Journal of Virological Methods 200 (2014) 35–40

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of reverse transcription loop-mediated isothermal amplification assay for rapid detection of an emerging potyvirus: Tomato necrotic stunt virus☆

Rugang Li, Kai-Shu Ling*

USDA-Agricultural Research Service, U.S. Vegetable Laboratory, Charleston, SC 29414, USA

Article history:
Received 27 September 2013
Received in revised form 18 January 2014
Accepted 24 January 2014
Available online 3 February 2014

Keywords:
Solanum lycopersicum
qRT-PCR
LAMP assay
Field disease diagnosis

A B S T R A C T

Tomato necrotic stunt virus (ToNStV) is an emerging potyvirus that causes severe stunting to infected tomato plants. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for sensitive detection of ToNStV. The sensitivity of RT-LAMP was comparable to that of conventional RT-PCR, with detection of ToNStV in a reaction containing only 8 pg of total tomato RNA or with 1:20,000 dilution of crude tissue extract. This assay was able to detect ToNStV in a broad range of solanaceous plant species. The RT-LAMP for ToNStV was specific with no cross-reactivity to other potyviruses (i.e. *Potato virus Y* and *Tobacco etch virus*), as well as several other common tomato viruses. RT-LAMP should complement RT-PCR and real-time RT-PCR assays reported previously, with a potential to provide a simple, rapid, and sensitive field diagnostic method for ToNStV.

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1. Introduction

Tomato necrotic stunt virus (ToNStV), a new potyvirus infecting tomato plants in Mexico, was identified recently using small RNA deep sequencing and assembly (Li et al., 2012). ToNStV has a genome of 10,057 nucleotides excluding the 3′ polyA tail. It encodes a single large open reading frame (ORF) and a short ORF (*pipo*) embedded in the P3 cistron. A second isolate with only 2 nucleotide substitutions was identified in another location in Mexico (Li et al., 2014). In host range studies using mechanical inoculation, ToNStV was found to infect numerous species in the family *Solanaceae* (Li et al., 2014).

Although a sensitive qRT-PCR system for ToNStV detection has been developed (Li et al., 2014), it requires a sophisticated thermal cycling instrument, and must be performed by a skilled technician. The loop-mediated isothermal amplification (LAMP) originally developed by Notomi et al. (2000) has been adapted for rapid identification of numerous plant viruses and viroids (Fukuta et al., 2003a, 2003b, 2004; Nie, 2005; Viganó and Stevens, 2007; Boubourakas et al., 2009; Kuan et al., 2010; Le et al., 2010; Tsutsumi et al., 2010; Hadersdorfer et al., 2011; Lee et al., 2011; Zhang et al., 2011; Zhao et al., 2012; Zhou et al., 2012; Ling et al., 2013). With the special designed primers, LAMP assay offers great sequence-specificity and high sensitivity. The DNA synthesis using a strand-displacing DNA polymerase can be carried out under an isothermal condition (Notomi et al., 2000; Nagamine et al., 2002). There are several ways to detect the LAMP amplified DNA products. Traditional detection is to separate DNA products by electrophoresis on an agarose gel which is stained with a DNA binding or intercalating dye, then visualized under U.V. light. The magnesium pyrophosphate, a byproduct produced during RT-LAMP reaction is proportional to the amplified product. Although direct visualization may judge the presence of white byproduct as turbidity of precipitate in the reaction tube, observation of the byproduct precipitate may be difficult, especially at lower target DNA concentration. A DNA intercalating dye such as SYBR Green I (Invitrogen, USA) could be used to improve the visibility with green fluorescence. We have used this method to achieve a genotype-specific detection of *Pepino mosaic virus* (Ling et al., 2013).

In the present study, we described the development of a RT-LAMP method specific for ToNStV and demonstrated its usefulness for practical disease diagnosis.

2. Materials and methods

2.1. Virus sources

ToNStV isolate MX9354 was identified originally in a sample from Mexico and maintained on tomato cultivar 'Moneymaker'

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* Corresponding author at: USDA-ARS, U.S. Vegetable Laboratory, 2700 Savannah Highway, Charleston, SC 29414, USA. Tel.: +1 843 402 5313; fax: +1 843 573 4715.
E-mail addresses: Rugang.Li@ars.usda.gov (R. Li), Kai.Ling@ars.usda.gov (K.-S. Ling).

0166-0934/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2014.01.017

in a growth chamber at 25 °C with a photoperiod of 14 h. Other common tomato viruses and viroids used in this work, including PepMV, *Potato virus Y* (PVY), *Tobacco etch virus* (TEV), *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), *Potato spindle tuber viroid* (PSTVd), and *Mexican papita viroid* (MPVd) were from collections stored at the USDA-ARS, U.S. Vegetable Laboratory in Charleston, South Carolina.

2.2. Virus inoculation

Seeds from tomato and other indicator plants were germinated in soilless plant growth media (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA) in a greenhouse with a temperature range in 18–30 °C and a natural photoperiod of 12–14 h. Virus inoculum was prepared (1:10, w/v) using ToNStV-infected leaf tissue in 0.01 M phosphate saline buffer, pH 7.0. Seedlings (2- to 3-leaf stage) were dusted with carborundum (600-mesh) and inoculated mechanically through gentle rubbing on the leaf surface. After inoculation, plants were transferred to a growth chamber at 25 °C with a photoperiod of 14 h. Leaf tissue was collected in 3–4 weeks post inoculation and used for RNA preparation.

