Development of a TaqMan Probe-Based Insulated Isothermal Polymerase Chain Reaction (iiPCR) Assay for Detection of \textit{Fusarium oxysporum} f. sp. \textit{cubense} Race 4

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Abstract

This study developed a novel and inexpensive detection method based on a TaqMan probe-based insulated isothermal polymerase chain reaction (iiPCR) method for the rapid detection of Panama disease caused by \textit{Fusarium oxysporum} f. sp. \textit{cubense} (Foc) race 4, which is currently among the most serious fungal vascular diseases worldwide. By using the portable POCKIT™ device with the novel primer set iiFoc-1/iiFoc-2, the Foc race 4 iiPCR assay (including DNA amplification and signal monitoring) could be completed within one hour. The developed Foc race 4 iiPCR assay is thus a user-friendly and efficient platform designed specifically for the detection of Foc race 4. The detection limit of this optimized Foc iiPCR system was estimated to be 1 copy of the target standard DNA as well as 1 fg of the Foc genomic DNA. This approach can serve as a rapid detection method for in planta detection of Foc race 4 in field-infected banana. It was concluded that this molecular detection procedure based on iiPCR has good potential for use as an efficient detection method.

Introduction

Bananas and plantains, members of the \textit{Musa} genus, are now cultivated in humid tropical areas worldwide, making them one of the most economically important and popular fruits crops in the world. However, the soil-borne fungal disease, banana Fusarium wilt (FW), commonly known as Panama disease, which is caused by \textit{Fusarium oxysporum} f. sp. \textit{cubense} (Foc), is a highly-lethal vascular fungal disease in banana plants. When Foc attacks a banana plant, it leads to yellowing and wilting of the older leaves that then progresses to the younger leaves until the whole plant is killed. This lethal effect makes Panama disease the major limiting factor for banana production worldwide [1].
Foc is not easy to eradicate from an infected field because the pathogen can produce thick-walled chlamydospores, which are highly resistant to fungicides or chemical fumigation [2] and can survive in infected soil as resting spores for more than 30 years [1,3–5]. One of the best means of managing FW at present is based on the breeding of FW-resistant lines [6–8], though this approach is very time-consuming [9,10]. In short, few effective, economical, environmentally safe, and curative management options are available for FW control [11].

Four races of Foc are recognized on banana based on the specificity of pathogenicity against various banana cultivars [12]. Race 1 is pathogenic to Gros Michel (genome type = AAA), while race 2 infects ABB cooking bananas, such as Bluggoe [12]. Race 4 (including tropical race 4, TR4; and subtropical race 4, ST4) affect Cavendish cultivars as well as those susceptible to race 1 and race 2 [12–14]. Foc TR4 has been found to cause severe damage to almost all presently popular cultivars. Hence, it has spread worldwide and brought about huge economic losses to the banana industry [15].

The timely eradication of any plants initially discovered to be infected by Foc and the avoidance of Foc-contaminated areas are important strategies for reducing the dissemination of the pathogen and the economic impacts of FW on the banana industry. Crucial control strategies targeting Foc race 4 could be implemented more effectively if early detection could be carried out to provide rapid, sensitive and specific test results for pathogen monitoring.

Molecular detection methods, including conventional polymerase chain reaction (PCR) [7], loop-mediated isothermal amplification (LAMP) [16], real-time PCR [10], and real-time LAMP (RealAmp) [17,18] assays, have been developed to specifically and rapidly detect Foc race 4 or Foc TR4. These detection methods offer high degrees of detection sensitivity and specificity.

Recently, insulated isothermal PCR (iiPCR), which is based on Rayleigh-Bénard convection PCR, has been described as being capable of sensitively and specifically detecting both RNA and DNA [19]. In addition, it can be performed with a single copper ring-wrapped capillary tube (R-tube™) heated by an isothermal heating source, making it relatively simple and inexpensive while foregoing the need for a relatively costly thermocycler [19–26].

The three steps of iiPCR, namely, denaturation, annealing, and extension, are completed in the copper ring-wrapped capillary tube by cycling reactions through a temperature gradient established by the Rayleigh-Bénard convection. An iiPCR instrument, the POCKIT™ Nucleic Acid Analyzer (GeneReach Biotechnology Corp., Taichung, Taiwan) [21,22], is now commercially available and allows the performance of a TaqMan probe-based iiPCR assay within a copper ring-wrapped capillary tube (R-tube™), in addition to completing the iiPCR assay automatically (Fig 1).

This paper describes a TaqMan probe-based iiPCR for rapid in planta detection of Foc race 4. The method should be sensitive, reproducible, rapid, and user-friendly, and should not require a costly thermocycler. The Foc race 4 iiPCR assay could potentially be a useful tool for routine quarantine detection of Foc race 4 to avoid further dissemination.

