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ORIGINAL ARTICLE

Rapid and quantitative detection of *Fusarium oxysporum* f. sp. *cubense* race 4 in soil by real-time fluorescence loop-mediated isothermal amplification

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Keywords
detection, *Fusarium oxysporum* f. sp. *cubense* (Foc) race 4, loop-mediated isothermal amplification, real-time fluorescence loop-mediated isothermal amplification, soil, subtropical race 4 (STR4),tropical race 4 (TR4).

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Abstract

Aims: In this study, a real-time fluorescence loop-mediated isothermal amplification (RealAmp) was developed and evaluated for the rapid and quantitative detection of *Fusarium oxysporum* f. sp. *cubense* race 4 (R4) in soil.
Methods and Results: The LAMP primer set was designed based on previously verified RAPD marker sequences, and the RealAmp assay could specifically detect and distinguish R4 isolates from other related species. The detection sensitivity of the RealAmp assay was approx. $3{\cdot}82 \times 10^3$ copies of plasmid DNA or 10^3 of spores per gram in artificially infested soil, indicating that the method is highly tolerant to inhibitor substances in soil compared to real-time PCR. Combining previously published TR4-specific detection methods with the newly established R4-specific RealAmp assay, an indirect approach to detect and differentiate ST4 isolates was achieved by comparing the detection results of R4 and TR4 simultaneously. The existence of ST4 isolates in China was subsequently confirmed through the developed approach.
Conclusion: The developed RealAmp assay has been confirmed to be a simple, rapid and effective method to detect R4 in soil, which facilitates to further identify and distinguish ST4 isolates through the comparative analysis of detection results between TR4 and R4 simultaneously.
Significance and Impact of the Study: The technique is an alternative quantitative detection method, which will be used for a routine detection service for the soil-borne pathogen in China.

Introduction

Fusarium wilt of banana, caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* (Foc), was first reported in Australia in 1874 and continues to be a major constraint and serious threat to banana (Musa spp.) production worldwide (Bancroft 1876). Based on the pathogenicity to specific banana cultivars, Foc has been classified into four races. Race 1 is virulent to AAA genomic cultivar 'Gros Michel' and AAB Rasthali (Waiter and Stover 1960); race 2 is virulent to 'Bluggoe' of the ABB genomic group (Moore *et al.* 1995); race 3 does not affect banana and are considered as distinct *forma specialis*, *heliconiae*; and race 4, which is divided into tropical (TR4) and subtropical (ST4) groups according to where it was collected (Groenewald *et al.* 2006; Dita *et al.* 2010), mainly causes disease in Cavendish cultivars and in those cultivars susceptible to race 1 and race 2 (Hwang and Ko 2004). Almost all currently popular cultivars are susceptible to race 4, which is considered as one of the major threats to banana production (Lin *et al.* 2009; Dita *et al.* 2010). The disease is now prevalent in all banana-growing regions (Moore *et al.* 1995; Butler 2013). In China, the Fusarium wilt of banana (AAA, Cavendish bananas) was first reported in Guangdong Provence in

2001 and all the isolates were identified as R4 (Li *et al.* 2011).

No fungicides are currently available to effectively control the disease once plants are infected. In infected soils, the pathogen persists by colonizing on nonsusceptible hosts and produces chlamydospores that act as reservoirs of inoculums (Schippers and Van Eck 1981). It has been reported that the Fusarium wilt-resistant banana cultivars were acquired through somaclonal variation (Hwang and Ko 2004) and transgenic bananas (Paul *et al.* 2011; Vishnevetsky *et al.* 2011; Yip *et al.* 2011; Koch *et al.* 2013; Ghag *et al.* 2014). However, no effective varieties or hybrids have been released so far. Therefore, Fusarium wilt of banana is still a major threat to banana production worldwide (Butler 2013). Thus, it is necessary to develop an efficient and reliable technique to detect Foc in soil before bananas are planted.

