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BRIEF REPORT

Rapid detection of genetically diverse tomato black ring virus isolates using reverse transcription loop-mediated isothermal amplification

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Abstract A reverse transcription loop-mediated isothermal amplification assay (RT-LAMP) has been developed for detection of tomato black ring virus (TBRV) isolates collected from different hosts. One-step RT-LAMP was performed with a set of four primers, the design of which was based on the coat protein gene. Results of RT-LAMP were visualized by direct staining of products with fluorescent dyes, agarose gel electrophoresis, and analysis of amplification curves. The sensitivity of RT-LAMP was 100-fold greater than that of RT-PCR. The RT-LAMP assay developed here is a useful and practical method for diagnosis of TBRV.

Keywords Detection · RT-LAMP · TBRV

Tomato black ring virus (TBRV) is a member of the genus *Nepovirus* in the family *Secoviridae* [1]. The genome is organized into two single-stranded RNAs with a poly(A) tail at the 3′ end and a viral protein genome (VPg) linked to the 5′ end. RNA1 encodes the RNA-dependent RNA polymerase and a proteinase. RNA2 encodes the coat and movement proteins [2–4]. TBRV is distributed worldwide and is known to infect a wide range of crop species, including grapevine, cherry, apricot, peach, berry fruits, solanaceous plants, and a number of weed and ornamental species [5–8]. In Poland, the virus is a quarantined pathogen of raspberry and strawberry. Therefore, rapid, reliable, sensitive, and specific detection of TBRV is essential especially for implementation of the control measures.

Previous studies have reported that the TBRV population is highly diverse, with isolates collected from the same host clustering in separate branches of phylogenetic trees [8, 9]. Genetic diversity has a great impact on molecular assays for diagnosis of TBRV [10]. Several methods have been developed for detection of TBRV, including serological assays such as enzyme-linked immunosorbent assay (ELISA) and nucleic acid-based method such as conventional RT-PCR and real-time PCR [10–12]. However, nucleic-acid-based methods – especially those performed under real-time conditions – are sensitive but require expensive equipment and the use of specific probes. Recently, reverse transcription loop-mediated isothermal amplification (RT-LAMP) of nucleic acids has been developed for rapid and efficient detection of different plant RNA viruses [13–15]. The RT-LAMP assay amplifies a target sequence with high sensitivity and specificity under isothermal conditions (63–65 °C) and exhibits sensitivity similar to or even greater than conventional RT-PCR. RT-LAMP is a cost-effective alternative to RT-PCR, as the reaction can be carried out in a heat block or a water bath. Taking into account the advantages of the RT-LAMP assay, our objective was to develop and evaluate RT-LAMP for rapid and specific detection of genetically diverse TBRV isolates.

In this study, we used 16 isolates of TBRV, including 14 Polish isolates and two from The Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH. The isolates were collected from different hosts during 2003–2015 (Table 1). To verify its specificity, RT-

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Table 1 Isolates of tomato black ring virus, non-target nepoviruses and healthy host plants used in this study

Target	Species	Isolate	Orginal host	Source	RT-LAMP
Target virus	*Tomato black ring virus*	DSMZ PV-0070	*Solanum tuberosum*	DSMZ, Braunschweig, Germany	+
		DSMZ PV-0191	*Rubus idaeus*	DSMZ, Braunschweig, Germany	+
		TBRV-B1	*Sambucus nigra*	IPP-NRI, Poznan, Poland	+
		TBRV-CK	*Cucurbita pepo*	IPP-NRI, Poznan, Poland	+
		TBRV-K5	*Cucurbita pepo*	IPP-NRI, Poznan, Poland	+
		TBRV-K8	*Cucurbita pepo*	IPP-NRI, Poznan, Poland	+
		TBRV-KC	*Cucurbita pepo*	IPP-NRI, Poznan, Poland	+
		TBRV-L1	*Robinia pseudoacacia*	IPP-NRI, Poznan, Poland	+
		TBRV-MJ	*Robinia pseudoacacia*	IPP-NRI, Poznan, Poland	+
		TBRV-O1	*Cucumis sativus*	IPP-NRI, Poznan, Poland	+
		TBRV-P1	*Solanum lycopersicum*	IPP-NRI, Poznan, Poland	+
		TBRV-Pi1	*Solanum lycopersicum*	IPP-NRI, Poznan, Poland	+
		TBRV-Z1	*Solanum tuberosum*	IPP-NRI, Poznan, Poland	+
		TBRV-S1	*Lactuca sativa*	IPP-NRI, Poznan, Poland	+
		TBRV-AG	*Tagetes patula*	IPP-NRI, Poznan, Poland	+
		TBRV-AY	*Tagetes erecta*	IPP-NRI, Poznan, Poland	+
Non-target nepovirus	*Arabis mosaic virus*	ArMV-1	*Laburnum anagyroides*	IPP-NRI, Poznan, Poland	-
	Beet ringspot virus	BRSV-1	*Robinia pseudoacacia*	IPP-NRI, Poznan, Poland	-
Healthy host species	*Chenopodium quinoa*			IPP-NRI, Poznan, Poland	-
	Cucumis sativus			IPP-NRI, Poznan, Poland	-
	Cucurbita pepo			IPP-NRI, Poznan, Poland	-
	Robinia pseudoacacia			IPP-NRI, Poznan, Poland	-
	Rubus idaeus			IPP-NRI, Poznan, Poland	-
	Sambucus nigra			IPP-NRI, Poznan, Poland	-
	Solanum lycopersicum			IPP-NRI, Poznan, Poland	-
	Solanum tuberosum			IPP-NRI, Poznan, Poland	-