Materials and Methods
Pathogen isolates and growth condition
The Foc isolates and other isolates used in this study are listed in Table 1. All the tested Foc were isolated from banana pseudostems. A single spore culture of each tested Foc isolate was grown on a Nash-PCNB plate (1.5% peptone, 2% agar, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.1% pentachloronitrobenzene, 0.03% streptomycin, and 0.1% neomycin) [27]. Other fungal isolates were grown on a potato dextrose agar (PDA) plate (200 g/l of potato extracts, 1%...
glucose, and 2% agar). Single colonies of *Ralstonia solanacearum* were grown on peptone sucrose agar (PSA) plate (1% peptone, 1% sucrose, 0.1% glutamic acid, pH 7.0, and 2% agar).

**Primer and TaqMan probe design**

The TaqMan probes and iiFoc primer used for the Foc race 4 iiPCR assays were designed on the basis of the recommended principles for iiPCR [20]. The iiFoc primer set iiFoc-1/iiFoc-2 and TaqMan probe iipFoc-1 (5’-6-FAM-ACCACGCGGATGAGATT-MGB-NFQ-3’) were designed according to the sequence of the Foc race 4-specific marker Foc242. The Foc242 marker has been confirmed to have high specificity for Foc race 4 [7,10]. The conserved primer set ITS1/ITS4 was used to amplify and sequence the ~500-bp rDNA regions, including the internal transcribed spacers 1 (ITS1), 5.8S rDNA, and ITS2 [28], for the purpose of identifying the isolates tested in this study. The other PCR-based identification techniques utilizing the Foc TR4-specific primer set FocTR4-F/FocTR4-R (designed by Dita et al. [15]) and the Foc race 4-specific primer set FocSc-1/FocSc-2 (designed by Lin et al. [10]) were used to confirm the specificity of the Foc race 4 iiPCR assay. All the sequences of the primer sets used in this study are listed in Table 2. The PCR conditions and protocols used for primer sets ITS1/ITS4, FocTR4-F/FocTR4-R, and FocSc-1/FocSc-2 were those described previously by White et al. [28], Dita et al. [15], and Lin et al. [10], respectively.

**Sample DNA preparation**

Dried fungal mycelium (100 mg), overnight-grown bacterial culture (1.5 ml), and field-infected banana tissues (300 mg) were frozen in liquid nitrogen and finely ground using a mortar and pestle. Genomic DNA (gDNA) was extracted according to Lin et al. [7], dissolved in a 0.1× TE
| Isolate code numbers | Diseases/species                                                   | Original hosts/tissues                                    | Geographic locations | PCR-based identification methods used in this study |
|----------------------|-------------------------------------------------------------------|----------------------------------------------------------|---------------------|--------------------------------------------------|
|                      | **Fusarium wilt of banana (FWB)/Fusarium oxysporum f. sp. cubense race 1 (Foc R1)** | Banana (Musa. sp.) cv. Lady Finger/Pseudostem (P)        | Queensland, Australia | **iiPCR** | **ITS1/ITS4** | **FocSc-1/FocSc-2** | **FocTR4-F/FocTR4-R** |
| ATCC96285            | **Fusarium wilt of banana (FWB)/Fusarium oxysporum f. sp. cubense race 1 (Foc R1)** | Banana (Musa. sp.) cv. Lady Finger/Pseudostem (P)        | Queensland, Australia | -         | +             | -                  | -                   |
| ATCC76257            | FWB/Foc race 2 (Foc R2)                                           | Banana cv. Bluggoe/P                                     | Honduras            | -         | +             | -                  | -                   |
| ATCC38741            | FWB/Foc subtropical race 4 (Foc ST4)                              | Banana cv. Cavendish/P                                   | Taiwan              | +         | +             | +                  | -                   |
| ATCC76262            | FWB/Foc ST4                                                       | Cavendish/P                                               | Taiwan              | +         | +             | +                  | -                   |
| ATCC96289            | FWB/Foc ST4                                                       | Cavendish/P                                               | Queensland, Australia | +         | +             | +                  | -                   |
| ATCC96290            | FWB/Foc ST4                                                       | Cavendish/P                                               | Queensland, Australia | +         | +             | +                  | -                   |
| TYC-F015             | FWB/Foc tropical race 4 (Foc TR4)                                 | Cavendish/P                                               | Kaohsiung, Taiwan   | +         | +             | +                  | +                   |
| TYC-F016             | FWB/Foc TR4                                                       | Cavendish/P                                               | Kaohsiung, Taiwan   | +         | +             | +                  | +                   |
| TYC-F017             | FWB/Foc TR4                                                       | Cavendish/P                                               | Kaohsiung, Taiwan   | +         | +             | +                  | +                   |
| TYC-F020             | FWB/Foc TR4                                                       | Cavendish/P                                               | Kaohsiung, Taiwan   | +         | +             | +                  | +                   |
| TYC-F021             | FWB/Foc TR4                                                       | Cavendish/P                                               | Kaohsiung, Taiwan   | +         | +             | +                  | +                   |
| TYC-F022             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F023             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F025             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F026             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F028             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F029             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F030             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F031             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F032             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F033             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F034             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F035             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F036             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F037             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |
| TYC-F038             | FWB/Foc TR4                                                       | Cavendish/P                                               | Pingtung, Taiwan    | +         | +             | +                  | +                   |

