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Differential identification of three species of *Curtovirus* using loop-mediated isothermal amplification

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Summary. – Rapid and sensitive detection methods for three species of *Curtovirus* were developed using a loop-mediated isothermal amplification (LAMP) technique. A universal primer set for detecting the three main species of *Curtovirus* at the same time, and three kinds of species-specific primer sets were designed and used for LAMP reactions. Results from the LAMP reactions were visualized both by color changes after adding SYBR Green I staining dye and by DNA laddering on agarose gel electrophoresis. The optimal conditions for the curtovirus LAMP reaction were confirmed at 60°C for the universal primers and at 62°C for the three species-specific primer sets. Amplification of curtoviruses by LAMP reaction was ten-fold more sensitive than that by polymerase chain reaction. Primers designed for curtovirus detection in this study did not anneal to or amplify DNA from other DNA or RNA viruses (tomato yellow leaf curl virus, tomato spotted wilt virus, and potato virus Y). Taken together, the primer sets and reaction conditions developed in this study show that the LAMP technique could be a useful tool to detect the three species of *Curtovirus* simultaneously and distinguish them in the laboratory and the field.

Keywords: beet curly top virus; Curtovirus; detection; Geminivirus; loop-mediated isothermal amplification

Introduction

Curtovirus, a genus of the family *Geminiviridae*, has single-stranded circular DNA genome encoding several genes in opposite directions encapsidated by a twin icosahedral (geminate) capsid structure, which can be transmitted by leafhoppers (Stanley *et al*., 1986). Beet curly top virus (BCTV), a member of the genus *Curtovirus,* causes curly top disease (CTD) in more than 300 plant species from 44 different families (Bennett, 1971). The typical symptoms of BCTV are stunting, leaf yellowing and curling, and vein swelling in several hosts, including the sugar beet (Stanley and Latham, 1992). Three distinct strains of BCTV have been studied, California/Logan, CFH, and Worland (Stenger, 1998). These strains have the same wide host range and are transmitted by the same leafhopper species, but share about 80% nucleotide sequence identity and show differential pathogenicity in sugar beet plants (Stanley *et al*., 1986; Stenger and Ostrow, 1996; Stenger, 1998; Soto and Gilbertson, 2003). Stenger studied differences among the three main BCTV strains and reported taxonomic and nomenclatural problems with the three strains (Stenger and Ostrow, 1996; Stenger, 1998). Because of their differential pathogenicity in plants and the more divergent sequences of nucleotides in complementary sense genes and the intergenic region compared to those of the virion sense, they became three separate species and were renamed (Stenger *et al*., 1998, 1990; Fauquet and Stanley, 2003; Soto and Gilbertson, 2003). The California/Logan strain retained its name as BCTV, the CFH strain became beet severe curly top virus (BSCTV), and the Worland strain

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Abbreviations: BCTV = beet curly top virus; BMCTV = beet mild curly top virus; BSCTV = beet severe curly top virus; LAMP = loop-mediated isothermal amplification

was designated beet mild curly top virus (BMCTV) (Stenger, 1998; Soto and Gilbertson, 2003).

The number of reported curtoviruses has increased continuously; therefore, methods to detect and distinguish curtoviruses are required for further studies. Even though some studies on curtoviruses in the field have dealt with PCR primer sets for distinguishing the three main species of *Curtovirus* (BCTV, BSCTV, and BMCTV) (Strausbaugh *et al*., 2008; Chen *et al*., 2010), application of other methods for detecting and distinguishing curtoviruses has not yet been reported. Several methods based on nucleic acid amplification or antibody-antigen-specific interactions have been used to detect viruses from infected plants. Both methods have been used in complementary ways to diagnose viral diseases (Crowther, 1998). Although rapid, specific, and sensitive tools for detecting plant pathogens have been developed due to recent advances in molecular biotechnology, all of these techniques have some disadvantages such as high cost, multiple steps and possible false-negatives and positives (Miller and Martin, 1988; Weiss, 1995). Serological detection methods have been utilized for on-site detection, because these techniques do not demand long periods of time, multi-steps, complicated machines, or specialists for the experiments (Torrance and Jones, 1981; Weiss, 1995). For example, strips based on the antibody-antigen interaction have been used for testing plant viruses (Danks and Barker, 2008). However, they lack sensitivity, so PCR-based methods, which are 100-fold more sensitive than ELISA, have been used for more sensitive detection in the laboratory (Minsavage *et al*., 1994). In most cases, the disadvantages of one technique may be strengths of other methods and vice versa; thus, minimizing the number of disadvantages has been a perennial problem of detection methods.

