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ARTICLE

Establishment of a loop-mediated isothermal amplification (LAMP) assay for the detection of phytoplasma-associated cassava witches' broom disease

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Abstract Cassava (*Manihot esculenta* Crantz) is one of the most important food crops in the tropics; however, bacterial phytopathogens pose a serious threat to its farming. Cassava Witches' Broom Disease (CWB) is caused by the infection of phytoplasma and is manifested as reduction in tuber yield and starch content at harvest of 10 and 30 %, respectively. Although polymerase-chain reaction provides the gold standard in diagnostics, this method requires significant investments in infrastructure and training. Here, we developed a loop-mediated isothermal amplification (LAMP) assay that allows specific detection of phytoplasma from field-collected samples. Three primer sets were designed, of which two detected phytoplasma DNA sequence encoding 16S rRNA (16S rDNA), the other detected cassava actin. Following a 1 h LAMP reaction at 63 °C, a positive reaction can be visualized by agarose gel electrophoresis, hydroxynaphthol blue color change, or the presence of a precipitate. In a pilot field study, the assay was able to rapidly distinguish between healthy and CWB-infected cassava. With further development, a LAMP for routine on-site screening of cassava crops can be envisioned.

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Keywords Cassava · Cassava witches' broom disease · Loop-mediated amplification · Loop-mediated isothermal amplification · Phytoplasma

Introduction

Cassava (*Manihot esculenta*) is one of the most important food crops in tropical Southeast Asia. However, the crop is under serious threat from bacterial phytopathogen-induced Cassava witches' broom disease (CWB). CWB impacts crop production levels, resulting in no harvest or significant reduction in yield (10–20 %) and starch content (20–30 %) (Alvarez et al. 2013). Typical symptoms include the presence of adventitious shoots and buds on infected plants, smaller and rougher leaves, a shorter internode, withered shoots, and black necrotic spots. Phytoplasma (*Candidatus phytoplasma*), the pathogen of witches' broom disease, are cell wall-lacking bacteria first described in the scientific literature in 1967 (Doi et al. 1967). Phytoplasma reside in the phloem of plants and spread through the saliva of leafhopper, planthopper, or insects belonging to *Cicadellidae, Cixiidae, Psyllidae, Delphacidae*, and *Derbidae*. Transmitted by sap-sucking insects, the bacteria cause more than 700 diseases in 300 plant species belonging to 38 different families, including crops, vegetables, fruit trees and ornamental plants, plant timber, and shade trees (IRPCM 2004; Weintraub and Beanland 2006).

Assays employing polymerase-chain reaction (PCR) are effective tools for phytoplasma identification and classification (Mondal and Shanmugam, 2013). PCR combined with restriction fragment length polymorphism (RFLP) analysis, known as PCR–RFLP, was applied to analyze more than 60 samples of cassava Witches' broom disease collected from Brazil and obtain phylogeny based on 16S

rDNA sequences (Flôres et al. 2013). A similar approach was applied to identify the phytoplasma groups causing a 2010 outbreak of Witches' broom disease in Vietnam where more than 60,000 hectares of cassava were infected (Alvarez et al. 2013). Other techniques, such as quantitative real-time PCR, variations of conventional PCR, and microarrays have also been developed to identify phytoplasma by 16S rDNA sequences (Parmessur et al. 2002; Hadidi et al. 2004; Torres et al. 2005). Major drawbacks of these methods include significant investments in infrastructure and training as well as a requirement for a relative high abundance of phytoplasma for detection, rending early identification of infection difficult (Razin et al. 1998).

