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Detection of *Tomato black ring virus* by real-time one-step RT-PCR

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ABSTRACT

A TaqMan-based real-time one-step RT-PCR assay was developed for the rapid detection of *Tomato black ring virus* (TBRV), a significant plant pathogen which infects a wide range of economically important crops. Primers and a probe were designed against existing genomic sequences to amplify a 72 bp fragment from RNA-2. The assay amplified all isolates of TBRV tested, but no amplification was observed from the RNA of other nepovirus species or healthy host plants. The detection limit of the assay was estimated to be around nine copies of the TBRV target region in total RNA. A comparison with conventional RT-PCR and ELISA, indicated that ELISA, the current standard test method, lacked specificity and reacted to all nepovirus species tested, while conventional RT-PCR was approximately ten-fold less sensitive than the real-time RT-PCR assay. Finally, the real-time RT-PCR assay was tested using five different RT-PCR reagent kits and was found to be robust and reliable, with no significant differences in sensitivity being found. The development of this rapid assay should aid in quarantine and post-border surveys for regulatory agencies.

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1. Introduction

Tomato black ring virus (TBRV) is a member of the genus *Nepovirus* (Secoviridae: Comovirinae) and infects a wide range of economically important crop species, including grapevine (*Vitis* sp.), *Prunus* spp., berryfruit (from the genera *Rubus*, *Ribes*, and *Fragaria*), solanaceous species, and a number of weed and ornamental species (Brunt et al., 1996; Harrison, 1957, 1958; Pospieszny et al., 2004). Symptoms include systemic chlorotic ringspot, leaf mottle and deformation, vein yellowing and stunting as well as necrotic spotting and flecking on indicator species (Brunt et al., 1996). Economic losses for this virus are difficult to quantify as it is mild to asymptomatic in some species such as sugar beet (*Beta vulgaris*) and strawberry (*Fragaria* sp.) (Heathcote, 1973; Martin and Tzanetakis, 2006), while causing yield losses of up to 40% on artichoke (*Cynara cardunculus*) (Gallitelli et al., 2004).

TBRV is transmitted both through seed (Lister and Murant, 1967), and by the nematodes *Longidorus elongatus* and *Longidorus attenuatus* (Brown et al., 1989; Harrison et al., 1961). The virus has been reported in Europe, North and South America, India and Japan (Brunt et al., 1996; Harrison, 1958). TBRV remains however, a regulated pest in the North American Plant Protection Organisation territory, as well as in Australia and New Zealand. In countries where vectors of TBRV are present, it is possible that TBRV could spread quickly if introduced given the wide host range of the virus (Alten and Ebsary, 1988). As such, early detection of the virus is important in the event of an incursion.

Detection and diagnostic methods for TBRV are limited, with enzyme-linked immunosorbent assay (ELISA) used most commonly in diagnostic laboratories (Martin and Tzanetakis, 2006; Rowhani et al., 2005). There are, at the time of writing, few published reverse-transcription polymerase chain reaction (RT-PCR) based protocols for TBRV detection either using specific primers or nepovirus subgroup B general assays (Digiaro et al., 2007; Wei and Clover, 2008).

More recently, real-time RT-PCR has been used successfully for the testing of nepoviruses, including *Grapevine fanleaf virus* (GFLV), *Tomato ringspot virus* (ToRSV), and *Tobacco ringspot virus* (TRSV) (Bertolini et al., 2010; Finetti-Sialer and Ciancio, 2005; Osman et al., 2008; Stewart et al., 2007; Yang et al., 2007). Real-time RT-PCR provides significantly greater specificity and sensitivity than ELISA or conventional RT-PCR (Bertolini et al., 2010; Kogovsek et al., 2008), and with the advent of portable real-time thermocyclers, it is transferred easily between the field and laboratory, making it ideal for diagnostic purposes. In this study, a TBRV-specific one-step real-time RT-PCR assay is compared to extant methods of TBRV detection, firstly to RT-PCR using nepovirus subgroup-B primers (Wei and Clover, 2008) and TBRV-specific primers (Digiaro et al., 2007), and secondly to ELISA.