(Continued)
buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 8.0), and stored at -20°C for further Foc race 4 iiPCR and PCR assays.

### Standard DNA preparation

The 404 bp RAPD marker (accession number EU379562) specific to Foc race 4 was published in an earlier study [7]. The specific primer sets FocSc-1/FocSc-2 (nt79-105/nt294-320) had

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**Table 1. (Continued)**

| Isolate code numbers | Diseases/species | Original hosts/tissues | Geographic locations | PCR-based identification methods used in this study |
|----------------------|------------------|------------------------|---------------------|-----------------------------------------------------|
| ATCC42007            | Fusarium wilt of watermelon (FWW)/F. oxysporum f. sp. niveum (Fon) | Watermelon (Citrullus lanatus (Thunb.) Matsum & Nakai)/Vine (V) | Taiwan | - + - - |
| ATCC42007            | FWW/Fon          | Watermelon/V           | Taiwan              | - + - - |
| ATCC62940            | FWW/Fon          | Watermelon/Seed        | Texas, USA          | - + - - |
| P33139               | Bacterial wilt of eggplant/Ralstonia solanacearum (Rs) | Eggplant (Solanum melongena L.)/Stem | Taiwan | - - - - |
| YJL-B001             | Bacterial wilt of cucumber/Rs | Cucumber (Cucumis sativus L.)/V | Pingtung, Taiwan   | - - - - |

*a The insulated isothermal polymerase chain reaction (iiPCR) method used for Fusarium oxysporum f. sp. cubense (Foc) race 4 detection was carried out in the R-tube™ within the POCKIT™ Nucleic Acid Analyzer designed by GeneReach Biotechnology Corporation (Taichung City, Taiwan).

*b The conserved primer set ITS1/ITS4 was used to amplify and sequence the ~500-bp rDNA region used for the identification of the internal transcribed spacers 1 (ITS1), 5.8S rDNA, and ITS2 of the fungal pathogens used in this study.

*c The Foc race 4-specific primer set FocSc-1/FocSc-2 designed by Lin et al. [10] was used to confirm the specificity of the Foc race 4 iiPCR assay.

*d The Foc tropical race 4-specific primer set FocTR4-F/FocTR4-R designed by Dita et al. [15] was used to confirm the specificity of the Foc race 4 iiPCR assay.

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been designed from the RAPD marker for rapid and specific detection of Foc race 4 by using PCR [7] and real-time PCR [10], respectively. To generate the standard template (named as pFoc242) used for iiPCR, the 242-bp DNA sequence amplified by the Foc race 4-specific primer set FocSc-1/FocSc-2 was gel-purified, cloned into pGEM®-T Easy vector (Promega Co, Madison, WI, USA), and sequenced. The copy number calculation of the standard template pFoc242 was based on the concentrations determined by a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific Inc., Cleveland, OH, USA). The standard template pFoc242 was dissolved in a 0.1× TE buffer and stored at -20°C for further Foc race 4 iiPCR assay.

**Foc race 4 iiPCR assay**

The Foc race 4 iiPCR was designed on the basis of the hydrolysis TaqMan probe-based POCKIT™ method described previously by Tsai et al. [22]. For this Foc race 4 iiPCR assay, each 50 μl iiPCR mixture contained tested gDNA, 1X Uni-ii HS Buffer (GeneReach Biotechnology Corp., Taichung, Taiwan), 0.5 mM of forward primer (iiFoc-1), 0.5 mM reverse primer (iiFoc-2), 200 nM of TaqMan probe (iipFoc-1) (Applied BioSystem, Life Technologies, Carlsbad, CA, USA), and 1 U of KAPA Taq DNA Polymerase (Kapa Biosystems, Boston, MA, USA). The iiPCR was carried out in the R-tube™ within the POCKIT™ Nucleic Acid Analyzer (GeneReach Biotechnology Corp., Taichung, Taiwan) following the instruction manuals. The fluorescent signal-to-noise ratios (signal after/signal before) collected by the POCKIT™ analyzer were converted automatically to positive (+ symbol) or negative (- symbol) according to the default S/N thresholds (S/N ratio ≥ 1.3) and then shown on the display screen of the POCKIT™ analyzer.