It is difficult to establish taxonomy systems for *Fusarium* species solely based on morphological characters. However, genetic polymorphisms in DNA sequences within the *Fusarium* species can provide a helpful tool for a more precise identification (Bentley *et al.* 1998). Random amplified polymorphic DNA (RAPD) is one of the PCR-based techniques that is widely used to analyse genetic diversity. Lin *et al.* (2009) firstly identified a RAPD marker specific to R4 isolate (referred as $OPA02_{404}$). The R4-specific PCR (Lin *et al.* 2009) and real-time PCR (Lin *et al.* 2013) were subsequently developed based on the RAPD marker sequence, respectively. Compared to RAPD, single nucleotide polymorphism (SNP) is more effective in differentiating the *Fusarium* species. Therefore, a large number of SNPs in the intergenic spacer region (IGS) of the nuclear ribosomal operon of Foc isolates provide a rich source of genetic diversity to develop TR4-specific PCR detection methods (Dita *et al.* 2010). Thangavelu *et al.* (2012) reported that presence of high level of genetic diversity among the Foc isolates in India using the inter inter-simple sequences repeats (ISSR) analysis, indicating ISSR is a powerful tool for clearly distinguishing the Foc isolates for subsequent management strategies and quarantine purposes (Thangavelu *et al.* 2012). China is the biggest producer of Cavendish bananas (AAA) in the world. The diversity of Foc present in the five banana-growing provinces of mainland China has been clarified by VCG analysis, molecular methods and pathogenicity testing. A new VCG 01213/16-specific primer set was developed to determine occurrence of TR4 in China (Li *et al.* 2013b). Furthermore, Li *et al.* (2013c) identified a TR4-unique 3′-UTR sequence from mutant W2987 isolate, indicating that this genetic locus is possibly associated with virulence and reliable for diagnosis and detection of Foc TR4 (Li *et al.* 2013c).

These marker-based tools are helpful for providing rapid and reliable detection and monitoring of Foc isolates. Besides these reported PCR-based methods mentioned above, an alternative technique, termed loop-mediated isothermal amplification assay (LAMP), is also widely used for the detection of plant pathogens. 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 are complementary to six distinct sequences on the target DNA to be amplified (Notomi *et al.* 2000). Subsequently, the approach to detect the amplified products after staining by fluorescence dye using an ESE-Quant tube scanner (ESE GmbH, Stockach, Germany) was developed for a real-time fluorescence loop-mediated isothermal amplification assay (LAMP). The assay can be performed without expensive equipments or reagents, and therefore, it is a simple and cost-effective technology compared to other DNA-based tests (Lucchi *et al.* 2010; Peng *et al.* 2013; Zhang *et al.* 2013). Based on the established DNA markers of Foc, LAMP was developed to detect the R4 in banana tissues (Li *et al.* 2013a) and RealAmp assay was established for TR4 detection in soil (Zhang *et al.* 2013), respectively. However, no molecular detection technology was reported for ST4 diagnosis due to no effective DNA marker identified so far. As the R4 includes ST4 and TR4, one feasible approach is to distinguish ST4 indirectly by comparing the detection results of R4 and TR4 simultaneously.

The objective of this study is to develop and evaluate a RealAmp assay for rapid and quantitative detection of R4 directly from soil samples. Previously, our group developed the method for detecting tropical race 4 (TR4) isolates in soil (Zhang *et al.* 2013). In combination with the newly established R4-specific RealAmp assay, an indirect detection system was successfully developed to differentiate ST4 isolates through comparative analyses of the detection results on R4 and TR4 isolates. Additionally, a survey was conducted in the banana-growing areas of South China to further determine the occurrence of R4, TR4 and ST4 isolates in China, respectively.

Materials and methods

Fungal isolates and microconidia preparation

Fusarium oxysporum isolates and other isolates used in this study were listed in Table 1. A single spore culture of each tested isolate was grown on potato dextrose agar (PDA) medium plates for 5–7 days at 25°C in the dark. The isolates were maintained in a collection at the Institute of Environmental and Plant Protection, Chinese Academy of Tropical Agricultural Sciences, PR China.