In this study, we provide primer sets not only for detecting three species of *Curtovirus* simultaneously, but also for distinguishing among these three species of *Curtovirus*. We also optimized the LAMP reaction conditions using total genomic DNA isolated from virus-infected plants. We show that the LAMP reaction provides higher specificity and sensitivity of virus detection than PCR.

Materials and Methods

Virus species and plant inoculation. The three main *Curtovirus* species, namely BCTV (previously called the California/Logan strain), BSCTV (CFH strain), and BMCTV (Worland strain), were used in this study. Information on the infectious clones of these three species has been described previously (Stenger *et al*., 1990). The three kinds of clones were provided by Dr. Drake C. Stenger (Agricultural Research Service, United States Department of Agriculture, Parlier, CA, USA). Each clone was transferred to *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw procedure and incubated in YEP media at 28°C for 2 days. Two-week-old *Nicotiana benthamiana* and four-week-old *Arabidopsis*, which are model host plants, were pricked about 5–10 times on the apical meristem using fine needles, and inoculum was dropped onto the meristems to infect the plants (Elmer *et al*., 1988). Leaf samples were harvested 35 days after agro-inoculation. Virus agro-inoculations were conducted in a restricted area in the Plant Quarantine Technology Center, Department of Animal and Plant Health Research, Animal and Plant Quarantine Agency, Korea.

DNA extraction from infected plants. Total DNA was extracted from leaves of virus-infected *Nicotiana benthamiana* as previously described (Dellaporta *et al*., 1983).

LAMP primer design. Two inner primers and two outer primers are needed for the LAMP reactions, (Notomi *et al*., 2000); therefore, the LAMP primer sets used to detect the three virus species (BCTV [GenBank Acc. No. M24597.2], BSCTV [GenBank accession number U02311.1], and BMCTV [GenBank accession number U56975.1]) separately were designed based on the uploaded sequences of each virus from GenBank using PrimerExplorer version 4 (http://primerexplorer.jp/elamp4.0.0/index.html). The multiple sequence alignment ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) results of the nucleic acids of the three species were used as the input instead of each sequence to construct a universal primer set to detect the three virus species of *Curtovirus*.

Optimization of LAMP conditions. LAMP reactions were carried out in 20 µl reaction mixtures at 60, 62, and 65°C for 60 min, followed by heat inactivation at 80°C for 5 min. The reaction mixtures contained 0.8 µmol/l each of the inner primers of FIP and BIP, 0.2 µmol/l each of the outer primers of F3 and B3, 0.8 mmol/l of each dNTP, 1 mol/l betaine (Sigma-Aldrich, St. Louis, MO, USA), 4 mmol/l $MgSO_4$, 8 U *Bst* DNA polymerase large fragment (New England Biolabs, Beverly, MA, USA), 1× ThermoPol™ reaction buffer (10×, New England Biolabs), and the specified amount of the target viral DNA in a final volume of 20 µl. The results were analyzed by colorimetric changes and agarose gel electrophoresis. A color change to fluorescent green was detected in infected samples after adding 1 µl 10,000× SYBR® Green I Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA, USA). Every experiment was repeated at least three times.

LAMP specificity and sensitivity. LAMP primer specificity was evaluated using 100 ng of total DNA prepared from BSCTV- and tomato yellow leaf curl virus (TYLCV)-infected plants, and 100 ng cDNA synthesized from total RNA isolated from tomato spotted wilt virus (TSWV)- and potato virus *Y* (PVY)-infected plants as templates. Diluted DNA samples ($1–10^{-8}$) were used as LAMP and PCR templates to conduct the LAMP sensitivity assay, and to compare the results to those of PCR. PCR amplification was performed with 20 µl reaction mixtures comprising 0.5 µmol/l each of the F3 and B3 outer primers for LAMP, 375 µmol/l of each dNTP, Super *Taq* DNA polymerase (Rexgene Biotech Co., LTD, Seoul, Korea), and 1× the supplied reaction buffer (10×, Rexgene Biotech). PCR amplifications were carried out as follows: pre-denaturation at

94°C for 5 min, followed by 35 cycles each at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Each experiment was performed at least three times.