**Sensitivity and specificity determination**

The specificity of the Foc race 4 iiPCR assay was tested by using the genomic DNA of plant pathogens isolated from banana plants, including Foc TR4, Foc ST4, Foc race 2, Foc race 1, and non-Foc, as well as those of other pathogens from non-banana plants, including *F. oxysporum* f. sp. *cubense* race 4, *F. oxysporum* f. sp. *cubense* tropical race 4, *Fusarium oxysporum* f. sp. *niveum* (Fusarium of watermelon), *F. oxysporum* f. sp. *lactucae* (Fusarium wilt of lettuce), and *Ralstonia solanacearum* (bacterial wilt). Serial dilutions of genomic DNA (ranging from 10⁵ to 1 fg) of Foc race 4 and standard template pFoc242 (ranging from 10⁶ to 1 copies) per reaction were subjected to sensitivity evaluation of the Foc race 4 iiPCR assay.

**Detection of field-banana samples**

The Foc race 4 iiPCR assay was used to detect Foc in field-infected banana samples. Sampling criteria of infected banana pseudostems were adhered to according Lin et al. [10]. Specifically, necrosis covering less than 1/3 of the total area of a pseudostem, less than 2/3 but equal to or
more than 1/3 of the total area, and equal to or more than 2/3 of the total area were recorded as mild, moderate, and severe symptoms, respectively. The field-infected banana pseudostems showing varying symptoms were individually sampled, surface-sterilized, and air dried. The surface-sterilized dried banana pseudostems were cut into 1 cm² sections and put onto a Nash-PCNB agar medium for a plate-out assay. Simultaneously, a piece of the banana pseudostems (0.3 g) surrounding each section was used for DNA extraction according to Lin et al. [7]. The DNA samples (50 ng) of symptomatic and symptomless pseudostems were used for further Foc race 4 iiPCR.

Results
Optimization of Foc race 4 iiPCR assay
In order to develop a realizable iiPCR detection system for Foc race 4, the novel iiPCR primer set iiFoc-1/iiFoc-2 and TaqMan probe iipFoc-1 were developed according to the sequence of the Foc race 4-specific marker Foc242. Expected DNA bands were detected by the positive control ITS1/ITS4 in all isolates tested. The results of the Foc race 4 iiPCR detection were in agreement with the results produced using the other two PCR-based detection methods utilizing the Foc TR4-specific primer set FocTR4-F/FocTR4-R and the Foc race 4-specific primer set FocSc-1/FocSc-2 (Table 1). The concentration of TaqMan probe is known to influence the amplification and optical signal detection of iiPCR. Different concentrations (ranging from 0 to 500 nM) of the probe iipFoc-1 were tested to optimize the iiPCR for Foc amplification and signal detection with the POCKIT™ analyzer. The optimal iipFoc-1 probe concentration for the Foc race 4 iiPCR assay was 200 nM with a S/N ratio of 4.36 (standard deviation = 0.17) (Fig 2). Therefore, 200 nM of iipFoc-1 probe was the first priority choice for further Foc iiPCR purposes.

Sensitivity and specificity evaluation of Foc race 4 iiPCR assay
A detection evaluation was performed to determine whether the Foc race 4 iiPCR assay was suitable for the detection of Foc race 4. Plant pathogenic isolates from banana plants, including Foc TR4, Foc ST4, Foc race 2, Foc race 1, Alternaria alternata (Alternaria speckle), Botryosphaeria dothidea (crown rot), Cladosporium musae (Cladosporium speckle), Colletotrichum gloeosporioides (anthracnose), Cordana musae (Cordana leaf spot), F. sonani (fruit rot), F. subglutinans (fruit rot), and as well as other pathogens from non-banana plants, including F. oxysporum f. sp. lactucae (Fusarium wilt of lettuce), F. oxysporum f. sp. niveum (Fusarium of watermelon), and R. solanacearum (bacterial wilt), were subjected to testing in order to assess the specificity of the Foc race 4 iiPCR assay using the POCKIT™ analyzer. The Foc race 4 iiPCR assay was positive for all of the isolates of Foc race 4 tested and negative for all the other pathogens of non-Foc race 4 (Table 1).

In addition, the results of the Foc race 4 iiPCR assay were consistent with those of the rDNA identification method using the universal primer set ITS1/ITS4 and with those of the PCR-based identification techniques using the Foc TR4-specific primer set (FocTR4-F/FocTR4-R) and Foc race 4-specific primer set (FocSc-1/FocSc-2) (Table 1). These data indicated that the Foc race 4 iiPCR assay offers a novel method for detecting Foc race 4 with high specificity.