Table 1 Fungal species and isolates used to evaluate the specificity of RealAmp assay

Species/isolate	Original hosts	Origin
Fusarium oxysporum f. sp. *cubense* race 1	Banana	Fujian
F. oxysporum f. sp. *cubense* race 1	Banana	Hainan
F. oxysporum f. sp. *cubense* race 2	Bluggoe	NRRL
F. oxysporum f. sp. *cubense* ST4	Banana	Africa
F. oxysporum f. sp. *cubense* TR4	Banana	Hainan
F. oxysporum f. sp. *cubense*	Banana	Taiwan
F. oxysporum f.sp. *niveum*	Watermelon	Jiangsu
F. oxysporum f. sp. *cucumerium*	Cucumber	Jiangsu
F. oxysporum f. sp. *lactucae*	Lettuce	Jiangsu
F. oxysporum f. sp. *luffae*	Loofah	Jiangsu
F. oxysporum f. sp. *pisi*	Pea	ACCC
Fusarium mangiferae	Mango	NRRL
F. mangiferiae	Mango	MRC
Fusarium sterilihyphosum	Mango	NRRL
Fusarium subglutinans	Mango	M20020
Fusarium mexicanum	Mango	NRRL
Fusarium solani	Smoke tree	CFCC
Fusarium graminearum	Wheat	ACCC
Alternaria musae	Banana	CGMCC
Ascochyta fabae	Horsebean	Fujian
Colletotrichum gloeosporioides	Banana	CGMCC
Cordana musae	Banana	CGMCC
Curvularia fallax	Banana	Taiwan
Mycosphaerella fijiensis	Banana	Hainan
Mycosphaerella melonis	Watermelon	Fujian
Guignardia musae	Banana	Hainan
Rhizoctonia solani	Banana	ACCC
Erwinia chrysanthemi	Banana	ACCC
Bacillus subtilis	Soil	ACCC

NRRL is presented ARS Culture Collection; CGMCC is presented the China General Microbiological Culture Collection Center; MRC is presented Culture collection of the Medical Research Council, South Africa; M20020 from Shanghai Academy of Agricultural Sciences; CFCC is presented China Forestry Culture Collection Center; ACCC is presented Agricultural Culture Collection of China; KSU is presented Culture collection of Kansas State University, Manhattan, Kansas, USA; CATAS is presented Chinese Academy of Tropical Agricultural Sciences.

For microconidia preparation, the cultures were placed in 100 ml of potato dextrose broth (PDB, 20 g glucose and 200 g potato extract in 1 l H_2O) medium in a 250-ml flask. After incubation in dark at 25°C on a rotary shaker for 6 days, the spores were filtered and adjusted to the desired concentration (10^8 spores ml^{-1}) by counting them in a haemocytometer for soil inoculation.

Soil sampling and artificial inoculum

Soil samples were collected from representative banana-growing areas in South China in 2010–2011, including Hainan Island, Guangdong Province, Guang'xi Province and Yunnan Province. Fifteen to twenty soil samples were randomly taken under the infected banana root by shovelling soil below the ground surface adjacent to the root system. All the soil subsamples were taken within 100 mm depth and 10 mm in diameter (Ophel-Keller *et al.* 2008). Samples were transported to a storage room and stored for a maximum of 2 weeks at room temperature. A total of 136 soil samples were collected and used for extracting genomic DNA.

For the artificially inoculated soil samples, 1 ml of R4 spore suspension (about 10^8 spores) was inoculated onto 10 g twice-autoclaved soil substrate in 15-ml conical tubes. The tubes were vortexed at maximum speed for 30 s. The samples were air-dried at ambient temperature and ground in liquid nitrogen to produce a fine powder and stored at −70°C prior to DNA extraction.

Extraction of genomic DNA

Approximately 100 mg freeze-dried mycelium or conidia were ground in liquid nitrogen, and the total genomic DNA of pure cultures was extracted using the E-Z 96® Fungal DNA Kit (Omega Biotek, Norcross, GA) following the manufacturer's protocol.

DNA was extracted from soil samples using a 2% hexadecyltrimethylammonium bromide (CTAB) method, according to Zhang *et al.* (2013). The concentration of extracted DNA was determined using a NanoDrop spectrophotometer ND-2000 (Thermofisher Scientific, Loughborough, UK) by monitoring absorbance at 260 and 280 nm.

