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Rapid detection of *Cucumber green mottle mosaic virus* in watermelon through a recombinase polymerase amplification assay

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ABSTRACT

Cucumber green mottle mosaic virus (CGMMV), a member of the genus *Tobamovirus*, is an important quarantine plant virus worldwide, and often causes seriously damages to productions of watermelon, melon, cucumber and other cucurbit crops. In this study, we developed a novel isothermal recombinase polymerase amplification (RPA) technique for detection of CGMMV in watermelon samples. A pair of CGMMV specific RPA primers was prepared based on the conserved CGMMV coat protein gene sequences. The result showed that this RPA detection method can be performed at 38 °C and completed in about 30 min, and there was no cross-reactivity with other common cucurbit viruses. Sensitivity assay showed that this RPA method was more sensitive compared with the regular RT-PCR. Using field-collected watermelon tissue samples, we have demonstrated that this newly developed method is rapid, easy to use and reliable for CGMMV detection, especially in resource-limited laboratories or on-site facilities.

Cucumber green mottle mosaic virus (CGMMV), a member of the genus *Tobamovirus*, is an important viral pathogen infecting cucurbit crops worldwide (Adams et al., 2009; Dombrovsky et al., 2017; Li et al., 2017). CGMMV-infected plants often show leaf mottling or mosaic, fruit distortion and plant stunting, resulting in significant yield reductions and economic losses (Ali et al., 2010; Okkyung et al., 2010; Raychaudhuri and Varma, 2010). CGMMV-infected watermelon plants also show fruit internal flesh sponginess, rotting, and discoloration known as 'blood flesh' (Boubourakas et al., 2010). CGMMV was first reported in the United Kingdom in 1935 and, in the past 10 years, CGMMV spread quickly and has become a major threat to cucurbit industries worldwide (Ainsworth, 1935; Dombrovsky et al., 2017). China is the largest watermelon producer in the world and produces about 80% of the global watermelon in 2016 (http://faostat.fao.org/). CGMMV was first reported in China in 2006 and is now considered as the greatest threat to watermelon production in China (Chen et al., 2006). To prevent further spread of CGMMV in China, the Chinese government had declared the virus as a quarantine pest in the country in 2007 (Chen et al., 2008). Therefore, it is pivotal to establish an effective and accurate detection method for CGMMV for sustainable watermelon production in China and worldwide.

Although CGMMV has several modes of transmission, infected seeds and mechanical wounding are the main transmission routes (Lee et al., 1990; Liu et al., 2014). Several detection methods, including immunoelectron microscopy, enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR and loop-mediated isothermal amplification (LAMP), are currently used to detect CGMMV in plant tissues (Chen et al., 2008; Shang et al., 2011; Shargil et al., 2015). These methods are, however, time consuming and can not be used to detect CGMMV in field collected samples on-site. Recombinase polymerase amplification (RPA) is an isothermal gene amplification technique and has been considered as a rapid, sensitive, and cost-effective molecular diagnostic method (Piepenburg et al., 2006). RPA is a simple technique and does not require a thermal cycler. RPA reaction operates at a constant temperature ranging from 37 to 42 °C (Si et al., 2017). This method has now been used to detect many important animal-infecting viruses, bacteria and fungi (Ahmed et al., 2014; Jaroenram and Owens, 2014; Lutz et al., 2010). Detections of plant viruses using RPA are currently limited to *Little Cherry virus* 2 (LChV2) (Mekuria et al., 2014), *Yam mosaic virus* (YMV) (Silva et al., 2015), *Plum pox virus* (PPV) (Zhang et al., 2014), *Bean golden yellow mosaic virus* (BGYMV), *Tomato mottle virus* (ToMoV), *Tomato yellow leaf curl virus* (TYLCV) (Londoño et al., 2016), *Rose rosette virus* (RRV) (Babu et al., 2017, 2016), and *Banana bunchy top virus* (BBTV) (Kapoor et al., 2017). In this study, we have developed a reliable RPA assay for CGMMV detection.

