Recombinase Polymerase Amplification Assay for Simultaneous Detection of Maize Chlorotic Mottle Virus and Sugarcane Mosaic Virus in Maize

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ABSTRACT: Maize chlorotic mottle virus (MCMV) can cause maize lethal necrosis (MLN) when coinfected with potyvirids, such as sugarcane mosaic virus (SCMV), maize dwarf mosaic virus, or wheat streak mosaic virus. MLN is often caused by coinfection of MCMV and SCMV, which has been reported in China and several countries of Africa. In this study, a recombinase polymerase amplification (RPA) assay was established for simultaneous detection of MCMV and SCMV in maize. The RPA assay can be completed within 30 min at 38 °C. The primers for the RPA assay were specific since no crossreaction was detected with other selected viruses that infected maize in China. The detection limit of the RPA method was 10^2 copies μL⁻¹, which was about 10-fold more sensitive than that of the conventional PCR method. Moreover, the RPA assay can be successfully applied to detect maize samples collected in the field. These results demonstrated that the established RPA assay is a rapid and efficient method to conduct simultaneous detection of MCMV and SCMV, which provides an alternative technology for MLN diagnosis.

INTRODUCTION

Maize chlorotic mottle virus (MCMV) belongs to the genus *Machlomovirus* of the family *Tombusviridae,* which was first reported in Peru in 1974. MCMV can be transmitted by mechanical inoculation and insect vectors (mainly thrips and beetles). Particularly, seeds are the most predominant way for long-distance spread of MCMV. The maize infected by MCMV showed chlorotic and mottle symptoms, and in severe cases, necrosis. However, when coinfection occurs with other maize-infecting potyvirids, such as sugarcane mosaic virus (SCMV), maize dwarf mosaic virus, or wheat streak mosaic virus, MCMV causes maize lethal necrosis (MLN) that results in serious yield losses. MLN, also known as corn lethal necrosis, was first reported in Kansas in 1978. The occurrence of MLN in China and several countries of Africa was caused by mixed infection of MCMV and SCMV, which resulted in severe yield reduction or even no grain harvest. For example, it was estimated that MLN could cause more than 2 billion USD of direct economic losses in China, which was equivalent to about 10 million tons of maize. Therefore, it is very important to establish a rapid and effective MLN diagnosis method for the sustainable development of maize production.

Several methods for MCMV detection have been reported, including reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), real-time TaqMan RT-PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP), and next-generation sequencing. However, these methods still face technical limitations. For example, the ELISA assay for virus detection depends on antibodies with high quality and validity. RT-PCR is time-consuming and limitedly sensitive, while real-time RT-PCR needs expensive laboratory equipment. Next-generation sequencing involves high cost and requires complex data analysis, which is mainly used to identify unknown viruses in plants. RT-LAMP needs higher temperature of reaction conditions that is difficult and impractical in the field.

With the development of molecular biology, a variety of thermostatic techniques for nucleic acid-based amplification have been developed. Among them, recombinase polymerase amplification (RPA) presents several advantages as a rapid and sensitive detection technology. RPA can be
completed by only one pair of primers to avoid the complicated primer design process.\textsuperscript{21,22} The RPA assay can be carried out in a temperature range of 37–42 °C in 30 min without any special thermal cycling equipment.\textsuperscript{23} RPA can also be improved by combining it with other assays, such as agarose gel electrophoresis,\textsuperscript{21} fluorescence-based RT-RPA assay,\textsuperscript{24} and RPA-lateral flow dipstick (RPA-LFD).\textsuperscript{25} In particular, agarose gel electrophoresis is the most common method for detection visualization. Recently, RPA has been used to detect diverse pathogens\textsuperscript{22,26} in which plant virus detection is developing rapidly.\textsuperscript{20,29–31} A previous study has reported that RPA is an efficient and accurate method for the detection of MCMV alone.\textsuperscript{32} However, there is no report on MLN diagnosis using the RPA method. Therefore, a fast and effective technology is necessary to be established for the simultaneous detection of MCMV and SCMV to provide another approach for the rapid diagnosis of MLN. Moreover, due to the short-amplification fragment of RPA, it is rarely used for simultaneous detection of two or more viruses in plants. In this study, we developed a simple and efficient RPA assay for simultaneous detection of MCMV and SCMV, which provides technical support for field diagnosis and prevalence risk estimation of MLN.