Results

Optimization of the reaction conditions for detecting Curtovirus

A universal primer set (Curto-F3, Curto-B3, Curto-FIP, and Curto-BIP), based on conserved sequences of three species, was used to detect the three species of *Curtovirus* (Fig. 1, Table 1). Reactions with this primer set were carried out for 60 min at 60, 62, and 65°C, respectively, using DNA samples isolated from BCTV-, BSCTV-, and BMCTV-infected *N. benthamiana* (Fig. 2) and *Arabidopsis* Col-0 at 35 days after inoculation. The most conclusive results were shown when the reactions were performed at 62°C for 60 min using the three templates prepared equally from each virus-infected plant, whereas colorimetric changes after adding SYBR Green I and bands such as the DNA ladder markers on the agarose gel appeared faintly at 60°C, and did not appear at 65°C (Fig. 3).

LAMP reactions for the three species of *Curtovirus* using the specific primer sets (Table 1) were performed with the virus-specific primer sets, and the three viral genomic DNAs were isolated from the three virus-infected plants at 60, 62, and 65°C individually after 1 hour. Laddering and color changes to green occurred only in reactions with samples from BCTV-infected plants at 60 and 62°C, but better results were obtained at 60°C (Fig. 4a and b). The optimal LAMP reaction conditions with the BMCTV-specific primer set (Table 1) were assessed under the same conditions. Tubes containing gDNA from BMCTV-infected tobacco produced the brightest green color after adding SYBR Green I, as well as the most intense DNA fragments on an agarose gel when the reactions were conducted at 60°C for 60 min (Fig. 4c and d). The same results were obtained for BMCTV-specific primers (Table 1, Fig. 4e and f). Following

