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Development of a real-time fluorescence loop-mediated isothermal amplification assay for rapid and quantitative detection of *Fusarium mangiferae* associated with mango malformation

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ABSTRACT

Fusarium mangiferae is a major causal agent of mango malformation disease (MMD) worldwide. Rapid and accurate detection of the causal pathogen is the cornerstone of integrated disease management. In this paper, a real-time fluorescence loop-mediated isothermal amplification assay (RealAmp) was developed for quantitative detection of *F. mangiferae* in China. The LAMP primer set was designed based on a RAPD marker sequence and positive products were amplified only from *F. mangiferae* isolates, but not from any other species tested, showing a high specificity of the primer sets. The detection limit was approximately 2.26×10^{-4} ng/μl plasmid DNA when mixed with extracted mango DNA. Quantification of the pathogen DNA of MMD in naturally collected samples was no significant difference compared to classic real-time PCR Additionally, RealAmp assay was visual with an improved closed-tube visual detection system making the assay more convenient in diagnostics.

Introduction

Mango (*Mangifera indica* L.) is considered as one of the most popular fruits grown throughout the tropics and subtropics worldwide [1,2]. Mango malformation disease (MMD) is one of the most destructive diseases on mango and imposing a continuous threat to the future of mango industries [3,4]. Infected mango plants may display gross deformations of vegetative and floral tissues in mango [5]. The affected flowers become sterile or abort shortly after fruit have set [2], which cause substantial reductions in yield and quality [6]. The disease spreads between mango plants by the bud mite *Aceria mangiferae* [7–9] and from place to place through infected vegetative planting material, such as infected nursery stock [9] and grafting [10].

The identity of the causal agent has been controversial for many years and it is now widely accepted that several species of the fungus in genus *Fusarium* associated with MMD, include *F. mangiferae*, *F. sterilihyphosum*, *F. proliferatum* and *F. subglutinans* [4,11–15]. Among these species, *F. mangiferae* has been identified as a major causal agent of MMD worldwide [11,12,16–21]. Additionally, two novel etiological agent of MMD have been reported, *F. mexicanum* caused vegetative malformation on mango in Mexico [22], and *Fusarium tupiense* sp. nov., which caused mango malformation in Brazil [23] and Senegal [24], respectively. Recently, extensive sample tests showed that among the causal agent of MMD in Peninsular Malaysia, *F. proliferatum* was the dominant species, followed by *F. mangiferae* and then *F. subglutinans* [17,25]. In China, MMD was first reported in 1992 in Yuanjiang county of Yunan province [26] and the causal agent firstly identified as *F. proliferatum* [13], and subsequently re-identified as *F. mangiferae* [14].

It is difficult to establish systems of taxonomy for *Fusarium* species solely basing on morphological characters and the genetic polymorphism demonstrate the variation in DNA sequences within the *Fusarium* species can provide a helpful tool for a more precise identification. Random amplified polymorphic DNA (RAPD) is one of the PCR-based techniques that widely used to analyze genetic diversity. Zheng and Ploetz (2002) firstly assess the genetic variation between various MMD-associate species with RAPD, and then

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developed a *F. mangiferae* specific PCR method based on aRAPD marker sequence [2,11,27]. Comparison to RAPD, amplified fragment length polymorphism (AFLP) is more effective in bringing out the polymorphism among the *Fusarium*. Newman et al. (2012) have successfully developed an AFLP-derived PCR method for species-specific detection of *F. mangiferae* [20]. Following, AFLPs were utilized to differentiate various *Fusarium* species, such as *F. mangiferae* and *F. subglutinans* in Penisular Malaysia [25]. Although PCR-based methods are widely used, it requires a thermocycler to carry the DNA amplification through specific temperature phases and the results need to further analyzed by electrophoresis. Apart from PCR-based methods, an alternative technique, termed loop-mediated isothermal amplification assay (LAMP) is also widely used for plant pathogens detection [28,29]. The LAMP assay is performed under isothermal conditions and employs a DNA polymerase with strand-displacing activity and a set of four especially designed primers, which recognize a total of six distinct sequences on the target DNA to be amplified [30,31]. Following, the detection of the amplified products through fluorescence dye using an ESE-Quant tube scanner (ESE GmbH, Stockach, Germany) was developed. The method doesnt need expensive equipment or reagent and is a simple and cost-effective technology compared to other DNA-based tests [32,33].

To the best of our knowledge, there are currently no effective resistant cultivar released in *Mangifera*, and no effective approaches available to control the disease once plants have been infected. Thus, quarantine policies and MMD-free planting materials are the important approaches to prevent disease spreading. In China, MMD has been locally epidemic in orchards scattered along with hot-dry valley of Jinsha River, but not found in other mango growing regions until now. It is, therefore, necessary to develop an early and accurate detection method specific to MMD, which is an important trigger for establish certified nurseries and initiation of containment measures ensuring the plant materials and grafts free from MMD.