Loop-mediated isothermal amplification (LAMP) is a method to amplify nucleic acids which rely on the DNA strand displacement activity of *Bst* DNA polymerase and four different primers that recognize six independent distinct regions of a target sequence (Notomi et al. 2000). LAMP is considered superior to PCR and microarray-based methods due to its cost-effectiveness, high specificity, better sensitivity, and convenient procedure (conducted at constant temperature without the need for an expensive thermal cycler) and evaluation (Notomi et al. 2000). LAMP is approximately 10–100-times more sensitive than PCR and can amplify the original amount 10^9–10^{10} times in 45–60 min, significantly faster than PCR (Nagamine et al. 2002; Li et al. 2007; Le et al. 2010; Bhat et al. 2013). When two loop primers are used, the sensitivity of the reaction is typically increased tenfold and the reaction time reduced to 30 min (Li and Ling, 2014). LAMP amplicons can be easily visualized by color indicators, the turbidity of magnesium pyrophosphate formed during the reaction (precipitate) or by agarose gel electrophoresis (Goto et al. 2009; Le et al. 2012). LAMP assays have been successfully used to detect phytoplasma infecting papaya, potatoes, coconut, periwinkle, and some insect hosts (Tomlinson et al. 2010; Bekele et al. 2011; Ravindran et al. 2012), suggesting that it may prove useful for early detection of phytoplasma-associated cassava witches' broom disease (CWB). In this report, we therefore attempted to establish a LAMP assay for rapid detection of phytoplasma-associated CWB.

Materials and methods

Cassava sample collection

Cassava (*Manihot esculenta* Crantz) field samples, healthy and CWB-infected (based on visual symptoms), were collected from our experimental station in Dong Nai province (Vietnam). The sample collections did not require any permission and no endangered or protected species were involved. Disease-free cassava KM 94 cultivar was from an in vitro collection maintained at the Agricultural Genetics Institute (Vietnam).

LAMP primer design

Two primer sets targeting 16S rDNA sequence of phytoplasma were designed using PrimerExplorer V4 (available at http://primerexplorer.jp/e) and LAMP designer software (Primer Biosoft, USA), respectively. A primer set targeting the cassava actin gene sequence (cassava4.1_033108m.g) was also designed for use to gauge the quality of isolated DNA (internal control). All primers (Table 1) were ordered from Macrogen Inc. (Korea).

Genomic DNA isolation

Genomic DNA from healthy and diseased cassava plants was extracted using Exgene Plant SV kit following manufacturer protocol, while plasmid DNA was isolated using Exgene Plasmid SV mini kit (GeneAll Inc., Korea).

HNB preparation

Hydroxynaphthol blue (HNB) (CAS 63451-35-4) was purchased from Santa Cruz Biotechnology (USA) and dissolved in deionized water at a stock concentration of 20 mM.

LAMP assay optimization

0.5 µl DNA template was used in a LAMP assay mixture, with appropriate concentrations of primers, 6.0 mM $MgSO_4$ (Thermo Scientific, USA) and 8U *Bst* 2.0 DNA polymerase (NEB, USA). The amplification temperature was assessed at 60, 62, 63, 64, and 63 °C. When necessary, LAMP products were visualized on 2 % agarose gels. Reaction results were also assessed as color alternation of HNB and the turbidity in LAMP tubes (precipitation).

Detection of CWB from field samples using LAMP assay and nested PCR

A primer set detecting cassava actin was used as an internal control to test the quality of the DNA isolated from field samples. The LAMP assay condition for the internal control was similar to that of the assay for detecting phytoplasma. Nested PCR, following a previously published procedure (Flôres et al. 2013; Nguyen et al. 2014), was performed to confirm the identity of healthy and CWB-infected field samples. Three rounds of nested PCR were implemented with primers shown in Table 2 and temperature cycles in Table 3. The products of LAMP and nested

Table 1 LAMP primers for the detection of phytoplasma 16S rDNA and cassava actin-coding sequence