+, positive result; -, no amplification

LAMP also was carried out using other nepoviruses – beet ringspot virus (BRSV) and arabis mosaic virus (ArMV). RNA was isolated from leaf tissues either by precipitation with lithium chloride or by using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The concentration of each RNA sample was measured in a ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and adjusted to 500 ng/µl. The CP region was amplified for all tested isolates by conventional RT-PCR using CPF (5′ GCCTGTCTCTCTCGCAATG 3′) and CPR (5′ AAGGAG CCAAACTGAAATG) 3′ primers designed using Oligo Analyzer 1.0.3 software based on the TBRV-MJ sequence (accession no. AY157994.1) and a Transcriptor One-Step RT-PCR Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The RT-PCR products (763 bp) were cloned using a TOPO®-TA Cloning Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and sequenced. The obtained sequences (GenBank accessions KR139939-KR139952) and other sequences retrieved from GenBank were aligned using ClustalW. The nucleotide sequence identity among the Polish TBRV isolates ranged from 93.1 % to 100 %. The obtained alignment was analyzed to identify appropriate regions for RT-LAMP primer design. RT-LAMP primers were designed using the Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) based on the TBRV coat protein region (Supplementary file). Each 25-µl LAMP reaction consisted of 2 µl (10 µM) of each primer (FIP [F1c-F2], 5′ GGTTTCTGGTGCTAGG GGTG-CACGGCAAATTTTGGGGA 3′; and BIP [B1c-B2], 5′ ACATATCCATGGTATAAAGGCCAAG-CCAA GCAAAATGCTGGTTA 3′), 0.5 µl (10 µM) of each of primer (F3, 5′ ACTCTGCAAATACAGTCTCC 3′; B3, 5′ TGTATCGAGATGCTCAAGC 3′), 15 µl of Isothermal Master Mix (ISO-001nd) (OptiGene, Horsham, UK), 1 µl of RNA template, 0.25 µl (1 u/µl) of reverse transcriptase (Novazym, Poznan, Poland), and water. The mixture was incubated at 63 °C for 30 min in a Thermoblock (Biometra, Göttingen, Germany). Two methods were used to analyze RT-LAMP products – electrophoresis on 1.5 % agarose

gels with Midori Green (NIPPON Genetics, Düren, Germany) and direct visual inspection by addition of 3 µl of SYBR Green I (diluted 1:1000; Novazym, Poznan, Poland) prior to the reaction. The sensitivity of the TBRV-specific RT-LAMP assay was determined using tenfold dilutions of total RNA template from TBRV-P1-infected plants. Total RNA was adjusted to 1000 ng/µl and further used in RT-PCR and RT-LAMP as described above. The detection limit was verified by electrophoresis on a 1.5 % agarose gel.

The reactions also were performed using Isothermal Master Mix ISO-001 (OptiGene, Horsham, UK) in a LightCycler® 480 (Roche, Mannheim, Germany), which allowed monitoring of amplification curves under real-time conditions. Real-time fluorescence data were obtained on the FAM channel (excitation at 470 nm and detection at 510 nm) in 20 min.

As is typical for RT-LAMP, ladder patterns of multiple bands were observed in the agarose gel for RNA from samples infected with TBRV, but not in the virus-free samples and those with RNA from BRSV or ArMV (Fig. 1A). Positive reactions turned green on addition of SYBR Green I, while in negative reactions or those with RNA from BRSV or ArMV as template, the color remained orange (Fig. 1B). Analysis of real-time RT-LAMP showed amplification curves only for samples with RNA from TBRV (Fig. 1C). The sensitivity of the RT-LAMP was 100-fold greater than that of conventional RT-PCR, and the detection limit was 10 fg/µl of total RNA (Fig. 2A and B).