Primer design

LAMP primers were designed based on a random amplified polymorphic DNA (RAPD) marker sequence (accession number EF155535.1) using PrimerExplorer V4 software (Eiken Chemical Co. Ltd., Tokyo, Japan) (http://primerexplorer.jp/e/). A forward inner primer FIP (5′-GAGCGCGGTGGCTCAATACGATACCTGTGAAGTCGC-3′) consisted of F1c (the complementary sequence of F1, 5′-GAGCGCGGTGGCTCAATA-3′, nt 254–237) and F2 (5′-CGATACCTGTGAAGTCGC-3′, nt 181–197), and a reverse inner primer BIP (5′-CGCTGGCTTCCGAAAC-TACTTGACAAGAACACCAGAAGC-3′) consisted of B1c (the complementary sequence of B1, 5′-CGCTGGCTT CCGAAACTACT-3′, nt 276–295) and B2 (5′-TGACAA-GAACACCAGAAGC-3′, nt 349–331). The F3 (5′-CG AATGGCAAGAGTCTGTT-3′, nt 160–178) and B3 (5′-T GTTCTGCCAGTTTGACG-3′, nt 376–359) were used as outer primers in this study. All primers were purified by HPLC (Sangon Biotech, Shanghai, China). The primer sequences and their respective binding sites are shown in

Fig. S1. Primer specificity was checked using the basic local alignment search tool (BLAST) against other fungal sequences and human DNA in the nonredundant GenBank database.

RealAmp assay

The RealAmp reaction was performed in 25 μl total volume contained 1·6 μmol l^{-1} each of FIP and BIP, 0·2 μmol l^{-1} each of F3 and B3, 12·5 μl LAMP reaction buffer containing 1·6 mmol l^{-1} dNTPs, 1 mol l^{-1} betaine, 8 mmol l^{-1} $MgSO_4$, 20 mmol l^{-1} Tris-HCl (pH 8·8), 10 mmol l^{-1} KCl, 10 mmol l^{-1} $(NH4)_2SO_4$, 0·1% Tween 20 (Deao Biotechnology Co., Ltd, Guangzhou, China), 8 U of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 0·2 μmol l^{-1} SYTO-9 fluorescent dye (Invitrogen, Carlsbad, CA) and 1 μl of purified template DNA (10 ng μl^{-1}). Following, 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), which was set to collect fluorescence signals at 1-min intervals.

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), a fluorescence unit threshold value was used, and threshold time (*Tt*) was calculated as the time at which the fluorescence was equal to the threshold value. In the plot, the y axis denotes the fluorescence units in millivolts (mV) and the x axis shows the time in minutes.

After the reaction, LAMP products were detected directly by visualization of the solution colour by mixing the pre-added 1 μl of 1 : 10 dilution of SYBR Green I into the reaction solution by slightly centrifugation. Green fluorescence was clearly observed with the naked eye in the positive reaction, whereas the colour remained orange in the negative reaction. The LAMP products (5 μl) were analysed by electrophoresis on a 2% (w/v) agarose gel and subsequently stained with ethidium bromide.

Real-time PCR

The real-time PCR assay designed in this study was performed with the SYBR® Premix *Ex Taq*™ kit (Takara, Dalian, China) using the PRISM® 7500 Fast Real-Time PCR (Applied Biosystems, CA, USA) in a total volume of 25 μl, following the manufacturer's instructions. The real-time PCR with R4 specific FocSc-1/FocSc-2 (5′-CAGGGGATGTATGAGGAGGCTAGGCTA/5′-GTGACAGCGTCGTCTAGTTCCTTGGAG) was designed according to the sequence of a 242-bp DNA fragment (named as Foc_{242}) which was derived from the R4 specific OPA02404 RAPD marker sequence (Lin *et al.* 2009, 2013). The standard curve was constructed with eight 10-fold serial dilutions of plasmid DNA in triplicate as described above. The thermal cycling conditions consisted of an initial denaturation for 10 min at 95°C, followed by 40 cycles at 95°C for 15 s annealing at 52°C for 10 s and extension at 72°C for 15 s.

