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Rapid and sensitive detection of *Banana bunchy top virus* by loop-mediated isothermal amplification

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ABSTRACT

A sensitive loop-mediated isothermal amplification (LAMP) assay was developed for rapid detection of *Banana bunchy top virus* (BBTV) infection. The reaction was performed in a single tube at 63 °C for 90 min, with an improved closed-tube detection system by adding the SYBR Green I dye to the inside of the tube lid prior to amplification. The detection limit of the LAMP assay was approximately 1 pg/μl plasmid DNA when mixed with extracted DNA from healthy banana plant, and no cross-reaction with other banana-infected pathogens was observed. Real-time turbidimetry was used to monitor the amplification result in the tubes, and it was shown that this LAMP assay was about 100-fold more sensitive than PCR. The results demonstrated that this LAMP method should be useful for both banana disease monitoring and mass propagation of virus-free banana plantlets.

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1. Introduction

Banana (*Musa nana*) is one of the most important fruit crops, and bunchy top disease is a major threat to banana production in many tropical and subtropical regions (Dale, 1987; Dietzgen and Thomas, 1991). The causal virus, *Banana bunchy top virus* (BBTV) which belongs to the genus *Babuvirus*, family *Nanoviridae* (Vetten et al., 2012), spreads between banana plants by the banana aphid *Pentalonia nigronervosa* and from place to place by transporting propagative materials derived from infected plants (Dale, 1987; Dietzgen and Thomas, 1991). Banana plants infected by BBTV may display distorted bunches, and new growth from infected plants usually become stunted with a bunchy top-like appearance. No effective resistance is known in *Musa* spp. to this virus, thus control is still based largely on the use of virus-free propagative materials, roguing of infected plants and implementation of quarantine barriers. At present, banana plantlets from tissue culture are used widely, it is necessary, therefore, to develop efficient techniques for BBTV detection to obtain virus-free propagative materials.

Published methods for detecting BBTV include virus isolation and purification (Wu and Su, 1990a; Harding et al., 1991; Thomas and Dietzgen, 1991), enzyme-linked immunosorbent assay (ELISA) (Wu and Su, 1990b; Thomas and Dietzgen, 1991; Geering and Thomas, 1996), nucleic acid hybridization (Hu et al., 1996; Hafner et al., 1997), PCR (Xie and Hu, 1995; Hu et al., 1996; Hafner et al., 1997; Dietzgen et al., 1999), and immnocapture-PCR (Sharman et al., 2000). Although ELISA and PCR are applied widely, a nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) is more rapid and simpler, using only a water bath or heating block (Notomi et al., 2000). The LAMP assay is performed under isothermal conditions, employing a DNA polymerase with strand-displacing activity and a set of four designed primers which recognize a total of six distinct sequences on the target DNA to be amplified; the amplified products contain single-stranded loops, allowing primers to bind without the need for repeated cycles of thermal denaturation (Notomi et al., 2000). As the LAMP reaction progresses, the by-product pyrophosphate ions bind to magnesium ions and form a white precipitate of magnesium pyrophosphate, and an increase in the turbidity with the production of white precipitate correlates with the amount of DNA synthesized, monitoring of the LAMP reaction was achieved by real-time measurement of turbidity (Mori et al., 2001, 2004).

The objective of this research was to develop a LAMP method for the diagnosis of BBTV infection of banana plants. Additionally, an improved visual closed-tube procedure was developed for high-throughput detection. The application of LAMP for the detection of BBTV in field-grown banana plants is described.

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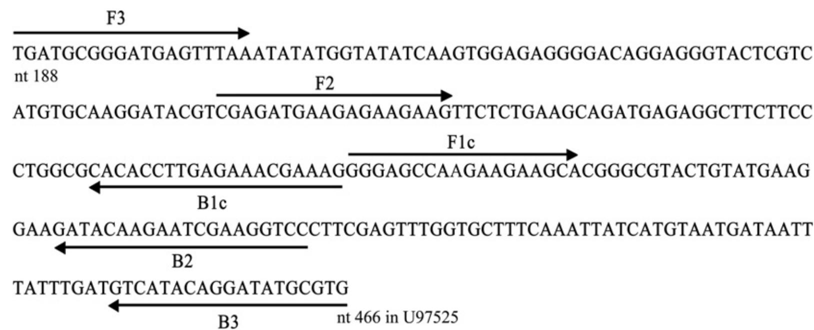

Fig. 1. Nucleic acid sequence of target fragment on DNA-R (containing the replication initiator protein gene) of the BBTV genome used for designing inner and outer primers. The specific sequences used for primer design and their relative positions in the viral genome are indicated by arrows.

2. Materials and methods

2.1. Virus sources and DNA extraction

Samples of plants showing BBTV or virus-like symptoms were obtained from different banana plantations on Hainan Island, China. The samples of leaves and suckers were collected, and genomic DNA was extracted using a modified CTAB DNA extraction method according to Gawel and Jarret (1991).