Serial dilutions of standard template pFoc242 (ranging from 10⁶ to 1 copies) and Foc gDNA (ranging from 10⁵ to 1 fg) were prepared to evaluate the sensitivity of the optimized Foc race 4 iiPCR assay. The Foc iiPCR data demonstrated that a standard template copy ranging from 10⁶ to 1 copies and a non-template-control (NTC) corresponded to average S/N ratios of 4.89±0.07 to 2.19±0.12 and 1.02±0.06, respectively (Fig 3A); and a Foc gDNA ranging from 10⁵ to 1 fg and an NTC corresponded to average S/N ratios of 4.74±0.17 to 1.43±0.08 and 1.02±0.02,
respectively (Fig 3B). In addition, the sensitivity of the Foc race 4 iiPCR assay was not affected with 50 ng banana gDNA added to the reaction mixture containing standard template pFoc242 or the Foc gDNA (S1 Fig). The data indicated that we were able to obtain positive S/N results with high reproducibility even when the template was as low as 1 copy of standard template pFoc242 and 1 fg of Foc gDNA.

Detection of field-infected banana samples by Foc race 4 iiPCR assay

A field detection evaluation was further performed to determine whether the Foc iiPCR was suitable for the detection of Foc race 4 in field-infected banana samples. For this purpose, varying symptomatic banana pseudostems were tested by the Foc iiPCR. The plate-out assay was used to confirm that the symptomatic bananas that were tested were Foc-infected while also indicating that the symptomless bananas that were tested were Foc-infected or Foc-free samples. Foc race 4 iiPCR assays showed average S/N ratios of 4.51±0.13, 4.56±0.05, 2.26±0.73, and 1.10±0.02 in severely symptomatic, moderately symptomatic, mildly symptomatic, and Foc-free pseudostems, respectively (Table 3). All the symptomatic banana pseudostem samples yielded positive Foc race 4 iiPCR results (Table 3), and the Foc race 4 iiPCR results agreed with the symptomatic detection and plate-out assay results, indicating that the Foc iiPCR is suitable for the detection of Foc race 4 in field-infected banana samples even if the infected bananas only exhibit mild symptoms.

We collected a total of 63 banana pseudostem samples (47 Foc-infected pseudostems with mild symptoms and 16 symptomless but Foc-infested pseudostems) from 8 different fields in
Fig 3. Sensitivity evaluation of Foc race 4 TaqMan probe-based insulated isothermal PCR (iiPCR) assay. Serial dilutions of (A) standard template pFoc242 (ranging from $10^6$ to 1 copies) and (B) *Fusarium oxysporum* f. sp. *cubense* (Foc) race 4 genomic DNA (ranging from $10^5$ to 1 fg) were subjected iiPCR assay. The S/N ratios (fluorescent intensity after/fluorescent intensity before) of Foc race 4 iiPCR assay were calculated. Mean S/N ratio of each reaction was plotted against standard template or Foc race 4 genomic DNA. Error bars represent the standard deviations from seven replicate reactions.

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the Pingtung County for Foc race 4 iiPCR detection. These Foc race 4 iiPCR results for detecting the 47 pseudostems with mild symptoms were in agreement with those produced using the plate-out assay (Table 4) and the PCR-based identification method with the Foc race 4-specific primer set (Table 4). The detection rates of the Foc race 4 iiPCR and the PCR-based identification method were 16/16 (100%) and 9/16 (56.25%), respectively, when the 16 symptomless pseudostems were used as test samples. These data indicated that the Foc race 4 iiPCR assay with the primer set iiFoc-1/iiFoc-2 and TaqMan probe iipFoc-1 has high sensitivity for the in planta detection of Foc race 4; and the field-diagnostic iiPCR results seem to be supported by the symptomatic characteristics and plate-out assay results, as well as by those of the other PCR-based identification method.

### Discussion

Foc race 4, which is the most virulent strain among all the races of Foc [5], is economically important as a destructive pathogen which causes Fusarium wilt on banana plants [10,12,15]. Thus far, Foc cannot be controlled with fungicides [15] because it can produce thick-walled chlamydospores which are highly resistant to chemical fumigation [2] and can survive in the infected soil as resting spores for numerous years [1,3–5]. Susceptible varieties cannot be successfully replanted in Foc-infected soil, while commercial banana cultivars resistant to Foc race

### Table 3. In planta detection of *Fusarium oxysporum* f. sp. *cubense* race 4 in field-infected banana by insulated isothermal polymerase chain reaction (iiPCR) method.