CGMMV lnxg strain (CGMMV-lnxg; GenBank Accession No. KY040049.1) was identified and maintained in our laboratory, and was

Abbreviations: CGMMV, Cucumber green mottle mosaic virus; RPA, recombinase polymerase amplification

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used as the positive control for RT-PCR and RT-RPA assays. *Watermelon mosaic virus* (WMV), *Squash mosaic virus* (SqMV), *Zucchini yellow mosaic virus* (ZYMV) were kindly provided by Dr. Qinsheng Gu (Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Zhengzhou, China). Total RNA was extracted from individual test samples using TRIzol Reagent (TaKaRa, Dalian, China) followed by first strand cDNA synthesis using the GoScript™ Reverse Transcriptase kit (Promega, Madison, WI, USA). CGMMV specific primers for RT-PCR and RT-RPA were designed according to the conserved CGMMV coat protein (CP) gene sequences available at the GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences of the primers are: forward primer, 5'- ACTTATTGCGTTTAGTGCTTCTTATGTTCC-3' and reverse primer, 5'- CGACAGACGAGGGTAACGCAGACAGGGACT-3'. The length of resulting amplification product was 140 base pair (bp).

The initial RT-RPA assay was performed at 38 °C using a TwistAmp Basic RT Kit (TwistDX, Cambridge, UK). Each 50 μL reaction contained 29.5 μL of a rehydration buffer, 2.4 μL of each CGMMV primer (10 μM), 2.5 μL of magnesium acetate (280 mM), 12.2 μL of nuclease-free water, and 1.0 μL of cDNA. To optimize the RPA reaction time, the reaction using CGMMV cDNA was allowed to continue for 10, 20, 30 and 40 min, respectively. The resulting amplicons were purified using the SanPrep Column PCR Product Purification kit (Sangon Biotech, Shanghai, China) and visualized in 2.0% agarose gels through electrophoresis and ethidium bromide staining. Results showed that only one clear DNA band of ~140 bp long was visualized in the gels under the UV light (Fig. 1). Analysis through Densitometry revealed that the concentration of DNA band from 30 min reaction was almost two fold higher than that from 20 min reaction, whereas no significant difference was observed between the products from the 30 or the 40 min reaction.

Although DNA products directly from RPA could be visualized in agarose gels through electrophoresis, better results could be achieved if the products were purified prior to gel loading, due mainly to the presence of proteins in the RPA reaction mixtures. It was reported that the presence of enzymes in the reaction mixtures could interact with amplicons and inhibit the migration of DNA in gels (Kersting et al., 2014; Piepenburg et al., 2006). Because usage of PCR purification kits increased the cost of RPA-based assays, we decided to try other methods for RPA replicon purifications. These methods included applications of a SanPrep Column PCR Product Purification kit (Sangon Biotech, Shanghai, China), incubation of DNA products with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, V: V: V), incubation of DNA products at 65 or 95 °C for 10 min, incubation of DNA products with loading buffer containing 10% sodium dodecyl sulfate (SDS), or mixing DNA products with a 15% formamide solution as previously described (Londoño et al., 2016). After electrophoresis in 2% agarose gels, the phenol/chloroform/isoamyl alcohol- or heat-treated RPA DNA products showed stronger DNA bands in the gels compared with that

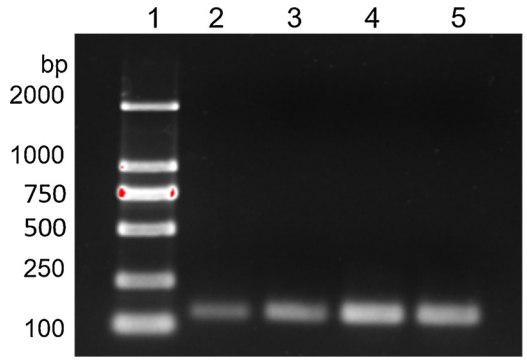

Fig. 1. Optimization of RPA reaction time. Lane 1, *Trans*2K DNA marker; lanes 2–5, DNA products amplified through 10 min, 20 min, 30 min or 40 min RPA reaction.