Specificity and sensitivity test of RealAmp assay

To test the specificity of the RealAmp assay, genomic DNAs isolated from other related fungi listed in Table 1 were used in the analyses. The specificity was also validated by conventional PCR using the specific R4 specific Foc-1/Foc-2 primer set (5′-CAGGGGATGTATGAGGAGGCT-3′/5′-GTGACAGCGTCGTCTAGTTCC-3′) previously described by Lin *et al.* (2009), which has been confirmed to be highly specific to Foc R4 and produced a specific 242-bp amplified band (Lin *et al.* 2009).

To determine the sensitivity of the RealAmp assay and construct standard curve, a 351-bp-specific DNA fragment containing the LAMP target region was amplified by PCR using the OPA02404-F/OPA02404-R (5′-ACTCGTGGCACGGTACTTG-3′/5′-GGAATGCGACGGTATTTTTG -3′) designed based on $OPA02_{404}$ marker sequence (accession number EU379562). The PCR products were subcloned into pMD18-T vector (Takara) and designated as pMD18-T-R4. The plasmid DNA of pMD18-T-R4 was adjusted to a concentration of 1·2 ng μl^{-1} (i.e. $3{\cdot}82 \times 10^8$ copies of plasmid DNA) and diluted in a 1 : 10 series before mixed with extracted Fusarium-free soil DNA. The mixed DNA was used as a reference to assess the detection sensitivity of the RealAmp assay which was compared with that of real-time PCR.

Field detection

To investigate the feasibility of the RealAmp assay for the detection of Foc R4 in the field, a systematic survey for Foc R4 was conducted in 136 field soil samples in banana-growing areas from South China in 2010–2011. The field-collected soil samples were quantitatively analysed through the RealAmp and real-time PCR assays. Furthermore, our group previously developed the method for detecting tropical race 4 (TR4) isolates in soil (Zhang *et al.* 2013). In combination with the newly established R4-specific RealAmp assay, an indirect approach to dif-

ferentiate ST4 isolates through comparative analyses of the detection results on R4 and TR4 isolates simultaneously was developed in this study. The statistical analyses were performed using the SPSS program ver. 17.0. Independent *t*-test and paired *t*-test were used to determine the significance ($P < 0{\cdot}05$) between two methods (Real-time PCR and RealAmp) for each sample and for all samples, respectively.

Results

Specificity test of the RealAmp assay

In the specificity test, the DNA ladder-like band pattern on agarose gel was only observed for products from DNAs of R4 isolates (both ST4 and TR4 isolates) but not from DNAs of any other tested isolates (Fig. 1a). The single 242-bp product was amplified only from R4 isolates, and no amplicons were observed from other pathogens or the control using Foc R4 specific Fon-1/Fon-2 primer pair (Fig. 1b). The colour of RealAmp reactions for Foc R4 isolates changed green but other reactions remained orange after SYBR Green I staining (Fig. 1c). The ESE-Quant Tube Scanner (ESE GmbH) was also used to monitor DNA synthesis in the reactions, by which fluorescence signals were only shown for R4 isolates (Fig. 1d). The sequence of RealAmp smallest amplicon is perfectly matched with the RAPD marker sequence (EF155535) that was used for the design of primers. Thus, the newly established RealAmp assay was proved to be a R4-specific detection method.

Standard curve analysis

Serial dilutions of pMD18-T-R4 plasmid DNA containing the R4 target region was used to evaluate the sensitivity of the newly established RealAmp assay. Gel electrophoresis showed that the RealAmp assay could detect as little as $3{\cdot}82 \times 10^3$ copies of plasmid DNA when mixed with Fusarium-free soil DNA (Fig. 2a), which was identical to the detection level by SYBR Green I staining (Fig. 2b). There was a good linear correlation between the threshold time (T_t) and the initial amount of plasmid DNA ($R^2 > 0{\cdot}99$), confirming that amplification was reliable and that RealAmp assay could be used for DNA quantification with traditional standard curves (Fig. 2c). A standard curve between the threshold time (T_t) against the amount of initial template DNA was thus constructed (Fig. 2d). We also determined that the detection limit of real-time PCR was approx. $3{\cdot}82 \times 10^2$ copies of target plasmid DNA (Fig. 3a). The standard curve produced by the real-time assay revealed a good linearity within the detection limit and a high correlation coefficient between C_t and the amount of template plasmid DNA ($R^2 > 0{\cdot}99$) (Fig. 3b). The sensitivity of real-time PCR was about 10-fold higher than that of the RealAmp assay. All the experiments were performed independently in triplicate with nearly identical results.