2.2. Primer design

LAMP primers were designed according to sequence of DNA-R (containing the replication initiator protein gene) of BBTV genome (accession number U97525) using PrimerExplorer V4 software (http://primerexplorer.jp/e/). A forward inner primer FIP (5′-*CTTTCGTTTCTCAAGGTGTGC*GTCGAGATGAAGAGAAGAAG-3′) consisted of F1c (the complementary sequence of F1, 5′-*CTTTCGTTTCTCAAGGTGTGC*-3′, nt 339–319 in the BBTV genome) and F2 (5′-GTCGAGATGAAGAGAAGAAG-3′, nt 265–284), and a backward inner primer BIP (5′-*GGGAGCCAAGAAGAAGCA*GGACCTTCGATTCTTGTATC-3′) consisted of B1c (the complementary sequence of B1, 5′-*GGGAGCCAAGAAGAAGCA*-3′, nt 340–357 in the BBTV genome) and B2 (5′-GGACCTTCGATTCTTGTATC-3′, nt 398–379). The outer primers F3 (5′-TGATGCGGGATGAGTTTA-3′, nt 188–205) and B3 (5′-CACGCATATCCTGTATGAC-3′, nt 466–448) were used for the initiation of LAMP reaction. The primer sequences and their respective binding sites were indicated in Fig. 1.

2.3. Reaction mixtures and optimal conditions

The LAMP reaction mix contained 1.6 μM each of the primers BBTV-FIP and BBTV-BIP, 0.2 μM each of the primers BBTV-F3 and BBTV-B3, 1.6 mM of dNTPs, 1 M of betaine (Solarbio, Beijing, China), 4 mM of $MgSO_4$, 10× ThermoPol reaction buffer (20 mM Tris–HCl, 10 mM KCl, 10 mM of $(NH_4)_2SO_4$, and 0.1% TritonX-100), 8 U of *Bst* DNA polymerase, and 1 μl of BBTV DNA template, and double-distilled water to a final volume of 25 μl. Then, an equal volume of paraffin oil was added to the tube to prevent evaporation, followed by adding 1 μl of 1:10-diluted SYBR Green I (Invitrogen, Carlsbad, CA) to the interior of the lid prior to amplification. The LAMP reaction was carried out in a Loopamp real-time turbidimeter (LA-320C; Teramecs, Kyoto, Japan) at 63 °C for 90 min and terminated at 80 °C for 10 min.

2.4. Analysis of LAMP products

Real-time turbidity readings at 650 nm were obtained and a turbidity threshold value of 0.1 was used. After reaction, LAMP products were detected directly by visual observation of the solution color by mixing the pre-added 1 μl of SYBR Green I to the reaction solution by gentle centrifugation (for about 5 s, up to 2000 × *g*). Green fluorescence was observed clearly with the naked eye with a positive reaction, whereas the color remained orange with the negative reactions. The LAMP products (5 μl) were analyzed by electrophoresis on a 2% (w/v) agarose gel and stained subsequently with ethidium bromide.

2.5. LAMP specificity

To confirm the specificity of the LAMP assay, the RNA of *Cucumber mosaic virus* (CMV), and the DNAs of *Banana streak OL virus* (genus *Badnavirus*, family *Caulimoviridae*), *Ralstonia solanacearum*, *Fusarium oxysporum* f. sp. *cubense* race 1 (Foc1) and race 4 (Foc4) were used in the analyses. For CMV RNA template, an additional 0.2 μl (8 U) of reverse transcriptase (Takara, Dalian, China) was added to LAMP reaction solution.

2.6. LAMP sensitivity

To determine the sensitivity of the BBTV LAMP assay, a DNA fragment of 748 bp containing the LAMP target region of the BBTV genome was amplified by PCR using primers BBTV-RP1 (5′-ATGTGG TATGCTGGATGTTC-3′) and BBTV-RP2r (5′-GGTTCATATTTCCCGCTTTGA-3′). The concentration of primers was adjusted to 10 μM for subsequent usage. The PCR mix was 50 μl in volume and contained 5 μl of 10× *EX Taq* buffer (Mg^{2+} Plus), 1 μl of dNTP mixture (10 mM each), 5 U of *EX Taq* DNA polymerase (Takara, Dalian, China) and 2 μl each of the primers. The thermal cycling program was as follows: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 7 min. PCR product was purified and cloned into the pMD18-T vector (Takara, Dalian, China) following the manufacturer's instructions. The recombinant plasmid, designated pMD18-T-BBTV, was adjusted to the concentration of 100 ng/μl, and diluted into a 10-fold serials (1×10^0 to 1×10^7 copies) before mixed with extracted DNA from healthy banana used as a reference to assess the detection limit of the LAMP assay.

To evaluate the feasibility of LAMP method for diagnosis of samples collected in the field, all samples collected from different geographic locations in Hainan Island were detected with PCR and LAMP assay, respectively.

3. Results

For the specificity test, no cross-reactivity with templates extracted, or from other banana-infecting pathogens was found. Only amplified DNA products from banana infected with BBTV

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