Journal of Virological Methods 240 (2017) 49–53

Contents lists available at ScienceDirect

Journal of Virological Methods

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

One-step reverse transcription loop-mediated isothermal amplification for the detection of *Maize chlorotic mottle virus* in maize

Ling Chen [a], Zhiyuan Jiao [a], Dongmei Liu [a], Xingliang Liu [b], Zihao Xia [a], Congliang Deng [b], Tao Zhou [a], Zaifeng Fan [a,*]

[a] *State Key Laboratory of Agro-biotechnology and Ministry of Agriculture Key Laboratory for Plant Pathology, China Agricultural University, Beijing 100193, China*

[b] *Beijing Entry-exit Inspection and Quarantine Bureau, Beijing 100016, China*

Article history:
Received 8 November 2015
Received in revised form 2 October 2016
Accepted 26 November 2016
Available online 27 November 2016

Keywords:
Maize chlorotic mottle virus (MCMV)
Loop-mediated isothermal amplification (LAMP)
Maize
Detection

A B S T R A C T

Maize chlorotic mottle virus (MCMV) is spreading in many regions worldwide, causing maize lethal necrosis when co-infected with a potyvirid. In this study, one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed to detect MCMV in maize. A set of four specific primers was designed based on the conserved coat protein gene sequences of MCMV. The RT-LAMP could be completed within 60 min under isothermal condition at 63 °C. The sensitivity test showed that the RT-LAMP was about 10-fold more sensitive than RT-PCR and no cross-reactivity was detected with other viral pathogens infecting maize in China. Moreover, the results of RT-LAMP could be visually inspected by SYBR Green I staining in a closed-tube, facilitating high-throughput application of MCMV detection. This method was further verified by testing field-collected samples. These results suggested that the developed MCMV RT-LAMP technique is a rapid, efficient and sensitive method which could be used as a routine screen for MCMV infection.

1. Introduction

Maize (*Zea mays* L.) is an important cereal crop worldwide. Recently, *Maize chlorotic mottle virus* (MCMV) has been found in southwest China as a quarantine virus (Wu et al., 2013; Xie et al., 2011). MCMV in the genus *Machlomovirus* of the family *Tombusviridae* can infect maize and lead to typical symptoms like mild mosaic, severe stunting, and leaf necrosis (Niblett and Claflin, 1978; Uyemoto et al., 1981). Moreover, MCMV causes corn lethal necrosis (CLN) disease by synergistic infection with a maize-infecting potyvirid such as *Maize dwarf mosaic virus* (Goldberg and Brakke, 1987), *Wheat streak mosaic virus* (Scheets, 1998; Stenger et al., 2007) or *Sugarcane mosaic virus* (Adams et al., 2013; Wangai et al., 2012; Xia et al., 2016), resulting in severe yield losses. CLN was first described in maize from Peru in 1974 (Castillo and Hebert, 1974), thereafter, this disease was reported on maize plants in the United States (Niblett and Claflin, 1978). MCMV mainly distributed in America (Jensen et al., 1991; Jiang et al., 1992), but it has been found in several maize planting regions in Asia and Africa (Deng et al., 2014; Lukanda et al., 2014; Mahuku et al., 2015), suggesting that MCMV is spreading in major maize-planting regions worldwide. Meanwhile, there is a high risk of MCMV rapidly spread throughout China because of a large scale planting of its natural hosts (such as maize and sorghum) and its diverse transmission by thrips/beetles, seed and mechanical inoculation (Jiang et al., 1992; Jensen et al., 1991; Nault et al., 1979; Zhang et al., 2011). Consequently, there is a great potential threat of MCMV to maize production in many regions where this virus is emerging.