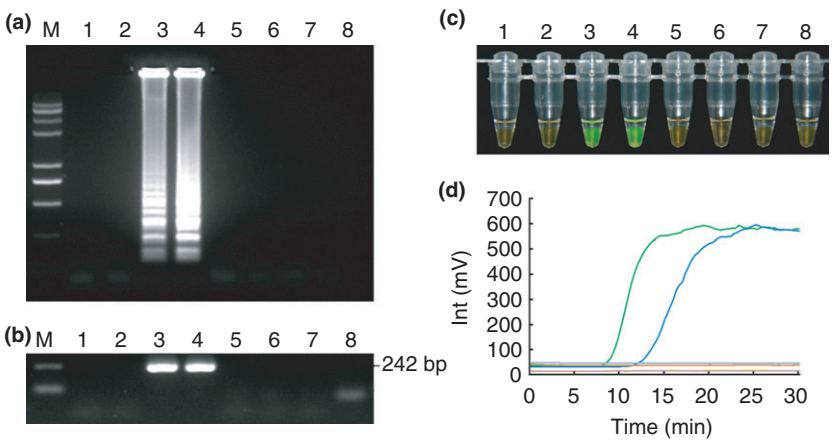

Figure 1 Specificity test of RealAmp assay. (a) Agarose gel electrophoresis analysis of RealAmp amplicons from DNAs of several species. Lanes 1-4 is genomic DNAs of *Fusarium oxysporum* f. sp. *cubense* (Foc) race 1, race 2, subtropical race 4 (ST4) and tropical race 4 (TR4), respectively; Lanes 5-8 is DNAs of *Mycosphaerella melonis*, *F. oxysporum* f. sp. *cucumerium* and *F. oxysporum* f. sp. *niveum*, respectively. Lane M is Trans 2K *plus* II DNA marker. Samples of number 1–8 in Fig. 1b–d is the same as in Fig. 1a. (b) Agarose gel electrophoresis of R4-specific PCR amplification products. (c) Visual inspection of the RealAmp amplicons. The original orange colour of products turned green in a positive reaction and remained orange in negative reaction. (d) The fluorescence unit *vs* time was plotted automatically by the ESE-Quant Tube Scanner. The graph depicts the fluorescence in millivolts (mV) on the y axis and time in minutes on the x axis. (—) 1; (—) 2; (—) 3; (—) 4; (—) 5; (—) 6; (—) 7 and (—) 8.