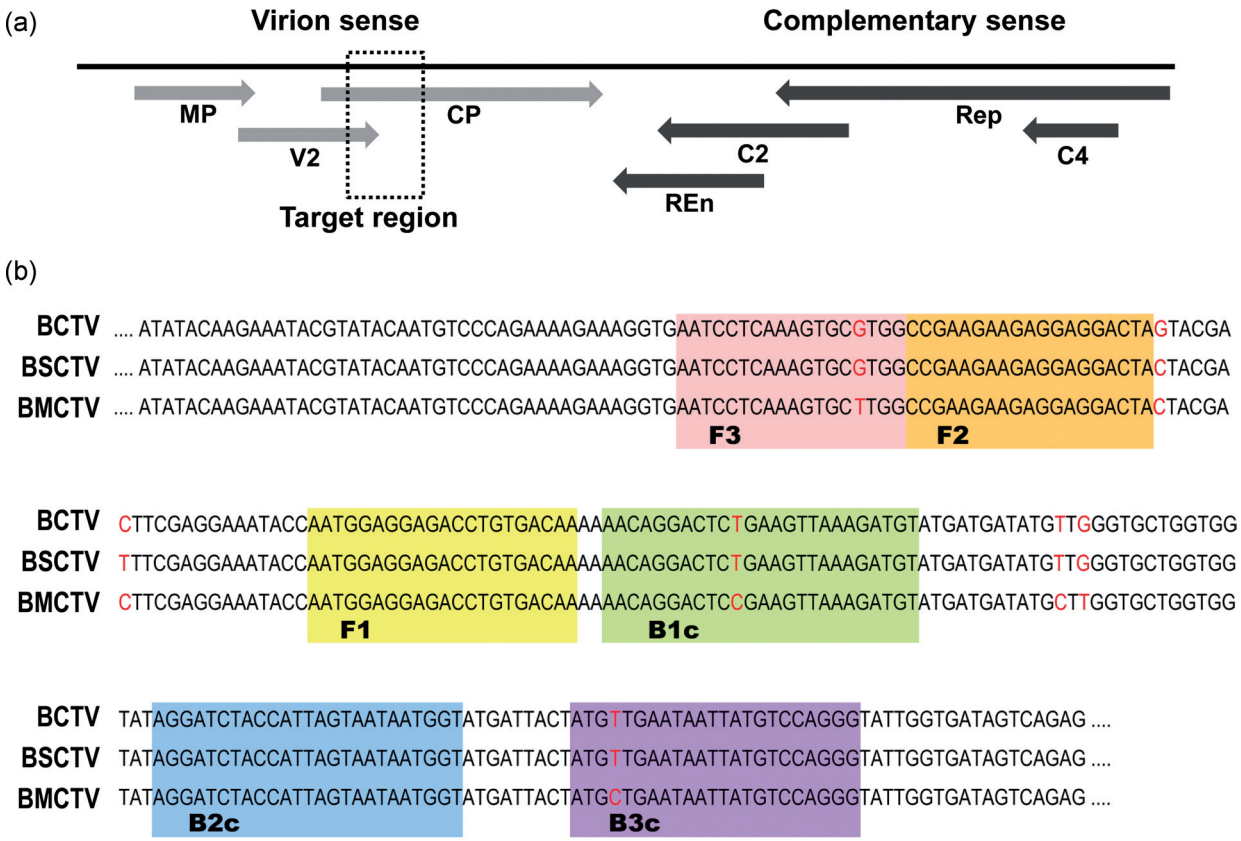

Fig. 1
Primers and their target sequences for LAMP of three species of *Curtovirus* (beet severe curly top virus [BSCTV], beet curly top virus [BCTV], and beet mild curly top virus [BMCTV])
(a) Full-length viral genome of three curtoviruses and the target region of the LAMP reaction (dotted box). (b) Multiple alignment of three curtovirus sequences near the target sequence and primer sets (colored boxes).

Table 1. Primer sets used for the LAMP detection of curtoviruses

Primer name	Sequence (5′– 3′)
Curtovirus (Universal)	
Curto-F3	AAT CCT CAA AGT GCK TGG
Curto-B3	CCC TGG ACA TAA TTA TTC ARC AT
Curto-FIP	TTG TCA CAG GTC TCC TCC ATT CCG AAG AAG AGG AGG ACT A
Curto-BIP	AAC AGG ACT CYG AAG TTA AAG ATG TAC CAT TAT TAC TAA TGG TAG ATC CT
BCTV	
BCTV-F3	GTT TGT CTG CCA CTC CTT
BCTV -B3	AGA GGG TGA TTC AAG AAC AG
BCTV -FIP	TCA TTG ATG ACG TAG ATC CCA CTT ATT GTG CTC CAA TAA GGT GT
BCTV -BIP	CGT TCT AGG GCT AAA ATC TAA GTG CGG AAG ACT ATG TGG GCT AG
BSCTV	
BSCTV-F3	CCT GCA TTT AAT GCC TCT G
BSCTV -B3	AAG TAC CAG CAA ACA ATT CC
BSCTV -FIP	GTG AAT TTC AGG TCG ATG GAA GAC TGC AGC ATC ATT AGC CG
BSCTV -BIP	CCA GTC GAT GTG ATC TCC GTA CTG CAA TAT TCA GGG AGC
BMCTV	
BMCTV-F3	ACT AAA ATC TAA ATG CCC TGA A
BMCTV-B3	ACC TCC ATT CCC ACA ATC
BMCTV-FIP	GCG CGG CCT TTT AGA TAT AAT AGT AGG TCC TAA TGA TCT AGC CCA
BMCTV-BIP	CGG CAT CCA CCC CGA AAT AAT CTT TCA CTC AAG TTC CAG A

these results, optimal conditions for the three primer sets were confirmed to be 60°C for 60 min for each set.

Comparison of the sensitivity of LAMP and PCR methods for Curtovirus

Serial dilutions of DNA extracted from BSCTV-infected leaf tissues of *N. benthamiana* and *Arabidopsis* Col-0 were used as a template to compare the amplification sensitivity between LAMP and PCR. The F3 and B3 primers (Fig. 1 and Table 1) were used for PCR instead of other primers to achieve the same targeted sequence position and length. The results showed that DNA at the 10^{-6} dilution was detectable by both the LAMP and PCR methods (Fig. 5). The LAMP reaction was more sensitive than PCR, a product was obtained for the more diluted template DNA (at 10^{-7} dilution), as shown by agarose gel electrophoresis (Fig. 5).