| Samples                  | Symptoms | Plate-out assay | Foc race 4 iiPCR results | Mean$^c$ of S/N ratio$^d$ |
|--------------------------|----------|-----------------|--------------------------|---------------------------|
|                          |          | No. positive/total results | No. positive/total results |                           |
| Symptomatic pseudostems  | Severe   | 6/6             | 6/6                      | 4.51 ± 0.13               |
| Symptomatic pseudostems  | Moderate | 6/6             | 6/6                      | 4.56 ± 0.05               |
| Symptomatic pseudostems  | Mild     | 6/6             | 6/6                      | 2.62 ± 0.76               |
| Foc-free pseudostems     | No symptom | 0/6             | 0/6                      | 1.10 ± 0.02               |
| Amplification positive control (10$^3$ copies of clone DNA) | 6/6 | 4.65 ± 0.18 |
| Amplification no template control | 0/6 | 1.07 ± 0.03 |

$^a$ Mild symptoms = less than 1/3 area of pseudostem necrosis; moderate symptoms = less than 2/3 but equal to or more than 1/3 area of pseudostem necrosis; severe symptoms = equal to or more than 2/3 area of pseudostem necrosis.

$^b$ A total of 24 varying symptomatic banana pseudostems were used for plate-out assay.

$^c$ Mean ± standard deviation is presented from six replicate samples collected from six separate pseudostems.

$^d$ S/N ratios were calculated from the values of the fluorescent intensity after/fluorescent intensity before of the Foc race 4 iiPCR assay.

### Table 4. Comparison of Foc race 4 iiPCR and PCR-based identification for field detection.

| Foc-infected pseudostems | Foc race 4 iiPCR | PCR-based identification |
|--------------------------|-----------------|--------------------------|
|                          | Positive | Negative | Total | Positive | Negative | Total |
| Mild symptom samples$^a$ | 47       | 0        | 47    | 47       | 0        | 47    |
| Symptomless samples$^a$  | 16       | 0        | 16    | 9        | 7        | 16    |
| Total                    | 63       | 0        | 63    | 56       | 7        | 63    |

$^a$ A total of 63 *Fusarium oxysporum* f. sp. *cubense* (Foc)-infected samples (including 47 pseudostems with mild symptoms and 16 symptomless but pathogen-infested pseudostems) collected from 8 different fields in Pingtung County were used to test the Foc race 4 iiPCR assay and the PCR-based identification using the Foc race 4 identification primer set FocSc-1/FocSc-2.
4 and TR4 have also not been extensively planted [10]. Therefore, refraining from planting any susceptible banana cultivars in Foc-infected fields, as well as preventing the introduction and dissemination of Foc from diseased to healthy banana plants as much as possible, are the top priorities in disease management strategies of FW.

Thus far, a variety of PCR-based methods which can be used to detect Foc race 4 have been made available [7]. However, gel electrophoresis, which is the standard procedure for analyzing PCR products, is relatively time-consuming. In this study, a TaqMan probe-based race 4 iiPCR assay for rapid in planta detection of Foc race 4 was developed. TaqMan probes derive fluorescence signals from the hydrolysis of a probe by 5’ to 3’ exonuclease activity of Taq polymerase, resulting in a significant increase in S/N ratios (signal intensity after/signal intensity before). The iiPCR fluorescent signals are detectable through use of the relatively simple iiPCR device, the POCKIT™ Nucleic Acid Analyzer. The major advantage of this assay is that, compared with conventional PCR, the Foc race 4 iiPCR assay does not require separate equipment to perform PCR and signal detection.

In this study, a Foc race 4 iiPCR assay was developed using the primer set iiFoc-1/iiFoc-2 and TaqMan probe iipFoc-1 according to the Foc race 4-specific marker OPA02404 (referred to as accession number EU379562) published by Lin et al. [7]. The sequence EU379562 is identical to the other sequence of a F. oxysporum f. sp. cubense isolate FOC-FT marker (referred to as accession number EF155535). The sequence EF155535 had been used for developing a Foc race 4 LAMP assay by Li et al. in 2013 [16]. The DNA marker should be specific to Foc race 4 and previously was tested with over a hundred isolates of F. oxysporum [7,10,16]. Hence, we used the Foc race 4-specific marker for developing the Foc race iiPCR assay. The optimal iiFoc-1 probe concentration for the Foc race 4 iiPCR assay was determined (Fig 2). A variety of diseases affecting banana plants were used for detection evaluation to determine whether the Foc iiPCR assay was suitable for the clinical detection of Foc race 4. The results of the clinical Foc iiPCR detection were supported by those of two reference PCR-based detection methods (Table 1).

The Foc iiPCR is a simple and accurate method for detecting Foc race 4 in a variety of symptomatic pseudostem samples (Table 3). High field-detection reproducibility in Foc race 4 detection by the Foc race 4 iiPCR assay was observed among the results generated from the random 63 field-collected pseudostems with mild symptoms or symptomless. These field-detection reproducibility results were supported by those of traditional plate-out assays and molecular detection assays with the reference primer sets (Table 4).