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2. Materials and methods

2.1. Isolate collection and RNA extraction

Isolates of TBRV and other nepoviruses were obtained from a range of commercial and academic sources (Table 1). Healthy plant samples, known to be hosts of TBRV, were obtained from the Ministry of Agriculture and Forestry collection. Total RNA was extracted from all samples using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. The validity of each TBRV isolate was confirmed by amplifying a PCR product using the published primers (digf and digr) and protocol of Digiaro et al. (2007). Each PCR product was then directly sequenced (EcoGene™, Auckland).

2.2. Primer design

The primers and TaqMan probe for real-time RT-PCR amplification of TBRV were designed using Biosearch RealTimeDesign online software (Biosearch Technologies, Novato, CA, USA) from an alignment of three extant TBRV complete genome sequences (Jończyk et al., 2004; Le Gall et al., 1995; Meyer et al., 1986) against the P2 ORF between nucleotides 759 and 831, annotated as per TBRV isolate ED (NCBI Accession No. X80831). The melting temperature, self-dimer and heterodimer properties of the selected set, TBRV-70F: 5′-GCTCGTAACAGTTGCGGAGATAT-3′, TBRV-70R: 5′-TGTCCACACTGTCATGGGA-3′, and TBRV-70P (labelled with 6′ carboxyfluorescein (FAM) and Black Hole Quencher-1 (BHQ-1)): 5′-TGCATAGGCTCACTCCTTGGGA-3′ were examined *in silico* using Oligo Analyzer (IDT Technologies, Coralville, IA, USA) to determine primer concentration and annealing temperature ranges for *in vitro* testing.

2.3. Reaction optimisation

The primers were evaluated initially without the FAM-labelled probe using SuperScript III Platinum SYBR Green One-Step qRT-PCR reagents (Invitrogen, Carlsbad, CA, USA). Reactions were run on a BioRad CFX96 real-time thermocycler (BioRad Laboratories, Hercules, CA, USA) and each reaction contained 10 μl SYBR Green RT-PCR master mix (Invitrogen), 0.4 μl Superscript III and Platinum *Taq* enzyme mix, 300 nM of forward and reverse primers, 3 mM $MgCl_2$ and 250 μg/μl bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO, USA). Thermocycling conditions used were reverse transcription for 15 min at 50 °C, initial denaturation of 94 °C for 2 min, then 40 cycles of denaturation at 94 °C for 10 s and annealing and extension at between 58 and 62 °C for 40 s. Amplification products were confirmed by generation of melt curves between 65 and 95 °C at 0.5 degrees/s, and by agarose gel electrophoresis.

After suitable reaction conditions were identified for target amplification using the primers, the FAM-labelled probe was then added to the reaction at a final concentration of 100 nM. The $MgCl_2$ concentration (3–5 mM), primer concentration (200 vs. 300 nM) reverse transcription temperature and length (10 vs. 15 min and 50 vs. 55 °C), and annealing/extension temperature (60 vs. 62 vs. 64 °C) were optimised further using the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen). Final optimised reaction conditions were as follows: 10 μl RT-PCR master mix, 0.4 μl Superscript III and Platinum *Taq* enzyme mix, 300 nM of forward and reverse primers, 100 nM of FAM-labelled probe, additional $MgCl_2$ to a final concentration of 4 mM, 250 μg/μl BSA, and 2 μl of total RNA extract in a reaction volume of 20 μl. Thermocycling conditions were reverse transcription at 55 °C for 10 min, initial denaturation at 94 °C for 2 min, then 40 cycles of 94 °C for 10 s, and 62 °C for 40 s.