Specificity of LAMP detection

Specific amplification of the target sequence is one of the most important aspects of a detection method. We selected a representative DNA virus belonging to the family *Geminiviridae* (TYLCV), and two RNA viruses (TSWV and PVY) as targets for reactions using genomic DNA or cDNA synthesized from total RNA samples from BSCTV, TYLCV, TSWV, and PVY-infected leaf tissues in order to test the specificity of the LAMP reaction primer set, which was designed only for *Curtovirus* using the previously confirmed conditions for the universal primer set. After the amplification reactions, SYBR Green I was added, and the samples were loaded on a 1% agarose gel. No green color or DNA laddering was observed in the TYLCV, TSWV, or PVY samples, but the reaction with genomic DNA isolated from BSCTV-infected tissue was bright green in color (Fig. 6a) and showed laddering (Fig. 6b). These results indicate that the LAMP reaction with the universal primer set was specific for detecting curtoviruses.

Discussion

Although many powerful techniques modified from PCR and ELISA have been developed to detect viruses and other pathogens, reports are available about detecting curtoviruses using only classical methods such as PCR (Strausbaugh *et al.*, 2008; Chen *et al.*, 2010). Most studies on *Curtovirus* focused mainly on phenotypic and genotypic characterization, viral protein functions, comparisons of symptom severity and sequences among the three main species (originally strains), and plant-virus interactions (Stanley *et al.*, 1986; Stenger, 1998; Soto and Gilbertson, 2003; Hur *et al.*, 2008; Park *et al.*, 2010). However, new technologies are needed to detect and identify *Curtovirus* species, which will help protect economically important crops. Among the many detection techniques reported, LAMP technology can be used for on-site detection, producing specific and sensitive results from a small amount of sample. The LAMP assay is a time- and cost-saving, accurate method to amplify nucleic acids (Notomi *et al.*, 2000). Many studies have, therefore, introduced primer sets and LAMP methods for detecting plant DNA and RNA viruses

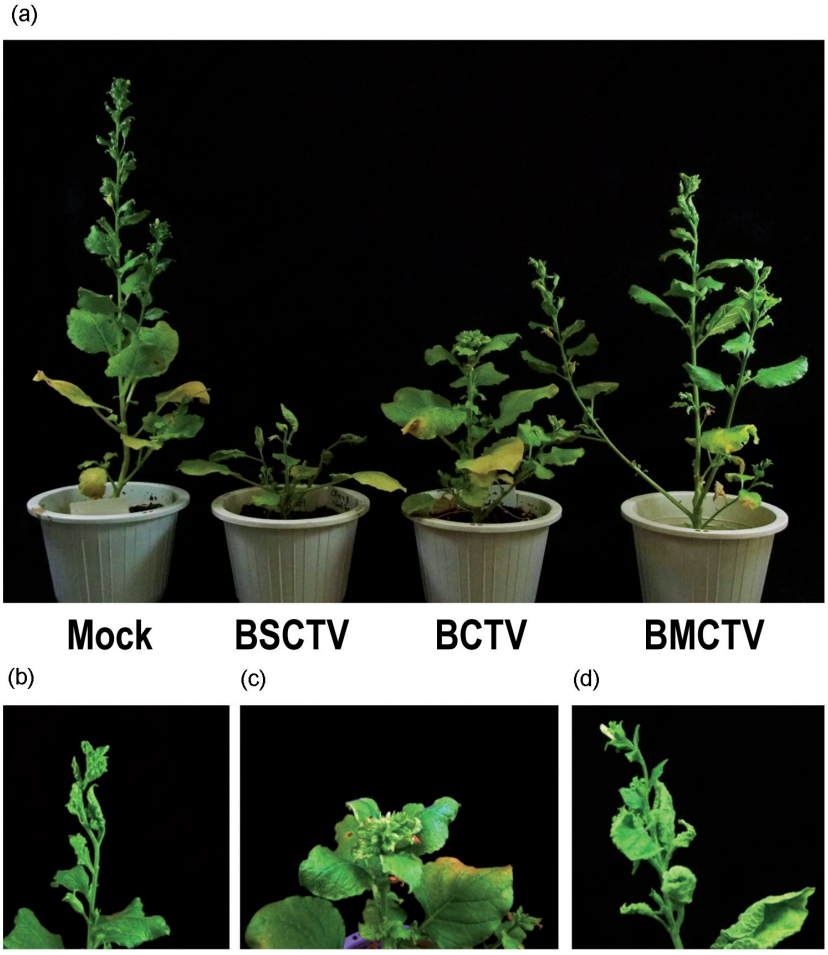

Fig. 2
Symptoms of infected *N. benthamiana* by (b) BSCTV, (c) BCTV, and (d) BMCTV at 35 days after inoculation and (a) a comparison of the plants

