of 10 ($10^{-1}$–$10^{-7}$) in RNA extracted from healthy *Vitis vinifera* to account for the effects of inhibitors from *Vitis* species. The copy number for each RNA dilution series was extrapolated from the standard curve.

### 2.5. Comparison of reagents

The ToRSV RT-PCR was performed using the following reagents: Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System, Quanta qScript™ XLT One-Step RT-qPCR ToughMix® (Quanta BioSciences, Gaithersburg, MD, USA), Roche RealTime Ready RNA Virus Master (Roche Applied Science, Mannheim, Germany) and Stratagene Brilliant III Ultra-Fast QRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). Reactions were performed as per manufacturer's recommendations with the exception for the Invitrogen kit where optimal reaction conditions were used as determined from the experiments carried out in Section 2.3. The Ct values and reaction efficiencies of each of the reagents were compared using the ToRSV-positive serial dilutions as described in Section 2.4.

### 2.6. Comparison to extant assays

Both specificity and sensitivity of the TaqMan real-time RT-PCR assay was compared with previously published assays for ToRSV detection, including a conventional RT-PCR (Griesbach, 1995), a SYBR Green-based (Stewart et al., 2007) and TaqMan probe-based (Osman et al., 2008) real-time RT-PCRs. The comparisons were made using RNA extracts from the 10 ToRSV isolates listed in