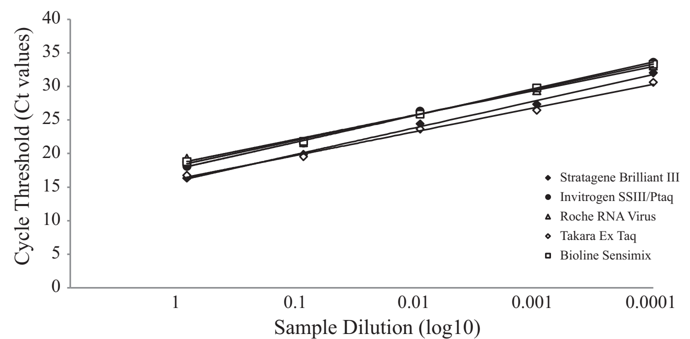

Fig. 1. A comparison of the standard curves of a ten-fold dilution series of TBRV-infected RNA amplified by real-time RT-PCR using five different reagent sets.

2.4. Specificity and sensitivity

Specificity was tested against RNA extracts from a panel of six TBRV isolates, and against RNA from five virus species from the nepovirus subgroups A, B and C, and from eight healthy host species of TBRV (Table 1). All samples were tested in triplicate and sample threshold and baseline values calculated automatically by the CFX manager software (BioRad). Samples with a crossing threshold (C_t) value greater than 38 were ignored, as were samples with an inter-replicate standard deviation of greater than 0.5 cycles. Reaction efficiency values were calculated using LinRegPCR software (Ramakers et al., 2003).

The sensitivity and detection limit of the TBRV real-time RT-PCR assay was explored by generating a standard curve using a plasmid with a known copy number of the TBRV insert (Fig. 1). The plasmid was serially diluted by a factor of 10 (1×10^7–1×10^3 copies/μl), in RNA from healthy *Prunus avium* to account for plant inhibition. 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 TBRV CFIA *Prunus persica* isolate in *Chenopodium quinoa* was serially diluted by a factor of 10 (10^{-1}–10^{-6}) in RNA extracted from healthy *P. avium* to account for plant inhibition from *Prunus* species. The copy number for each RNA dilution series was extrapolated from the standard curve. C_t values and reaction efficiencies were calculated as described.

2.5. Comparison of reagents

The TBRV RT-PCR was tested with the following reagents: Stratagene Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), Bioline SensiMix Probe One-Step Kit (Bioline, Sydney, Australia), Takara One-Step *Ex Taq* qRT-PCR Kit (Takara Bio Inc., Shiga, Japan), and Roche RealTime ready RNA Virus Master (Roche Applied Science, Mannheim, Germany) using the previously described optimised reaction conditions, with the exception that the manufacturer's recommended reverse-transcription and initial denaturation temperatures and incubation periods were followed. The C_t values and reaction efficiencies of each of the reagents were compared using the TBRV positive sample serial dilution described in Section 2.4.

2.6. Comparison to extant assays

Finally, both specificity and sensitivity of the real-time RT-PCR assay was compared with the TBRV-specific (Digiaro et al., 2007) and nepovirus group-B (Wei and Clover, 2008) conventional one-step RT-PCRs, performed as described in their respective publications, and to a TBRV-specific ELISA assay (Neogen Europe Ltd., Auchincruive, UK), performed as per the manufacturer's instruc-

Table 1
Isolates of *Tomato black ring virus*, non-target nepoviruses and healthy host plants used for the optimisation of the TBRV-specific real-time RT-PCR assay.