2.3. RNA isolation

Total RNA was extracted from plant leaf tissue using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol with some minor modifications. Approximately 0.1 g of fresh leaf tissue was collected in a 1.5 ml microfuge tube, 200 μl Trizol was added, and tissue was homogenized by hand with a mini pestle. An additional 800 μl of Trizol was added, followed by the addition of 200 μl of chloroform. The suspension was vortexed for 15 s, allowed to settle for 5 min, then centrifuged at 13,500 × *g* for 10 min at 4 °C. Six hundred microliters of supernatant were transferred to a new microfuge tube, mixed with 0.5 ml of isopropanol and stored for 10 min at room temperature. The RNA pellet was obtained after centrifugation at 13,500 × *g* for 10 min at 4 °C and then washed with 70% ethanol. After air-drying for 5 min at room temperature, the RNA pellet was dissolved in 200 μl of nuclease-free water and stored in a −80 °C freezer until use.

2.4. Crude tissue extract preparation

Approximately 100 mg of fresh leaf issue was homogenized with a mini pestle in a microfuge tube with 200 μl of 0.1 M Tris–HCl, pH 8.0 (Tsutsumi et al., 2010; Li et al., 2014). The suspension was centrifuged at 13,500 × *g* for 2 min. The supernatant was transferred into a new microfuge tube for making serial dilutions.

2.5. RT-LAMP assay

Four LAMP oligonucleotides specific for a selected 200-bp conserved coat protein-coding region (nt 9059 to nt 9258) were designed using PrimerExplorer V4 software (available at http://primerexplorer.jp/elamp4.0.0/index.html) including two inner primer (FIP, BIP) and two outer primers (F3, B3). Two loop primers (Loop F, Loop B) were added according to loop sequences (Fig. 1). Primers were synthesized and purified as standard molecular grade (desalting) by Sigma Genosys (USA). The RT-LAMP assay was conducted using a RNA amplification kit from Eiken Chemical (Japan) following the manufacturer's instructions. For each 25 μl reaction, it consisted of 12.5 μl of 2× reaction buffer [containing 40 mM Tris–HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, and 2.8 mM each of dNTPs], 2 μl of 20 μM FIP, 2 μl of 20 μM BIP, 1 μl of 20 μM of Loop-F primer, 1 μl of 20 μM of Loop-B primer, 0.25 μl of 20 μM of F3 primer, 0.25 μl of 20 μM of B3 primer, 1.0 μl of enzyme mix (AMV reverse transcriptase and *Bst* DNA polymerase) and 1.0 μl of RNA preparation. The mixture was incubated at 65 °C for 60 min for both reverse transcription and amplification, followed by denaturation for 2 min at 95 °C to terminate the reaction. Products were checked by electrophoresis on a 2% agarose gel containing 1:10,000 diluted SYBR Safe DNA Gel Stain (Invitrogen, USA) or visualized directly by addition of 1.0 μl of 1:10 diluted SYBR Green I (Invitrogen, USA). To determine the sequence specificity, two microliters of products were digested with 24 units of restriction enzyme *Eco*RI and the resulting two bands were isolated, cloned and sequenced by Sanger sequencing (Functional Biosciences, Madison, WI).

2.6. RT-PCR

For validation, a RT-PCR was performed using Takara One Step Ex Taq qRT-PCR Kit (Clontech, USA) with the forward primer F3 and the reverse primer B3. The 10 μl reaction contained 5 μl of 2× master mix, 0.25 μl Ex Taq HS mix (5 U/μl), 0.25 μl RTase mix (5 U/μl), 0.25 μl F3 primer (20 μM), 0.25 μl B3 primer (20 μM), and 0.5 μl RNA template. The RT-PCR was carried out in a PTC-200 Peltier thermal cycler (MJ Research), with a reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 2 min, and then 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The reaction was completed after a final extension at 72 °C for 10 min. RT-PCR products were analyzed by electrophoresis on a 2% agarose gel stained with 1:10,000 SYBR Safe DNA Gel Stain.

3. Results

3.1. RT-LAMP detection of ToNStV

To maximize DNA production in RT-LAMP, it was necessary to optimize reaction conditions, such as the incubation temperature and duration. Using Trizol-purified total RNA preparation as template, the amount of DNA production with 60 min incubation under seven temperature points (58 °C, 60 °C, 61 °C, 63 °C, 65 °C, 67 °C and 69 °C) were compared. Amplified DNA products appeared as a characteristic ladder on a 2% agrose gel, indicating that various sizes of stem-loop DNA products with inverted repeats of the target sequence were produced. The minimum size of amplified DNA products was ~160 bp (the distance between primers F2 and B2). The highest concentration of DNA was produced at 65 °C. A much weaker amplified product was observed at 67 °C, but no visible amplified product was generated at 69 °C (Fig. 2A). The similar results were observed in two additional experiments. To determine the minimum time that was required for a reaction to generate sufficient amount of DNA products that could be visible through staining on an agarose gel after electrophoresis, six incubation time points (30 min, 40 min, 50 min, 60 min, 70 min and 80 min) were evaluated, and repeated two more times, using 65 °C as the incubation temperature (Fig. 2B). The DNA products were visible after 30 min of incubation and accumulated gradually as the incubation time extended. However, after incubation for 60 min, amplified DNA products reached a plateau, with no significant improvement in accumulation with longer reaction time. Therefore, reaction conditions with incubation temperature of 65 °C for 60 min were considered optimum for RT-LAMP using the designed primers.

3.2. Specificity of RT-LAMP for ToNStV

To determine the specificity of RT-LAMP assay for detection of ToNStV, we examined its potential cross-reactivity to two closely

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