The objectives of this study were to develop a RealAmp assay for rapid and quantitative detection of *F. mangiferae* in planting materials to aid in the establishment of the disease-free nurseries for future supply of certified mango nursery stocks and grafts. The usefulness of the RealAmp assay was verified by testing filed collected samples in comparison to AFLP-derived real-time PCR. Furthermore, the visual inspection of RealAmp products using an improved closed-tube detection system by adding SYBR Green I to the interior of the tube lid prior to amplification makes it more convenient in field surveys.

Materials and methods

Source of isolates and mycelium preparation

F. mangiferae and other isolates used in this study were listed in Table 1. The isolates were maintained in a collection at the Institute of Environmental and Plant Protection, Chinese Academy of Tropical Agricultural Sciences, PR China.

For genomic DNA extraction, 10 pieces of agar culture (ca. 1 × 1 × 2 mm) obtained from the margin of 3-day-old colonies growing on potato dextrose agar (PDA) plates were placed in 100 ml of potato dextrose broth (PDB) liquid medium in a 250-ml flask. After incubation in the dark at 25 °C on a shaker for 6 days, the mycelia were collected on filter paper and stored at −70 °C until use.

Extraction of genomic DNA from mycelium and mango

Approximately 100 mg of freeze-dried mycelium or conidia were ground in liquid nitrogen, and the total genomic DNA was isolated according to the manufacturer's instructions of E-Z 96® Fungal DNA Kit (Omega, USA). Mango genomic DNA was extracted using CTAB method as reported previously [20,34].

All DNA samples were eluted with 100 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C until required. DNA concentration of extracts was determined by monitoring absorbance at 260 and 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

LAMP primer design

LAMP primers were designed according to the sequence of an RAPD marker sequence of the *F. mangiferae* genome using PrimerExplorer V4 software (Eiken Chemical Co. Ltd., Tokyo, Japan) (http://primerexplorer.jp/e/). A forward inner primer FIP (5′-*TGACACCTGAGCCTCCGA*GAGAATGTGAAGAGGCTCAG-3′) consisted of F1c (the complementary sequence of F1, 5′-*TGACACCTGAGCCTCCGA*-3′) and F2 (5′-GAGAATGTGAAGAGGCTCAG-3′), and a backward inner primer BIP (5′-GAGGCTGCACATGTGGCTAT*TGTGTACGTATGGATGTTACAG*-3′) consisted of B1c (the complementary sequence of B1, 5′-GAGGCTGCACATGTGGCTAT-3′) and B2 (5′-*TGTGTACGTATGGATGTTACAG*-3′). The outer primers F3 (5′-ACTTATGCTGGAGGTGGT-3′) and B3 (5′-*CCAGGCATGTAAGTACTAACG*-3′) were used for the initiation of RealAmp reaction. All the primers were purified by HPLC (Sangon Biotec, Shanghai, China). The primer sequences and their respective binding sites were indicated in Fig. 1.

RealAmp assay

The RealAmp method was performance using the commercially available DNA thermostatic amplification kit (Guangzhou Diao Biotechnology Co., Ltd., Guangdong, China) following the manufacturer's instructions. Reactions containing 2× reaction buffer (40 mM Tris–HCl pH8.8, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH4)_2SO_4$, 0.2% Tween-20, 1 M Betaine, 2.8 mM of dNTPs each), 1.6 μM of each inner primers of FIP and BIP, 0.2 μM of each outer primers of F3 and B3, 8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 0.2 μM SYTO-9 fluorescent dye (Invitrogen, Carlsbad, CA), 1 μl of template DNA, and double-distilled water to a final volume up to 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 dilution SYBR Green I (Invitrogen, Carlsbad, CA) to the inside of the lid prior to amplification with an improved close-tube visual detection system. The RealAmp assay was carried out at 63 °C for 90 min using the ESE-Quant Tube Scanner (ESE GmbH, Stockach, Germany), which was set to collect fluorescence signals at 30 s intervals [32].

The Threshold validation test is used to identify that the signal has increased sufficiently to be deemed as positive. During the real-time amplification, the fluorescence data were obtained on the 6-carboxyfluorescein (FAM) channel (excitation at 487 nm and detection at 525 nm), and a fluorescence units threshold value was used, and threshold time (*Tt*) calculated as the time at which the fluorescence equaled the threshold value. The threshold value is 10 times standard deviation of the fluorescence signal during initial 5 min. In the plot, the *Y*-axis denotes the fluorescence units in millivolts (mV) and the *X*-axis shows the time in minutes.

Specificity and sensitivity test of RealAmp assay

To confirm the specificity of the RealAmp assay, the DNAs of previously confirmed MMD-associate *Fusarium* species, *Fusarium* relative pathogens, mango-infecting pathogens and other relative fungi were used in the analyses (Table 1). In addition, the smallest fragment from RealAmp amplification products were cloned and