Several traditional methods for detecting MCMV have been reported including enzyme-linked immunosorbent assay (Uyemoto, 1980), immunofluorescence (Nault et al., 1979), RT-PCR, Real-time TaqMan RT-PCR (Zhang et al., 2011), and the next generation sequencing (Adams et al., 2013). However, these existing methods have limitations to detect MCMV. The results of immunological assay depend on the quality and availability of antibodies and false positives often make the results unreliable. RT-PCR is time consuming and the sensitivity is limited. Real-time RT-PCR is a relatively sensitive technique to detect MCMV, but it also requires expensive equipment in a laboratory setting. Next generation sequencing was mainly applied in the identification of plants infected with unknown virus because of its high costs and complex data analysis. Therefore, it is necessary to establish a rapid and effective technique to detect MCMV for controlling the spread of this virus.

* Corresponding author.
E-mail address: fanzf@cau.edu.cn (Z. Fan).

http://dx.doi.org/10.1016/j.jviromet.2016.11.012
0166-0934/

One-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay is suitable for detecting virus and measuring field samples because of its characteristics of rapidity, efficiency, simplification, high sensitivity and specificity (Nagamine et al., 2002; Notomi et al., 2000).

This study was to develop a one-step RT-LAMP assay for rapid and sensitive detection of MCMV in field-grown maize.

2. Materials and methods

2.1. Virus sources, virus inoculations and RNA isolation

MCMV was multiplied from the full-length cDNA clone (pMCM41) provided by Dr Kay Scheets. *Sugarcane mosaic virus* (SCMV), *Pennisetum mosaic virus* (PenMV) and *Rice black streaked dwarf virus* (RBSDV) were from previously published sources (Fan et al., 2003, 2004; Jia et al., 2012). Field maize samples were collected from Yuxi region of Yunnan Province.

Maize inbred line B73 and cv. Va35 plants were grown in a growth chamber (28 °C day and 22 °C night, 16 h light and 8 h night cycles) for virus inoculation. Crude extracts were prepared by homogenizing the MCMV-, SCMV- or PenMV-infected maize leaf tissues in 0.01 M phosphate buffer (0.01 M KH_2PO_4: 0.01 M Na_2HPO_4 = 49: 51 (V/V), pH 7.0) at a ratio of 1:10 (*w/v*). The crude extracts were rub-inoculated to the first true leaves of one-week-old B73 maize seedlings and the systemically infected leaves were harvested at 10 days post inoculation (dpi). For RBSDV inoculation, one-week-old Va35 seedlings were exposed to small brown planthoppers carrying RBSDV (five insects on each seedling) for 3 days in specific inoculation chambers and the systemically infected leaves were harvested at 30 dpi.

Total RNA was extracted from infected and healthy maize leaves using Trizol reagent (Invitrogen, Carlsbad, CA, USA).

2.2. Primer design

Available sequences of MCMV isolates were aligned to obtain the conserved coat protein (CP) gene sequences (accession nos. NC_003627, JQ982468, KF010583, JQ982470, JQ982469, GU13867, KJ782300, KP772217, EU358605, KF744394, KP851970) and then the RT-LAMP primers were designed according to the conserved CP gene sequences using the program Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). A forward inner primer (FIP, 5′-GCGCACACTGGAATCTCGAGAATT-CTCCATGTCCGAAATTCTGC-3′, nt 3598-3575/nt 3527-3546) consisted of F1c (the complementary sequence of F1) and F2, and a reverse inner primer (BIP, 5′-CAAATGGCTGGCAGCACAA-GAATTC-GATACGCACAGAGTTGAACA-3′, nt 3647-3665/nt 3707-3688, GAATCC represents the *Eco*R I sites) consisted of B1c (the complementary sequence of B1) and B2. The outer primers F3 (5′-GAGCTATTCGAGCCAACC-3′, nt 3451-3468) and B3 (5′-TAGTGGTGTCTGCTGTGA-3′, nt 3738-3721) were used for the initiation of the RT-LAMP reaction. The conventional RT-PCR primers were also designed according to the same and expanded conserved CP gene sequences of MCMV compared with that of RT-LAMP (Gong et al., 2010). The forward primer MCMV F (5′-ATGGCGGCAAGTAGCCGGTCTACCCGAGGTAGAA-3′, nt 3384-3417) and reverse primer MCMV R (5′-TCAATGATTTGCCAGCCCTGGCCTGGAACCAGG-3′, nt 4094-4061) were used for RT-PCR reaction. Genome position listed according to the complete genome sequence of MCMV (accession no. JQ982468).