Target	Species	Isolate	Original host	Source
Target virus	*Tomato black ring virus*	DSMZ PV-0070	*Solanum tuberosum*	DSMZ, Braunschweig, Germany
		DSMZ PV-0191	*Rubus idaeus*	DSMZ, Braunschweig, Germany
		DSMZ PV-0521	*Pelargonium* sp.	DSMZ, Braunschweig, Germany
		CPVC-289	*Nicotiana clevelandii*	Canadian Plant Virus Collection, Agriculture Agri-Food Canada, Summerland, BC, Canada
		CFIA	*Prunus persica*	CFIA, Sidney, BC, Canada
		ATCC PV-554	Unknown	ATCC, Manassas, VA, USA
Non-target Nepoviruses	*Arabis mosaic virus*	Unknown	*Chenopodium* sp.	MAF Collection, Auckland, New Zealand
	Cycas necrotic stunt virus	Unknown	*Paeonia* sp.	MAF Collection, Auckland, New Zealand
	Peach rosette mosaic virus	Agdia LPC-87700	Unknown	Agdia, Elkhart, IN, USA
	Tobacco ringspot virus	Agdia LPC-64000	Unknown	Agdia, Elkhart, IN, USA
	Tomato ringspot virus	DSMZ PV-0049	*Pelargonium* sp.	DSMZ, Braunschweig, Germany
Healthy host species	*Fragaria × ananassa*			MAF Collection, Auckland, New Zealand
	Ipomoea batatas			MAF Collection, Auckland, New Zealand
	Prunus avium			MAF Collection, Auckland, New Zealand
	Ribes nigrum			MAF Collection, Auckland, New Zealand
	Rubus idaeus			MAF Collection, Auckland, New Zealand
	Solanum lycopersicum			MAF Collection, Auckland, New Zealand
	Solanum tuberosum			MAF Collection, Auckland, New Zealand
	Vitis vinifera			MAF Collection, Auckland, New Zealand

tions using the panel of TBRV positive, negative and non-target samples listed in Table 1. It should be noted that three of the non-target samples were not tested by ELISA due to sample availability.

3. Results

3.1. Reaction optimization

Using SYBR green reagents, it was found that there was negligible difference in C_t values between annealing and extension at 58, 60 and 62 °C for all six TBRV RNA extracts. However, at 58 °C a weak amplification signal, with C_t values of between 36 and 39 cycles, was observed with healthy host plant RNA. This was determined to be cross-reaction and dimer formation as the melting temperatures of these products was 73–73.5 °C, as opposed to the 78–79 °C observed for the TBRV amplification product. Increasing annealing/extension temperature to greater than 60 °C eliminated the cross reaction.

During assay optimisation using the FAM-labelled TaqMan probe, it was found that reducing primer concentration to 200 nM caused a loss of sensitivity of approximately 1–2 cycles, as did increasing the annealing/extension temperature to 64 °C. The TBRV positive RNA failed to amplify with a concentration of 3 mM $MgCl_2$ although amplification was achieved with 4 mM or greater; 4 mM $MgCl_2$ also gave a ~1 cycle increase in sensitivity to all TBRV RNA extracts. However, there was only a negligible difference between 4 and 5 mM. Finally, there was no difference in sensitivity observed by reducing the reverse-transcript incubation period from 15 to 10 min, or increasing incubation temperature to 55 °C.

3.2. Assay specificity

The optimised TaqMan RT-PCR assay was found to be specific to the six TBRV isolates tested, with no cross-reaction to other nepovirus species from subgroups A, B or C (Table 2). Both the nepovirus group B primers and the ELISA failed to detect the CPVC-0289 TBRV isolate, although it was detected by real-time RT-PCR. As expected, the nepovirus subgroup B primers amplified *Cycas necrotic stunt virus*, but did not amplify nepoviruses from subgroups A or C. This is in contrast to the TBRV-specific ELISA (Neogen), which reacted not only to TBRV but also to *Arabis mosaic virus* (ArMV), *Peach rosette mosaic virus* (PRMV) and ToRSV positive controls (Table 2). The TBRV-specific assay of Digiaro et al. (2007) amplified all six of the TBRV isolates and did not cross-react with any of the other nepovirus isolates tested. Finally, none of the assays cross-reacted with RNA from healthy host plants.