**RESULTS AND DISCUSSION**

**Reaction Time Optimization of RPA.** To optimize the RPA reaction time, the RPA assay was conducted for 10, 20, 30, 40, and 50 min, respectively. The results showed that two weak amplification bands with an expected size initially appeared for 10 min and they gradually became dense with more time as determined by the agarose gel electrophoresis analysis (Figure 1). In particular, the band density indicated that the yield of amplification products after 30 min of reaction was twice than that after 20 min of reaction, while no significant difference was observed after more than 30 min of reaction (Figure 1). Therefore, RPA assay was conducted for the optimized reaction time of 30 min in the subsequent analysis.

**Specificity of RPA.** To evaluate the specificity of RPA primers, cDNAs prepared from maize plants infected with rice black-streaked dwarf virus (RBSDV), maize yellow mosaic virus (MaYMV), or sorghum mosaic virus (SrMV) were used in this study. Results showed that two amplified fragments were detected in maize plants coinfected with MCMV and SCMV, but only one band was visualized in individually MCMV- or SCMV-infected maize plants (Figure 2). No amplification was detected in maize plants infected by other selected viruses (Figure 2). These results demonstrated that the RPA primers were specific to simultaneously detect MCMV and SCMV, thereby no crossreaction with other three maize-infecting viruses was detected.

**Sensitivity of RPA.** The primer sensitivity of RPA was analyzed using 10-fold serial dilutions of the constructed plasmids. The results revealed that the detection limit of the RPA assay was 1 × 10^5 copies μL^{-1} (Figure 3A), while that of PCR was 1 × 10^3 copies μL^{-1} (Figure 3B). These results demonstrated that the established RPA assay was 10-fold more sensitive than the PCR method.

**RPA Reliability for Field Samples.** To evaluate the reliability of the established RPA assay for simultaneous detection of MCMV and SCMV in sampled maize plants, RPA and PCR assays were independently performed to detect seven samples showing MLN or other virus-like symptoms, respectively. The RPA results showed that MCMV and SCMV were simultaneously detected in three of the assayed maize samples while two samples were found to be infected by either MCMV or SCMV alone (Figure 4A). In addition, neither MCMV nor SCMV was detected in other two samples. PCR assay was also performed using the same seven samples to confirm the detection results obtained by RPA assays (Figure 4B).

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**Figure 1.** Optimization of the RPA reaction time. Lane M, Trans2K Plus II DNA marker and lanes 1−5, DNA products amplified by the RPA reaction for 10, 20, 30, 40, and 50 min, respectively.

**Figure 2.** Specificity analysis of RPA for simultaneous detection of MCMV and SCMV. Lane M, Trans2K Plus II DNA marker; lane 1, maize plants coinfected with MCMV and SCMV; lane 2, maize plants infected with SCMV; lane 3, maize plants infected with MCMV; lane 4, maize plants infected with RBSDV; lane 5, maize plants infected with MaYMV; lane 6, maize plants infected with SrMV; lane 7, healthy maize plants; and lane 8, distilled water control.

**Figure 3.** Sensitivity of (A) RPA and (B) PCR for simultaneous detection of MCMV and SCMV. Lane M, Trans2K Plus II DNA marker; lanes 1−9, 10-fold diluted plasmids (10^9−10^1 copies μL^{-1}); and lane 10, distilled water control.
will reduce their sensitivity. Therefore, agarose gel electrophoresis is still suggested as the most common visualization method. In this study, the RT-RPA assay was established for rapid, sensitive, and simultaneous detection of MCMV and SCMV in maize plants without expensive laboratory equipment. Compared with other current detection techniques, the established RPA assay offered several advantages. The RPA method is time-saving and could be completed within 30 min. Moreover, positive results were obtained when the template plasmids were diluted to $1 \times 10^5$ copies $\mu$L$^{-1}$ with the RPA method, indicating its high sensitivity. Third, the RPA technology did not require a thermal cycle and was suitable for noninstrumented nucleic acid amplification platforms. In conclusion, it is a rapid and efficient method for simultaneous detection of MCMV and SCMV, which contributes to the diagnosis of MLN, especially in laboratories with limited resources. To our best knowledge, this study is the first report on utilization of RPA for the simultaneous detection of MCMV and SCMV in maize.