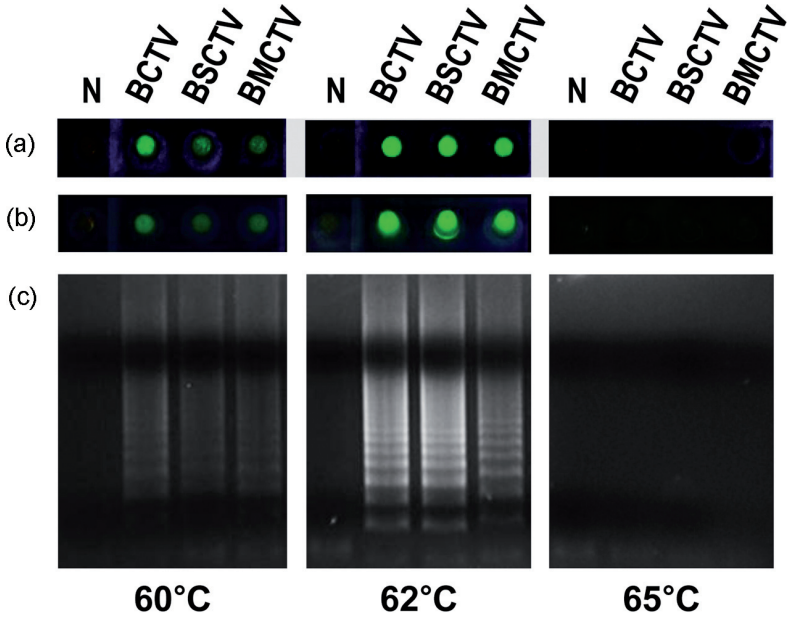

Fig. 3
LAMP of each DNA for detection of the three *Curtovirus* species at three different temperatures (60, 62, and 65°C) using universal primers that can be used to amplify all types of curtovirus DNA
Tubes for visual observations that have amplified products from (a) *N. benthamiana* and (b) *Arabidopsis* samples with SYBR Green I under UV light. (c) DNA gel staining with ethidium bromide. Lane N, no template control.

(Nie, 2005; Kuan *et al.*, 2010). Specifically, DNA viruses in the genus *Begomovirus*, such as TYLCV and squash leaf curl virus (SLCV), have been amplification targets using the LAMP method, and these viruses are detectable from infected plants and viruliferous whiteflies (*Bemisia tabaci*) (Fukuta *et al.*, 2003; Kuan *et al.*, 2010; Hsieh *et al.*, 2012).

This study investigated a universal LAMP primer set for detecting *Curtovirus* species simultaneously, and the three LAMP primer sets for distinguishing the three species of *Curtovirus* that proved useful in identifying and distinguishing the curtoviruses. The isothermal conditions required by LAMP could be met simply by using a water bath, so it is possible to instantly evaluate the virus detection results from many samples by just adding SYBR Green I staining dye to the amplified reaction solution without the gel electrophoresis step to visualize the reactions (Nie, 2005). These advantages will allow virologists, regardless of the background or work experience, to detect and diagnose *Curtovirus* correctly, easily, and rapidly in the field or laboratory. The primer sets and optimized LAMP reaction conditions developed in this study will be very helpful in detecting and distinguishing the *Curtovirus* species together and separately anywhere and anytime.