3.3. Assay sensitivity

As the amplification efficiency of RNA is likely to be less than a DNA plasmid as a result of the reverse-transcription step, it is difficult to compare these templates directly; as such, the sensitivity of the assay can only be estimated from the standard curve rather than being absolute. The highest dilution at which the TaqMan assay could still detect TBRV was 10^{-6} with an average C_t value of 36.19 ± 0.4; each reaction at this dilution was estimated to contain around 9 ± 0.3 copies of the TBRV target region. Greater reliability and consistency of detection was observed with the with 10^{-5} dilution with an average C_t value of 32.99 ± 0.3; at this dilution, each reaction was estimated to contain around 91 ± 3.0 copies of the TBRV target region. Amplification efficiency, by extrapolation from the standard curve was 2.006, $R^2 = 0.998$. The sensitivity of the assay compares favourably with both conventional RT-PCR assays, which both amplified approximately 91 copies of template (Table 3). The ELISA assay, while not directly comparable, detected up to a sample dilution of 10^{-5} after 2 h colour development (data not shown).

3.4. Comparison of reagents

The TBRV real-time assay was observed to be robust across the range of reagents tested. The Takara *Ex taq* and Stratagene Brilliant III one-step RT-PCR mixes produced C_t values on average 1.3 and 1.8 cycles earlier than the Invitrogen SSIII/Platinum *Taq*, Roche RNA virus master, and Bioline SensiMix reagents with the most concentrated RNA dilution, although the difference became less pronounced as the concentration of the template decreased. Overall, there was no significant difference in C_t values ($F_{(4,20)} = 0.21$, $p = 0.92$) or amplification efficiency ($F_{(4,20)} = 2.74$, $p = 0.057$), despite using different reverse-transcription temperatures for each reagent set. However, it was observed that the Takara *Ex Taq* reagents did give a lower overall relative fluorescence range, possibly due to high background fluorescence, for the TBRV positive samples (281–345 RFU) than average for the other reagents tested (797.25 ± 88.42–1256.25 ± 124.86 RFU).

Table 2
Specificity of the TBRV-specific real-time RT-PCR developed in this study, compared to the TBRV-specific and nepovirus-B general RT-PCRs and the TBRV-specific ELISA. Positive amplification or detection is indicated by '+', negative by '–', while samples not tested are indicated by 'NT'.

Species	Isolate	TBRV real-time RT-PCR	Digiaro et al. (2007) TBRV ELISA RT-PCR	Wei and Clover (2008) Nepovirus-B RT-PCR	TBRV (Neogen)
Tomato black ring virus	DSMZ PV-0070	+	+	+	+
	DSMZ PV-0191	+	+	+	+
	DSMZ PV-0521	+	+	+	+
	CPVC-289	+	+	–	–
	CFIA	+	+	+	+
	ATCC PV-554	+	+	+	+
Arabis mosaic virus	Unknown	–	–	–	+
Cycas necrotic stunt virus	Unknown	–	–	+	NT
Peach rosette mosaic virus	Agdia LPC-87700	–	–	–	+
Tobacco ringspot virus	Agdia LPC-64000	–	–	–	NT
Tomato ringspot virus	DSMZ PV-0049	–	–	–	+
Fragaria × *ananassa*		–	–	–	–
Ipomoea batatas		–	–	–	–
Prunus avium		–	–	–	–
Ribes nigrum		–	–	–	–
Rubus idaeus		–	–	–	–
Solanum lycopersicum		–	–	–	NT
Solanum tuberosum		–	–	–	–
Vitis vinifera		–	–	–	–