2.3. Conventional RT-PCR detection of MCMV

The first-strand cDNA of MCMV-infected maize leaves was synthesized using M-MLV reverse transcriptase according to the instructions of the manufacturer (Promega, Madison, WI, USA). An RT reaction mixture (20 μL final volume) included 5 μL of total RNA (2.5 μg), 4 μL of dNTP mixture (2.5 mM), 1 μL (200U) of M-MLV reverse transcriptase, 4 μL of M-MLV 5× reaction buffer, 1 μL of random primers (10 μM), 1 μL (40U) of recombinant RNasin ribonuclease inhibitor, and 4 μL of sterile, RNase-free water. The mixture was incubated at 37 °C for 60 min. After the RT reaction, 1 μL cDNA product was added to 24 μL of the PCR mixture which consisted of 12.5 μL of Premix (Takara Bio Inc., Dalian, China), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), and 10.5 μL of sterile distilled water. Thermal cycling conditions used for MCMV detection consisted of 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s. A final elongation step was performed at 72 °C for 10 min. The PCR products (5 μL) were analyzed by electrophoresis on 1% agarose gels, followed by ethidium bromide staining.

2.4. Optimized RT-LAMP conditions

RNA Amplification Kit (Eiken, Shanghai, China) was used for the RT-LAMP reactions. The concentration of total RNA was 500 ng/μL. The RT-LAMP reaction mixture contained 12.5 μL of 2 × Reaction Mix, 5 μL of total RNA template (2.5 μg), 1 μL of Enzyme Mix, 1 μL of Fluorescent Detection Reagent, 1 μL each of the FIP and BIP primers (10 μM), 1.25 μL each of the F3 and B3 primers (1 μM), 1 μL of distilled water. Six different temperatures (from 60 to 65 °C) and five different durations of reactions (15 min, 30 min, 45 min, 60 min, and 75 min) were performed to optimize the reaction conditions. The mixtures were incubated at 95 °C for 2 min to terminate the reactions. The optimized RT-LAMP was carried out at 63 °C for 60 min and terminated at 95 °C for 2 min.

2.5. Analysis of RT-LAMP products

The RT-LAMP products (0.5 μL) were analyzed by electrophoresis on a 2% agarose gel and subsequently stained with ethidium bromide. Lanes containing a laddered amplification pattern were considered positive, while lanes containing no visible bands were regarded as negative. Meanwhile, the reaction tubes were visualized under UV light (3UV Transilluminator, wavelength: 365 nm), and positive samples showed green fluorescence, whereas negative samples remained original orange.

2.6. Specificity of RT-LAMP

To confirm the specificity of the RT-LAMP, the reaction products were purified, digested with *Eco*R I, repurified, and cloned into the vector pUC-18, which was pre-digested with *Eco*R I, purified, dephosphorylated with Thermosensitive Alkaline Phosphatase (Promega, Madison, WI, USA) and repurified. The recombinant plasmids were subsequently sequenced. The specificity of the assay was also tested by RT-LAMP reactions that used maize-infecting viruses (SCMV, PenMV, RBSDV), healthy maize plants and distilled water. The presences of MCMV, SCMV, PenMV and RBSDV in the samples was determined by RT-PCR using total RNA extracted from the systemically infected leaves of MCMV-, SCMV-, PenMV- and RBSDV-inoculated maize plants, respectively, and the primers used are provided in Supplementary Table 1.

All of the RT-LAMP assays were performed as the optimized reaction system and conditions described above.