Primers	Primer's length (bp)	Sequences	Product's length (bp)
1-FIP	49	5′-GGT GTT CCT CCA TAT ATT TAC GCA TCT AGA GTA AGA TAG AGG CAA GTG-3′	223
1-BIP	39	5′-CTG ACG CTG AGG CAC GAA AGA GTA CTC ATC GTT TAC GGC-3′	
1-F3	23	5′-CAT TGT GAT GCT ATA AAA ACT GT-3′	
1-B3	20	5′-CAA CAC TGG TTT TAC CCA AC -3′	
2-FIP	42	5′- TGC ACC ACC TGT GCA ACT GAT AAG GTC TTG ACA TGC TTC TGC-3′	221
2-BIP	41	5′-TGG GTT AAG TCC CGC AAC GAG CTT GCT AAA GTC CCC ACC AT-3′	
2-F3	20	5′- AGG TAC CCG AAA AAC CTC ACC-3′	
2-B3	19	5′- TCC CCA CCT TCC TCC AAT T-3′	
Actin FIP	43	5′-GCT TCT CCT TCA TGT CAC GGA CTG ATG AAG ATC CTC ACT GAG A-3′	270
Actin BIP	44	5′-TGA ACA GGA ACT TGA GAC TGC CCA TCA GGA AGC TCA TAG TTC TT-3′	
Actin F3	18	5′-GCT CTT CCA CAT GCC ATT-3′	
Actin B3	20	5′-CTT CTG GAC AAC GGA ATC TT-3′	

Table 2 Primer sequences of nested PCR

	Primers	Sequences
Round 1	P1A	ACGCTGGCGGCGCGCCTTAATAC
	P7A	CCTTCATCGGCTCTTAGTGC
Round 2	R16F2n	GAAACGACTGCTAAGACTGG
	R16R2	TGACGGGCGGTGTGTACAAACCCCG
Round 3	R16(I)F1	TAAAAGACCTAGCAATAGG
	R16(I)R1	CAATCCGAACTGAGAATGT

PCR were visualized on 2 % agarose gel, stained with ethidium bromide and visualized under 254 nm UV light. The LAMP reactions were also assessed using HNB as a color indicator.

Results

Testing of LAMP primers

The first step in developing a LAMP assay, or any assay, is to determine its sensitivity and specificity. We tested the sensitivity, or limit of detection, of each primer set using a *pGEM-T* plasmid harboring the target phytopathogen sequence. Starting with 100 ng plasmid DNA and tenfold serial dilutions, we found that both primer sets could detect up to a 10^8-fold dilution of plasmid (Fig. 1), with primer set 2 exhibiting higher sensitivity. When the same DNA concentration was used in conventional PCR, it was found that PCR could detect the presence of target sequence up to 10^7-fold dilution. Thus, the LAMP assay was 10 times more sensitive. Specificity of the designed primers was also analyzed using the genomic DNA isolated from a healthy cassava plant (Fig. 2). Both primer sets failed to amplify, even when extremely high concentrations of DNA were used (1500 nanogram per reaction, data not shown).

Testing of HNB as a color indicator

Positive reactions of a LAMP assay can be visualized in many ways. The most reliable method is to separate the products on 2 % agarose gel and stain with ethidium bromide under UV light; however, this method is time-consuming and laborious. Previously, we successfully employed calcein as a fluorescent indicator for LAMP assays against nine rice viruses (Le et al. 2010). Again, this method requires an UV-lamp, hindering its usefulness in

Table 3 Cycling parameters of nested PCR

Round 1	94 °C	94 °C	55 °C	72 °C	72 °C	15 °C
	4 min	1 min	2 min	3 min	7 min	10 min
	1 cycle	38 cycles			1 cycle	1 cycle
Round 2	94 °C	94 °C	50 °C	72 °C	72 °C	10 °C
	1 min 30 s	30 s	50 s	1 min 20 s	10 min	10 min
	1 cycle	30 cycles			1 cycle	1 cycle
Round 3	94 °C	94 °C	50 °C	72 °C	72 °C	10 °C
	1 min 30 s	1 min	2 min	3 min	10 min	15 min
	1 cycle	34 cycles			1 cycle	1 cycle