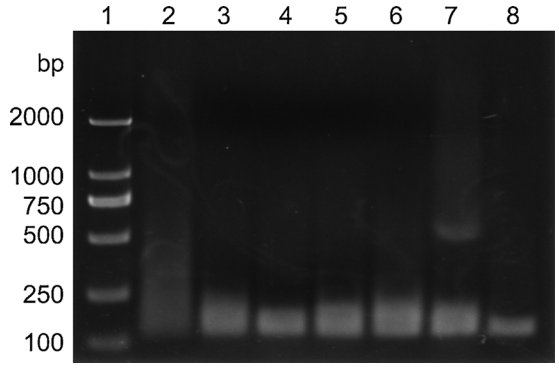

Fig. 2. Effects of different DNA extraction methods on visualizations of RPA amplified products. Lane 1, *Trans*2K DNA marker; lane 2, untreated RPA DNA products; lane 3, RPA DNA products mixed with the loading buffer containing 15% formamide; lane 4, RPA DNA products pre-treated with an equal volume of Phenol/chloroform/isoamyl alcohol; lane 5, RPA DNA products pre-treated at 65 °C for 10 min; lane 6, RPA DNA products pre-treated at 95 °C for 10 min; lane 7, RPA DNA products pre-treated with the loading buffer containing 10% SDS; lane 8, RPA DNA products purified using the SanPrep Column PCR Product Purification kit.

purified using the PCR product purification kit. The SDS-treated DNA products gave two DNA bands in the gels and a smear-like pattern above the top band. No clear DNA band was observed in the lanes loaded with the formamide-treated RPA DNA products (Fig. 2). These results indicated that, although purification of DNA products using the PCR Product Purification kit was expensive and time consuming compared with other methods tested in this study, it did give a sharper DNA band in the gel. Heat and Phenol/chloroform/isoamyl alcohol treatments also gave acceptable assay results. Considering these two methods are more cost effective and time-saving, we recommend these two methods for CGMMV detection in resource-limited laboratories and/or in on-site facilities. Researchers in these laboratories or facilities can choose the most appropriate method according to their need.

To evaluate the specificity of the RPA assay described above, we tested the CGMMV-, WMV-, SqMV- or ZYMV-infected watermelon leaf tissues. Tissues from buffer-inoculated healthy watermelon plants were used as negative controls in this study. Results showed that a strong and unique RPA product band was obtained from the CGMMV-infected watermelon leaf tissues. No RPA product band was obtained from the samples harvested from the WMV-, SqMV-, ZYMV-, or buffer-inoculated watermelon plants (Fig. 3). This finding indicated clearly that the RT-RPA method developed in this study for CGMMV detection is specific

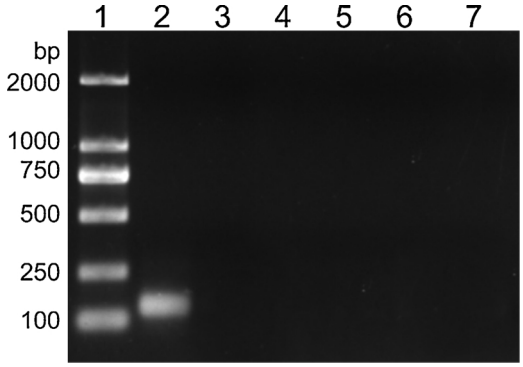

Fig. 3. Specificity of RPA assay in CGMMV detection. Lane 1, *Trans*2K DNA Marker; lane 2, RPA DNA products from total RNA extracted from CGMMV-infected watermelon leaf tissues; lane 3, RPA using distilled water as a negative control; lane 4, RPA using total RNA from healthy watermelon leaf tissues; lane 5, RPA using total RNA from WMV-infected watermelon leaf tissues; lane 6, RPA using total RNA from ZYMV-infected watermelon leaf tissues; lane 7, RPA using total RNA from SqMV-infected watermelon leaf tissues.

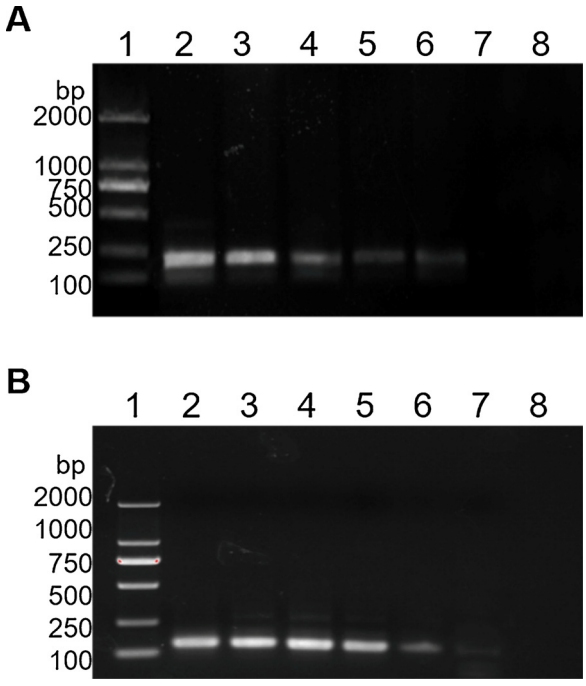

Fig. 4. Sensitivity of RT-PCR (A) and RT-RPA (B) in CGMMV detection. Lane1, *Trans*2K DNA marker; lanes 2–8, RT-PCR or RT-RPA using 10-fold diluted total RNA isolated from CGMMV-infected watermelon leaf tissues.