Several studies have reported the use of the real-time PCR [10], LAMP [16], and RealAmp [18] methods to sensitively detect Foc race 4. The results of the sensitivity evaluation showed that the detection limit of the Foc race 4 iiPCR assay was comparable to that of the published detection methods of Foc race 4. Specifically, the minimum detection concentrations required for our iiPCR, the real-time PCR [10], and LAMP [16] were 1 fg, 1 fg, and 10 fg of Foc race 4 genomic DNA, respectively. The detection sensitivity of the RealAmp was $3.82 \times 10^3$ copies of standard DNA in artificially infested soil [18], a sensitivity value that is difficult to compare with that of the Foc iiPCR assay.

In conclusion, to our knowledge, this is the first study to report the detection of phytopathogenic fungi using a TaqMan probe-based iiPCR assay. We have developed an efficient and sensitive Foc race 4 iiPCR assay for the detection of Foc race 4. The Foc race 4 iiPCR developed in this study provides an alternative to conventional PCR, real-time PCR, LAMP, and RealAmp assays in the detection of Foc race 4. This Foc race 4 iiPCR assay has the potential to serve as a rapid, specific, and sensitive tool for the routine in planta detection of Foc race 4 for the banana.
Supporting Information

S1 Fig. Effect of banana genomic DNA (gDNA) on the detection sensitivity of Foc race 4 TaqMan probe-based insulated isothermal PCR (iiPCR) assay. Fifty ng banana gDNA was added to Foc race 4 iiPCR reaction mixture containing (A) pFOc242 standard template (ranging from 10^6 to 1 copies) or Fusarium oxysporum f. sp. cubense (Foc) race 4 gDNA (ranging from 10^5 to 1 fg) for sensitivity evaluation. The S/N ratios (fluorescent intensity after/fluorescent intensity before) of Foc race 4 iiPCR assay were calculated. Mean S/N ratio of each reaction was plotted against standard template or Foc race 4 gDNA. Error bars represent the standard deviations from seven replicate reactions.

(PDF)

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Author Contributions

Conceived and designed the experiments: YHL. Performed the experiments: YHL YJL TDC LLH TYC. Analyzed the data: YHL YJL. Contributed reagents/materials/analysis tools: YHL YJL. Wrote the paper: YHL PFLC.