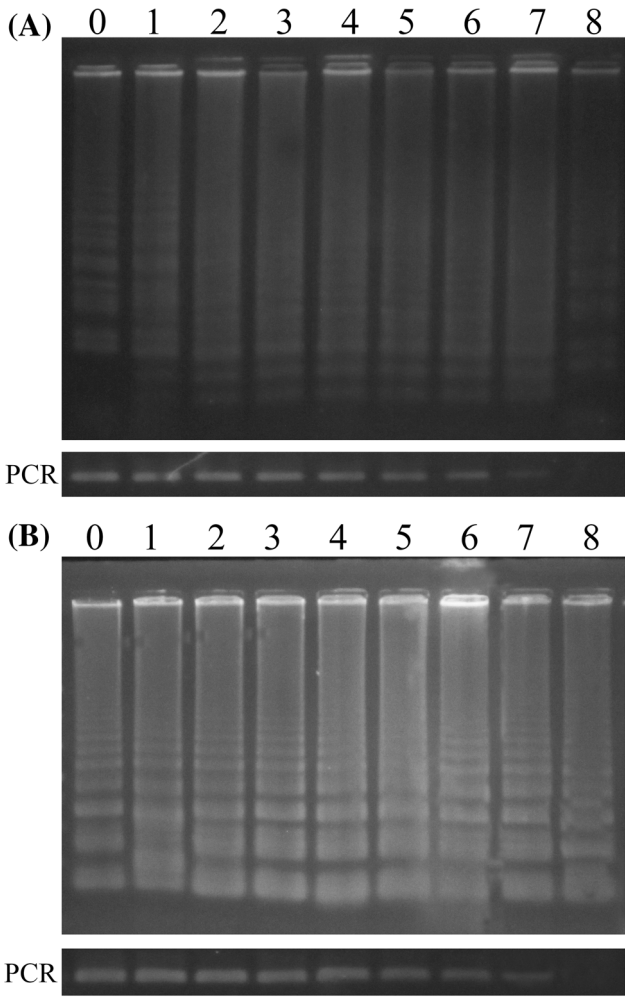

Fig. 1 Sensitivity of primer set 1 (**A**) and 2 (**B**) targeting phytoplasma DNA in LAMP reactions and PCR. *Lane 0–8* 10–10^8 dilutions of the 100 ng phytoplasma DNA

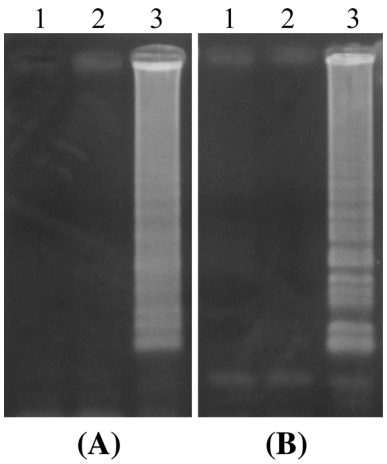

Fig. 2 Specificity of the LAMP assay. (**A**) Primer set 1, (**B**) Primer set 2. *Lane 1* negative control (no DNA template); *Lane 2* 50 ng of genomic DNA from healthy cassava (KM94 cultivar); *Lane 3* 50 ng genomic DNA of healthy cassava (KM94 cultivar) plus 50 ng DNA plasmid carrying phytoplasma 16S rDNA