In the present study, we were able to demonstrate successful amplification of TBRV within 25–30 min at 63 °C using an RT-LAMP assay. The LAMP assay can rapidly amplify target sequences with high sensitivity under isothermal conditions using a specific set of primers and a DNA polymerase with strand-displacement activity [16]. The amplification products were stem-loop DNA structures with several inverted repeats, cauliflower-like or dumbbell structure, and multiple loops. All positive samples detected by gel electrophoresis or in real-time could also be detected visually by addition of fluorescent dyes. Therefore, the results of RT-LAMP amplification can be observed directly under UV light, eliminating gel electrophoresis and greatly reducing the time required [17]. The RT-LAMP method can be widely employed in laboratories where resources are limited; however, to identify new evolving isolates,

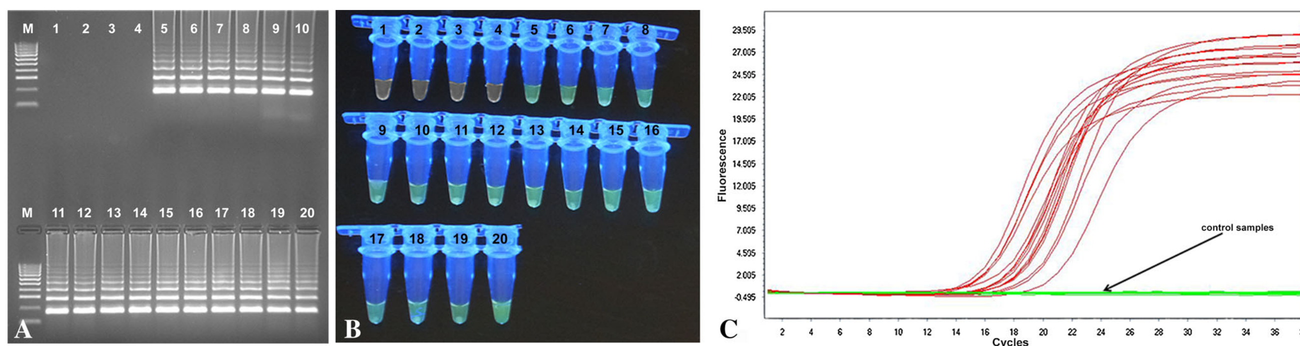

Fig. 1 Detection of TBRV isolates by RT-LAMP. **A.** Analysis of RT-LAMP products on agarose gels. Lane M, HyperLadder IV (Bioline, London, UK); lane 1, negative control (water); lane 2, negative control (healthy tomato sample); lane 3, BRSV; lane 4, ArMV; lanes 5-20, TBRV isolates **B.** Visual detection of RT-LAMP products using SYBR Green I. Numbers correspond to the gel lanes in panel A. **C.** Real-time monitoring of the RT-LAMP assay of the TBRV isolates. Red color, amplification curves; green lines, control samples (water and healthy plant samples)

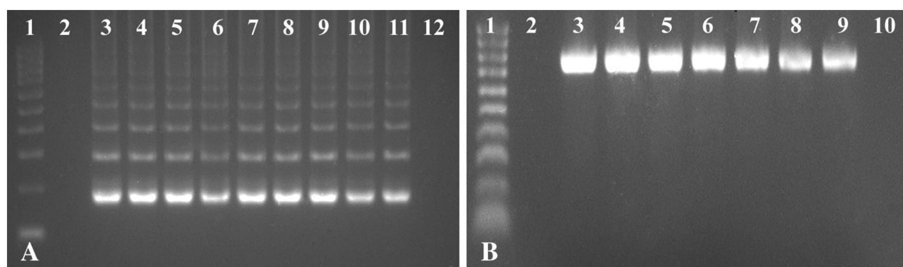

Fig. 2 A comparison of sensitivity of RT-LAMP and RT-PCR methods. **A.** Detection limit of the RT-LAMP assay using total RNA isolated from leaves of tomato infected with TBRV-P1. Lane 1, HyperLadder IV (Bioline, London, UK); lane 2, negative control (water); lanes 3-12, serial tenfold dilutions of RNA (ranging from 1000 ng/µl to 1 fg/µl). **B.** Detection limit of the RT-PCR assay using total RNA isolated from tomato infected with TBRV-P1. Lane 1, HyperLadder IV (Bioline, London, UK); lane 2, negative control (water); lanes 3-10, serial tenfold dilutions of RNA (ranging from 1000 ng/µl to 100 fg/µl)

conventional RT-PCR and further sequencing will be required. The dye can be added prior to amplification, thus reducing risk of cross-contamination. Further analysis of LAMP products through sequencing or restriction enzyme analysis would distinguish between false positive results and contamination. To reduce the chances of contamination, similar protocols to those followed for PCR are required [18]. The RT-LAMP developed here was capable of detection of TBRV in as little as 10 fg/μl of total RNA. Our results indicate that the TBRV LAMP assay is sensitive and specific and has the potential to be developed into a field or greenhouse diagnostic test.

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