## EXPERIMENTAL SECTION

### Materials

The sources of MCMV and SCMV were generously provided by Prof. Zaiying Fan (China Agricultural University, Beijing, China). Both viruses were propagated on maize inbred line B73 in a separate growth chamber (28 °C 16 h light and 22 °C 8 h night cycles). Maize leaf tissues infected with MCMV or SCMV were homogenized with 0.01 M phosphate buffer solution (0.01 M KH$_2$PO$_4$:0.01 M Na$_2$HPO$_4$ = 49:51 (v/v), pH 7.0) at a ratio of 1:10 (w/v) to prepare crude extracts. The crude extracts were rub-inoculated on the first true leaves of one-week-old B73 maize seedlings, and the systemically infected leaves were sampled at 10 days after inoculation. The maize plants collected in the fields were confirmed to be infected with RBSDV, MaYMV, or SrMV, and the cDNA templates of RBSDV-, MaYMV-, and SrMV-infected maize plants were generously provided by Dr. Mingjun Li (Southwest University, Chongqing, China).

### Primer Design

The design of RPA primers was consistent with the description on RPA manufacturers’ websites. In order to facilitate the formation of a complex between recombinases and primers, the length of the RPA primer is generally designed at 30–35 nt. The RPA primers should be free from long tracks of guanine residues at the 5’ end, palindromes and secondary structures, direct or inverted repeats. In order to obtain robust results, RPA primers should be used to amplify the shorter fragments, which are usually more than 70 bp and less than 500 bp. MCMV genomic sequences (accession numbers: MH645620.1, X14736.2, EU358605.1, MK491605.1, MN756483.1, MK684213.1, MK684203.1, MK684201.1, MF467375.1, and MH205605.1) and SCMV genomic sequences (accession numbers: KT736022.1, KR611106.1, AJ542184.1, AJ310109.1, AJ271085.1, AJ310111.1, AJ310106.1, AJ421461.1, AJ421463.1, and AJ310107.1) were obtained from the GenBank database. According to the design principle of RPA primers, the specific RPA primer pairs MCMV-F/MCMV-R and SCMV-F/SCMV-R for simultaneous detection of MCMV and SCMV were designed using Primer Premier 5 (PREMIER Biosoft International, Palo Alto, USA) and based on utilization of RPA for the simultaneous detection of MCMV and SCMV in maize.
Table 1. Primers Used in This Study

| virus  | primer | sequence (5'→3')               | fragment size |
|--------|--------|--------------------------------|---------------|
| MCMV   | MCMV-F | CTTCCGATTACATGTTATCAATACCCTCG  | 206 bp        |
|        | MCMV-R | CACAGTGAATGCTCTGGGAATACATTGT   |               |
| SCMV   | SCMV-F | CAAGATATATCAACACATAGGAACATAG   | 388 bp        |
|        | SCMV-R | GAAGTCATTCTATAGAAATCGAAGCATAC  |               |

on the abovementioned conserved regions of MCMV and SCVM genome sequences, respectively. The primers are listed in Table 1. The amplicons of MCMV and SCMV were expected to be 206 and 388 bp in length, respectively.

**Total RNA Extraction and cDNA Synthesis.** For total RNA extraction, maize leaf samples either infected by MCMV or SCMV or MCMV and SCMV in laboratory or collected from maize fields were ground to powder in liquid nitrogen. About 100 mg of powder samples were transferred into 2 mL RNase-free centrifuge tubes, and then 1 mL of TRNzol Universal reagent (Tiangen Biotech, Beijing, China) was added and mixed well. The homogenate was placed for 5 min at room temperature, and 200 μL of chloroform was added and agitated at 150 rpm for 15 s. Next, the homogenate was placed for 5 min at room temperature and then centrifuged at 13,000 g for 15 min at 4 °C. After collecting the supernatant, an equal volume of isopropanol was added. Then the well-mixed solution was placed for 10 min at room temperature. Subsequently, the solution was centrifuged at 13,000 g for 10 min at 4 °C. The supernatant was discarded, and the pellets were rinsed using 1 mL of 75% ethanol. After ethanol was removed, the pellets were air-dried at room temperature. The total RNA was dissolved in 20 μL of RNase-free H2O and quantified using a microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