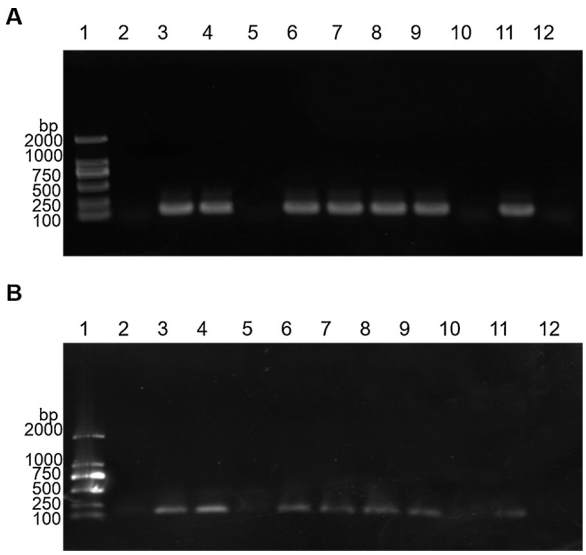

Fig. 5. Detection of CGMMV in field-collected watermelon leaf samples using RT-PCR (A) or RT-RPA (B). Lane 1, *Trans*2K DNA marker; lane 2, assays using total RNA from a healthy watermelon leaf sample; lanes 3–11, assays using total RNA from nine field-collected watermelon samples; lane 12, assay using distilled water as a negative control.

and reliable.

To further determine the sensitivity of the RT-RPA assay, we ten-fold (1.0 to 1.0×10^{-7}) diluted the total RNA (~1.3 μg/μL in the original sample) extracted from the CGMMV-infected watermelon leaf tissues and analyzed for CGMMV using RT-PCR and RT-RPA, respectively. Results showed that CGMMV was detected in the total RNA diluted up to 1.0×10^{-6} μg by RT-RPA, more sensitive than that detected by RT-PCR (1.0×10^{-5} μg) (Fig. 4). This result indicated that the RT-RPA assay was more sensitive than RT-PCR, based on the DNA band intensity in gels.

To determine the reliability of RT-RPA in detection of CGMMV in field-collected samples, nine watermelon leaf samples with or without virus-like symptoms were analyzed by RT-RPA and RT-PCR, respectively. Results revealed that CGMMV was detected by both RT-RPA and RT-PCR in seven assayed watermelon samples (Fig. 5), indicating that the RT-RPA assay can be reliably used to detect CGMMV infection in field-collected samples.

Multiple diagnostic techniques, including RT-PCR and several serological assays, have been used to detect viruses in various plant samples (Demeke and Adams, 1992). Although test results obtained through RT-PCR are reliable, this technique is too costly and time-consuming for people who have limited laboratory equipment, including thermal cycler, and conduct mostly field surveys. In addition, regular RT-PCR requires high quality polymerase and nucleic acid samples (Henson and French, 1993; Korschineck et al., 1991). To overcome these difficulties, we decided to develop an easy to use and reliable isothermal nucleic acid amplification technology that does not require a thermal cycler, and can be conducted in a relatively short period of time, and in resource-limited on-site facilities. Furthermore, this assay should be tolerant to various amplification reaction inhibitors in the test samples (Nassuth et al., 2000). We consider that this isothermal amplification can also be useful for amplifications of host messenger RNAs or RNAs that are bound with protein(s).

Because RPA can be done in about 30 min, does not require expensive instruments, easy to operate, and can be performed at low temperature (38 °C) using only a recombinant enzyme and a polymerase, we recommend this technology for amplifications of nucleic acids from plants, viruses or other biological materials in future.

In conclusion, a user-friendly RPA technology has been developed for on-site detection of CGMMV in watermelon tissues. This technology is easy to operate and highly specific and sensitive. We consider that this RPA method is ideal for rapid and reliable detection of CGMMV in plant tissues in under-equipped laboratories or in on-site facilities.

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