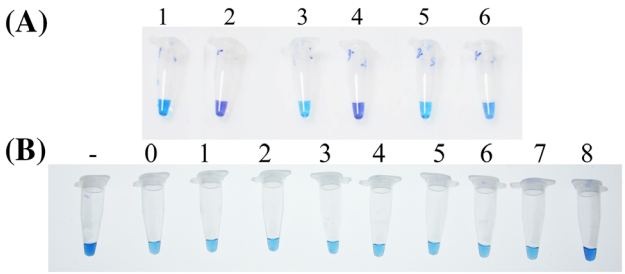

Fig. 3 Optimization of HNB as an indicator of positive LAMP reactions. (**A**) Effect of different HNB concentrations on its color reaction. Tube 1, 2: 120 μM HNB; Tube 3, 4: 100 μM HNB, Tube 5, 6: 80 μM HNB. Tube 1, 3, 5: Positive; tube: 2, 4, 6: Negative control. (**B**) Limit of detection of a positive reaction by HNB color change; tube (−): no DNA template; 0–8: 100–108 dilutions of the 100 ng initial phytoplasma DNA in each LAMP reaction

the field. Next, we tested HNB, as a color indicator of positive LAMP amplification (and assay progress). Different concentrations of HNB exhibited contrasting levels, with 100 μM HNB found most suitable (Fig. 3A). A easy-to-visualize color change, indicating a positive reaction, was observed at a 10^7-fold dilution of 100 nanogram plasmid template (Fig. 3B), equivalent to the sensitivity of PCR combined with agarose gel electrophoresis.

Detection of CWB-infected field samples

To test if the LAMP assay could detect infected field samples, we collected healthy and CWB-infected cassava from the field and isolated genomic DNA. To avoid false-negative/positive results in the assay due to poor DNA quality or other artifacts, we employed a separate primer set against an actin-coding gene in the cassava genome. A negative sample was called only if the actin-coding gene and the phytoplasma 16S rDNA reactions were positive and negative, respectively. Similarly, a positive sample was called only if positive in both assays (Fig. 4A, B). CWB infection was confirmed by a nested PCR procedure (Fig. 4C). Finally, positive LAMP reactions were successfully identified using HNB as color indicator (Fig. 5). In conclusion, our LAMP assay successfully distinguished between healthy and CWB-infected cassava from the field.

Discussion

CWB seriously affects on cassava yield and production value. Previously, a PCR-based technique was developed to detect the presence of phytoplasma in diseased cassava; however, it has numerous caveats: it requires several

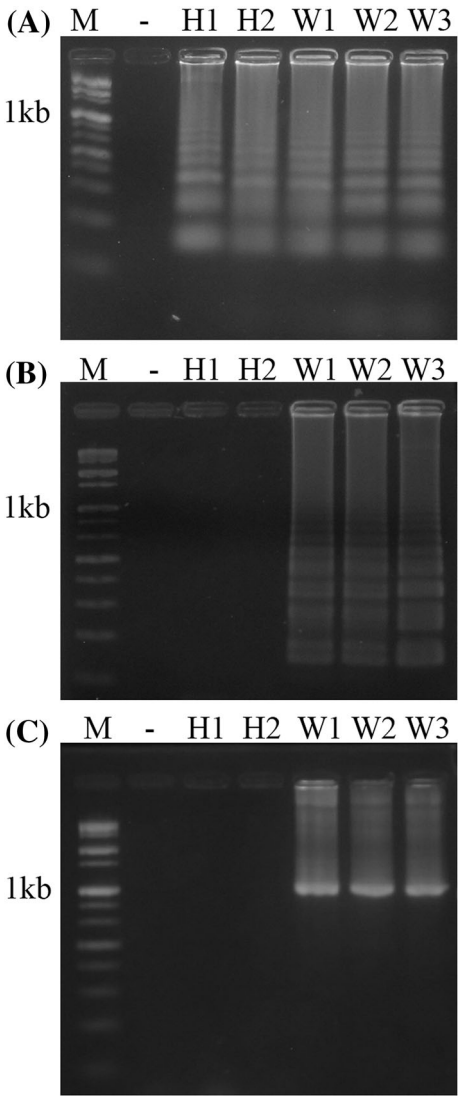

Fig. 4 Detection of CWB-infected cassava from field-collected samples. (**A**) LAMP assay detecting cassava gene encoding Actin. (**B**) LAMP assay (with primer set 2) detecting phytoplasma 16S rDNA. (**C**) Nested PCR detecting 16S rDNA. M: 1 kb DNA ladder; (−): No DNA template; *Lane* H1, H2: healthy cassava; W1, W2, W3: CWB-infected cassava