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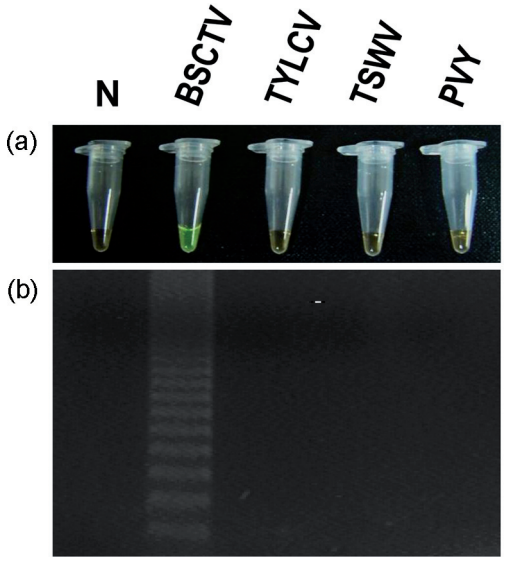

Fig. 6
LAMP specificity test using other viruses (beet severe curly top virus [BSCTV], tomato yellow leaf curl virus [TYLCV], tomato spotted wilt virus [TSWV], and potato virus Y [PVY]-infected plant samples)
(a) Tubes for visual observations that have amplified products with SYBR Green I under visible light. (b) DNA gel staining with ethidium bromide. Lane N, no template control.

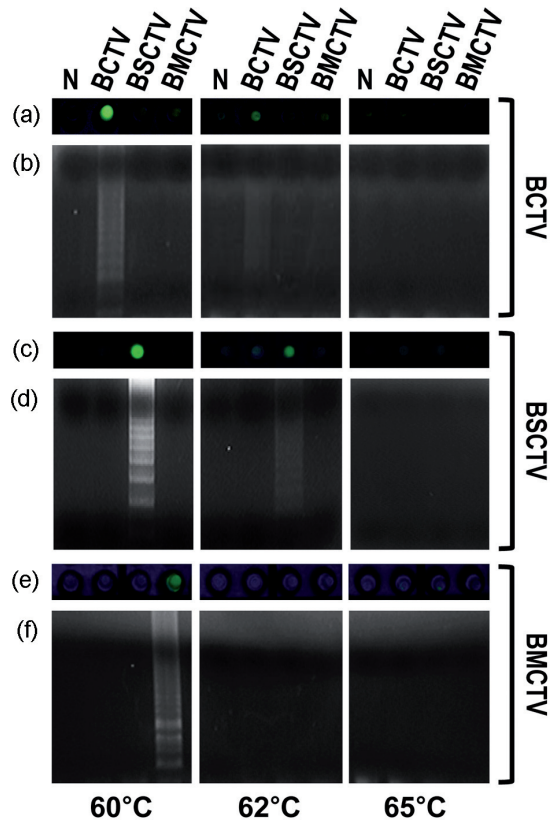

Fig. 4
LAMP at three different temperatures (60, 62, and 65°C) using three kinds of specific primers for each species (BCTV [(a) and (b)], BSCTV [(c) and (d)], and BMCTV [(e) and (f)])
(a), (c), and (e) Tubes for visual observation that have amplified products with SYBR Green I under UV light. (b), (d), and (f) DNA gel staining with ethidium bromide. Lane N, no template control.

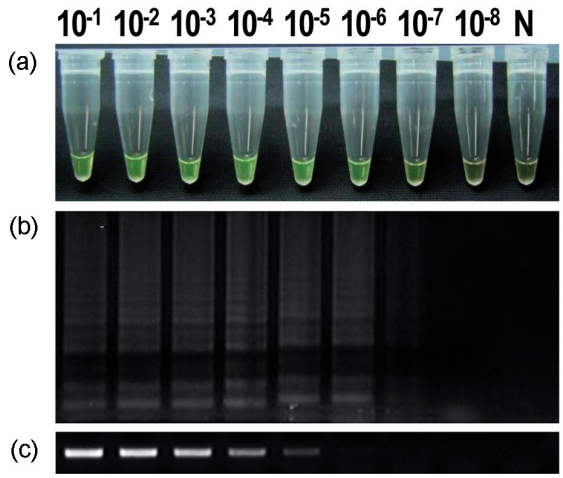

Fig. 5
The sensitivity of LAMP reaction for curtoviruses using universal primers
(a) Products from the LAMP reaction with SYBR Green I under visible light. DNA gel staining with ethidium bromide (b) LAMP products and (c) PCR products. Lane N, no template control.

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