References

1. Ploetz RC. Fusarium wilt of banana is caused by several pathogens referred to as Fusarium oxysporum f. sp. cubense. Phytopathology. 2006; 96: 653–656. doi:10.1094/PHYTO-96-0653 PMID: 18943184
2. Shi J, Mueller WC, Beckman CH. Ultrastructural responses of vessel contact cells in cotton plants resistant or susceptible to infection by Fusarium oxysporum f. sp. vasinfectum. Physiol Mol Plant Pathol. 1991; 38: 211–222.
3. Beckman CH, Roberts EM. On the nature and genetic basis for resistance and tolerance to fungal wilt diseases of plants. Adv Bot Res. 1995; 21: 35–77.
4. O’Donnell K, Kistler HC, Cigelnik E, Ploetz RC. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci, USA. 1998; 95: 2044–2049. PMID: 9482835
5. Ghag SB, Shekhawat UKS, Ganapathi TR. Fusarium wilt of banana: biology, epidemiology and management. Int J Pest Manag. 2015; 61: 250–263.
6. Wang CJ, LinYS, Lin YH, Chung WH. Modified primers for the identification of nonpathogenic Fusarium oxysporum isolates that have biological control potential against Fusarium wilt of cucumber in Taiwan. PLoS ONE. 2013; 8: e65093. doi: 10.1371/journal.pone.0065093 PMID: 23762289
7. Lin YH, Chang JY, Liu ET, Chao CP, Huang JW, Chang PFL. Development of a molecular marker for specific detection of Fusarium oxysporum f. sp. cubense race 4. Eur J Plant Pathol. 2009; 123: 353–365.
8. Lin YH, Chen KS, Liou TD, Huang JW, Chang PFL. Development of a molecular method for rapid differentiation of watermelon lines resistant to Fusarium oxysporum f. sp. niveum. Bot Stud. 2009; 50: 273–280.
9. Lin YH, Chen KS, Chang JY, Wen YL, Hsu CC, Huang JW, et al. Development of the molecular methods for rapid detection and differentiation of Fusarium oxysporum and Fusarium oxysporum f. sp. niveum in Taiwan. N Biotechnol. 2010; 27: 409–418. doi: 10.1016/j.nbt.2010.05.005 PMID: 20471505
10. Lin YH, Su CC, Chao CP, Chen CY, Chang CJ, Huang JW, et al. A molecular diagnosis method using real-time PCR for quantification and detection of Fusarium oxysporum f. sp. cubense race 4. Eur J Plant Pathol. 2013; 135: 395–405.
11. Lievens B, Rep M, Thomma BPHJ. Recent developments in the molecular discrimination of formae specialis of Fusarium oxysporum. Pest Manag Sci. 2008; 64: 781–788. doi: 10.1002/ps.1564 PMID: 18335499
12. Ploetz RC. Management of Fusarium wilt of banana: A review with special reference to tropical race 4. Crop Prot. 2015; 73: 7–15.
13. Hwang SC, Ko WH. Cavendish banana cultivars resistant to Fusarium wilt acquired through somaclonal variation in Taiwan. Plant Dis. 2004; 88: 580–588.
14. Su HJ, Hwang SC, Ko WH. Fusarial wilt of Cavendish bananas in Taiwan. Plant Dis. 1986; 70: 814–818.
15. Dita MA, Waalwijk C, Buddenhagen IW, Souza MT Jr, Kema GHJ. A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen. Plant Pathol. 2010; 59: 348–357.
16. Li B, Du J, Lan C, Liu P, Wang Q, Chen Q. Development of a loop-mediated isothermal amplification assay for rapid and sensitive detection of Fusarium oxysporum f. sp. cubense race 4. Eur J Plant Pathol. 2013; 135: 903–911.
17. Zhang X, Zhang H, Pu J, Qi Y, Yu Q, Xie Y, et al. Development of a real-time fluorescence loop-mediated isothermal amplification assay for rapid and quantitative detection of Fusarium oxysporum f. sp. cubense tropical race 4 in soil. PLoS ONE. 2013; 8: e82841. doi:10.1371/journal.pone.0082841 PMID: 24376590
18. Peng J, Zhang H, Chen F, Zhang X, Xie Y, Hou X, et al. (2014) Rapid and quantitative detection of Fusarium oxysporum f. sp. cubense race 4 in soil by real-time fluorescence loop-mediated isothermal amplification. J Appl Microbiol. 2014; 117: 1740–1749. doi: 10.1111/jam.12645 PMID: 25200557
19. Balasuriya UB, Lee PY, Tiwari A, Skillman A, Nam B, Chambers TM, et al. Rapid detection of equine influenza virus H3N8 subtype by insulated isothermal RT-PCR (iiRT-PCR) assay using the POCKIT™ Nucleic Acid Analyzer. J Virol Methods. 2014; 207: 66–72. doi:10.1016/j.jviromet.2014.06.016 PMID: 24992669
20. Wilkes RP, Tsai YL, Lee PY, Lee FC, Chang HFG, Wang HTT. Rapid and sensitive detection of canine distemper virus by one-tube reverse transcription-insulated isothermal polymerase chain reaction. BMC Vet Res. 2014; 10: 213. doi: 10.1186/s12917-014-0213-8 PMID: 25200113
21. Chang HFG, Tsai YL, Tsai CF, Lin CK, Lee PY. A thermally baffled device for highly stabilized convective PCR. Biotechnol J. 2012; 7: 662–666. doi:10.1002/biot.201100453 PMID: 22241586
22. Tsai YL, Wang HTT, Chang HFG, Tsai CF, Lin CK, Peng PH, et al. Development of TaqMan probe-based insulated isothermal PCR (iiPCR) for specific and sensitive on-site pathogen detection. PLoS ONE. 2013; 7: e45278.
23. Tsai YL, Lin YC, Chou PH, Peng PH, Lee PY. Detection of white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions. J Virol Methods. 2012; 181: 134–137. doi:10.1016/j.jviromet.2012.01.017 PMID: 22326658
24. Lung O, Pasick J, Fisher M, Buchanan C, Erickson A, Ambagala A. Insulated isothermal reverse transcriptase PCR (iiRT-PCR) for rapid and sensitive detection of classical swine fever virus. Transbound Emerg Dis. 2015; doi:10.1111/tbed.12318
25. Chua KH, Lee PC, Chai HC. Development of insulated isothermal PCR for rapid on-site malaria detection. Malar J. 2016; 15: 134. doi: 10.1186/s12936-016-1183-z PMID: 26931146
26. Carossino M, Lee PYA, Nam B, Skillman A, Shuck KM, Timoney PJ, et al. Development and evaluation of a reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) assay for detection of equine arteritis virus in equine semen and tissue samples using the POCKIT™ system. J Virol Methods. 2016; 234: 7–15. doi:10.1016/j.jviromet.2016.02.015 PMID: 27036504
27. Nash SM, Snyder WC. Quantitative estimations by plate counts of propagules of the bean root rot Fusarium in field soils. Phytopathology. 1962; 52: 567–572.
28. White TJ, Bruns T, Lee S, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols: A guide to methods and applications. Academic Press, Inc., New York; 1990. pp. 315–322.