rounds of PCR and three different primer pairs (nested PCR), is time-consuming, and requires a thermocycler–effectively rending it far from an ideal choice for the field. Recently, LAMP has been employed in various diagnostic assays for plants diseases (Goto et al. 2009; Le et al. 2010; Bekele et al. 2011; Le et al. 2012). Because LAMP relies on the strand displacement activity of *Bst* polymerase, it can amplify the target DNA at a constant temperature, providing several advantages: it does not require a thermocycler and can employ relatively inexpensive heat incubators and is highly robust, with a dynamic detection range surpassing PCR (Notomi et al. 2000). In the present study, LAMP was 10 times more sensitive than conventional PCR and could detect phytopathogen at as low as 10^{-6} ng DNA. Moreover, the specificity of the assay was proven through the identification target DNA in a mixture with large amount of non-target DNA, further demonstrating the superior specificity of LAMP.

HNB is an indicator for the alkaline earth metals such as Mg^{2+} and Ca^{2+}. LAMP reaction releases a large quantity of pyrophosphate which reacts with Mg^{2+} to form insoluble product magnesium pyrophosphate. Therefore, a positive reaction will decrease the concentration of Mg^{2+} in the solution significantly. In a master mix containing 1.4 mM dNTP and 6 mM Mg^{2+} or higher, HNB is purple, but when Mg^{2+} concentration decreased to below 6 mM, the color of HNB will change to blue and this can be taken as a signal of a positive LAMP reaction (Goto et al. 2009). In our study, the initial concentration of dNTPs of the reaction was 1.5 mM, rendering HNB an easy-to-visualize purple or deep blue color. In addition, the presence of a precipitate can reveal a positive LAMP reaction. Although both PCR and LAMP can produce magnesium pyrophosphate during their respective reactions, only magnesium pyrophosphate formed by LAMP can be observed by the naked eye (Mori et al. 2001). Mg^{2+} was added to a final concentration of 6 mM, allowing rapid detection of positive LAMP reactions once Mg^{2+} concentration decreases.

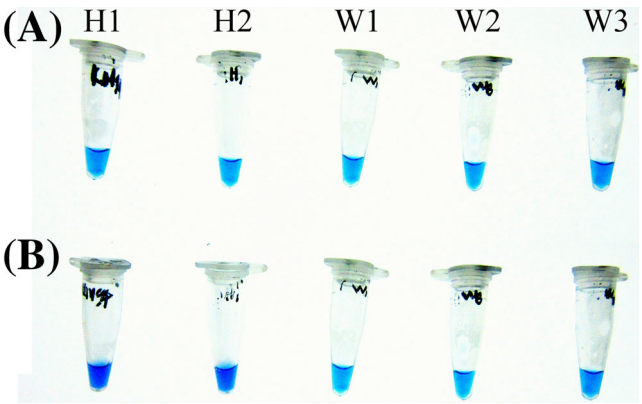

Fig. 5 Detection of CWB-infected field samples using HNB as an indicator. (A) LAMP assay detecting Actin-coding gene (internal control), (**B**) LAMP assay detecting phytoplasma 16S rDNA. H1, H2: healthy samples; W1, W2, W3: CWB-infected field samples

The appearance of magnesium pyrophosphate precipitate, together with a blue color, in a LAMP reaction greatly aids visual identification of infected plants. In conclusion, this pilot study revealed that our LAMP assay can distinguish between healthy and diseased cassava collected from the field with high sensitivity and specificity, allows rapid visual identification of Cassava Witches' Broom Disease, and should provide impetus for further translational research into this devastating pest.

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