4. Discussion

Despite the wide host range of TBRV, which includes many economically important fruit and solanaceous crops, there are few reliable or rapid molecular methods for the detection of TBRV. ELISA is the current standard technique, and is, with strain-specific antisera, a rapid means of strain differentiation in the absence of sequence data (Dodd and Robinson, 1984; Le Gall et al., 1995). However, the commercially available antisera used in this study showed a concerning lack of specificity to TBRV and cross-reacted with ArMV, PRMV and ToRSV; the ELISA (Neogen) also failed to detect one of the six TBRV samples. This is a cause for concern as ArMV, ToRSV and TBRV are all known to infect both grapevine (Digiaro et al., 2007), and strawberry (Martin and Tzanetakis, 2006), which could result in false positives during diagnosis. Conventional RT-PCR by contrast is more rapid, and shows much greater specificity, although of the two assays tested in this study, the Digiaro et al. (2007) primers were more effective, amplifying all six of the TBRV RNA extracts from the test panel, whereas the Wei and Clover (2008) nepovirus subgroup-B primers did not amplify one of the TBRV isolates. The relative paucity of RT-PCR-based diagnostic assays for TBRV is likely due to the relative lack of sequence data for this species, with only three extant and publicly available complete RNA-2 sequences at time of writing.

The real-time RT-PCR assay designed in this study showed comparable specificity to the Digiaro et al. (2007) primers, amplifying all of the TBRV isolates, while not cross-reacting with other nepovirus species or nucleic acid extracts from common hosts of TBRV. However, real-time RT-PCR has several advantages over conventional RT-PCR, being more rapid, and can also be used to screen large numbers of samples in a run-time of approximately 90 min, without the need to perform gel electrophoresis.

The detection limit of the TBRV real-time RT-PCR assay developed in this study, was approximately nine copies per reaction. This is a ten-fold increase in sensitivity compared with conventional RT-PCR using either the nepovirus subgroup B, or TBRV-specific primers (Digiaro et al., 2007; Wei and Clover, 2008). It is more difficult to directly compare sensitivity between the real-time RT-PCR and ELISA given different extraction methods were applied, and the larger sample volume used in ELISA, but it may be generally observed that the real-time RT-PCR is more sensitive than the TBRV ELISA and/or RT-PCR assays (Bertolini et al., 2010; Stewart et al., 2007). It is also difficult to compare the sensitivity of the TBRV real-time RT-PCR to real-time PCR assays for other nepovirus species (Bertolini et al., 2010; Finetti-Sialer and Ciancio, 2005; Osman et al., 2008; Osman and Rowhani, 2006; Stewart et al., 2007), as sensitivities are reported rarely in terms of target copy number, but in terms of sample dilution which are not directly comparable. However, a detection limit of approximately nine copies of target, with a reliable quantitation limit of 91 copies, as found for the TBRV assay, is consistent with assays for other, unrelated plant viruses (Agindotan et al., 2007; Gutierrez-Aguirre et al., 2009; Ruiz-Ruiz et al., 2007).

Finally, the TBRV-specific real-time RT-PCR assay designed in this study was found to be robust and reliable when tested using a range of common, commercially available one-step qRT-PCR kits. The assay performed best, as determined by C_t values, with the Stratagene Brilliant III and Takara *Ex Taq* reagents, although these results were not significantly greater (approximately 1–1.8 cycles) than the other suppliers. All kits, regardless of the supplier, could detect TBRV down to a dilution of approximately 10^2 copies per reaction, therefore there was no observable loss of sensitivity. This is particularly important for diagnosis as availability and cost often dictate reagent choices.

Table 3
Sensitivity of the TBRV-specific real-time RT-PCR assay developed in this study compared to two conventional RT-PCR assays. Positive: +, Negative: –. C_t values for the real-time RT-PCR assay are an average of three replicates; C_t values of greater than 38 cycles were considered negative.

TBRV RNA dilution	Estimated concentration	TBRV real-time RT-PCR	Wei and Clover (2008) conventional RT-PCR	Digiaro et al. (2007) conventional RT-PCR
10^{-1}	918,000 ± 81,504.5	19.76 ± 0.23	+	+
10^{-2}	82,566.6 ± 6104.4	22.87 ± 0.13	+	+
10^{-3}	9495.0 ± 120.2	26.32 ± 0.27	+	+
10^{-4}	1001.3 ± 86.6	29.53 ± 0.19	+	+
10^{-5}	91.1 ± 3.6	32.99 ± 0.30	+	+
10^{-6}	9.1 ± 0.3	36.19 ± 0.44	–	–

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