The first-strand cDNA was synthesized using HiScript III 1st Strand cDNA synthesis kit (+gDNA wiper; Vazyme, Nanjing, China) according to the instructions of the manufacturer. The reaction mixture contained 3 μL of total RNA (about 2 ng), and 5 μL of RNase-free ddH2O, which was incubated at 70 °C for 5 min and then placed on ice for 5 min. Then, 2 μL of 5X gDNA wiper Mix was added and mixed. The solution was incubated at 42 °C for 2 min. Subsequently, 4 μL of RNase-free ddH2O, 2 μL of 10X RT Mix, 2 μL of HiScript III Enzyme Mix, 1 μL of Oligo (dT) 20 VN, and 1 μL of random hexamers were added and mixed slowly, and the solution was incubated at 25 °C for 5 min, 37 °C for 45 min, and 85 °C for 5 s. The obtained cDNAs were kept at −20 °C until future analysis.

**Conventional PCR Detection.** The PCR system was composed of 1 μL of cDNAs or plasmids, 12.5 μL of Premix Taq (TaKaRa, Dalian, China), 0.5 μL (10 μM) of each primer of two primer pairs, and 9.5 μL of ddH2O. The PCR conditions used for MCMV and SCMV detection were 94 °C for 3 min, 32 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final step at 72 °C for 10 min. The obtained amplicons were visualized on 1.5% agarose gels through electrophoresis and ethidium bromide staining.

**RPA Assay for MCMV and SCMV Detection.** The prepared cDNAs or plasmids were used as the template. The RPA reaction mixture consisted of 29.5 μL of rehybridization buffer, 7.4 μL of nuclease-free water, 2.5 μL of magnesium acetate (280 mM), 2.4 μL (10 μM) of each primer, and 1 μL of cDNAs or plasmids. The RPA amplification product was purified using a SanPrep column PCR product purification kit (Sangon Biotech, Shanghai, China) and visualized on 1.5% agarose gels by electrophoresis.

**Reaction Time Optimization of RPA.** The cDNAs from maize leaf samples infected with MCMV and SCMV were used, and the reaction was carried out for 10, 20, 30, 40, and 50 min, respectively. The RPA assays were performed as described above.

**Specificity of RPA.** To determine the specificity for simultaneous detection of MCMV and SCMV using the RPA assay, maize plants infected with MCMV, SCVM, MaYMV, RBDSV, and SrMV were compared. The negative control was maize leaves inoculated with phosphate buffer. The fragments obtained by RPA reactions were cloned into pEASY-T1 vector (TransGen Biotech, Beijing, China). The constructed pEASY-T1-MCMV and pEASY-T1-SCMV plasmids were confirmed by sequencing (Sangon Biotech, Shanghai, China), and the sequences had more than 98% similarity with the known sequences of MCMV and SCMV, respectively.

**Sensitivity of RPA.** To compare the primer sensitivity of the RPA assay, the plasmids were 10-fold serial diluted. Briefly, the DNA concentrations of the constructed plasmids were initially 1.22 × 10^11 copies μL^−1 and 3.88 × 10^10 copies μL^−1, which were diluted to 1 × 10^9, 1 × 10^8, 1 × 10^7, 1 × 10^6, 1 × 10^5, 1 × 10^4, and 1 × 10^3 copies μL^−1, respectively. The minimum detection limits of RPA and PCR assays were determined using the diluted plasmid as the template. The RPA and PCR assays were performed as described above.

**RPA Reliability for Field Samples.** The reliability of the RPA assay was validated on maize leaf samples with MLN or other viral symptoms, which were collected from the Yuxi region of Yunnan Province, China. The field samples were analyzed by RPA and PCR assays.

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