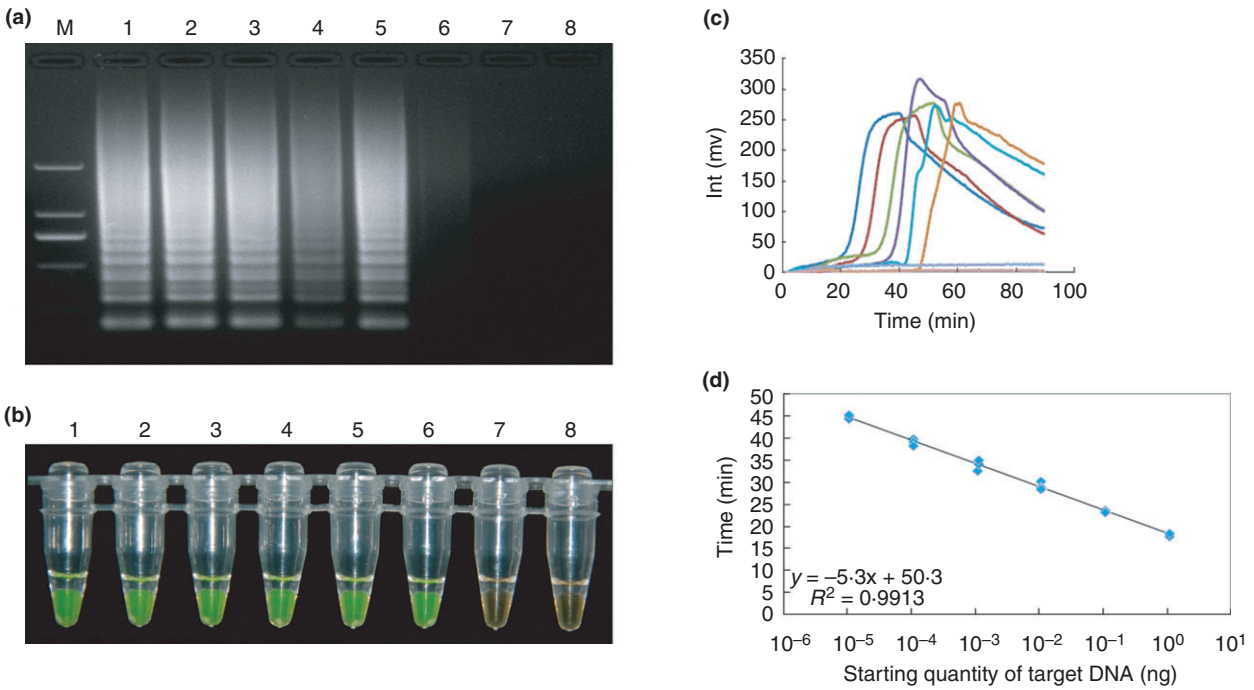

Figure 2 Sensitivity of the RealAmp assay and standard curve construction. (a) Sensitivity of the RealAmp assay using serial dilutions of template plasmid DNA. Lane M, Trans2K *Plus* II DNA marker, lanes 1-8 correspond to serial 10-fold dilutions of plasmid DNA ranging from $3{\cdot}82 \times 10^8$ copies to $3{\cdot}82 \times 10^2$ copies of target plasmid DNA. (b) Visual detection of the RealAmp amplification products. Concentration of template for number 1 to 8 in Fig. 2b is the same as in Fig. 2a. (c) The fluorescence units *vs* time amplification curves plotted automatically using an ESE-Quant Tube Scanner. Concentration of template for number 1 to 8 in Fig. 2c is the same as in Fig. 2a. (d) Standard curve for RealAmp assay. The threshold time (*Tt*) *vs* the amount of initial template plasmid DNAs were plotted using an ESE-Quant Tuber Scanner. (—) $3{\cdot}82 \times 10^8$ copies; (—) $3{\cdot}82 \times 10^7$ copies; (—) $3{\cdot}82 \times 10^6$ copies; (—) $3{\cdot}82 \times 10^5$ copies; (—) $3{\cdot}82 \times 10^4$ copies; (—) $3{\cdot}82 \times 10^3$ copies; (—) $3{\cdot}82 \times 10^2$ copies and (—) Negative Control.

Sensitivity of artificially inoculated soil samples

Both pure spores and artificially infected soil samples were used to evaluate the sensitivity of the newly established RealAmp assay as compared to the real-time PCR. The results showed that RealAmp assay could detect a minimum of DNAs extracted from 10^3 of pure spores (Fig. 4a) and also from 10^3 of spores per gram in artificially infested soil in a RealAmp reaction (Fig. 4b), respectively. The detection limit of real-time PCR could detect DNAs of 10 pure spores (Fig. 4c) and 10^3 of spores in artificially infested soil (Fig. 4d) in a real-time PCR reaction. The detection limit of real-time PCR was 100-fold higher than that of RealAmp assay using DNAs from pure spores. Importantly, the RealAmp assay and real-time PCR assays have the nearly same detection limit for soil samples, indicating that certain substances in soil might inhibit the activity of DNA polymerase in the real-time PCR.

Analysis of field-collected soil samples

wA total of 136 naturally infested soil samples were collected and tested to determine the occurrence of Foc R4 by the RealAmp and real-time PCR. The result showed the detection rates of RealAmp assay (128/136, 94·1%) were slightly lower than real-time PCR (129/136, 94·9%) for the field samples in this study. The quantification of Foc R4 DNA in soil between real-time PCR and RealAmp was statistically analysed with the SPSS software, and no significant difference between the RealAmp and real-time PCR was observed on quantitative detection results (paired *t*-test, $P > 0{\cdot}05$) (Fig. S2, Table S1).

Differentiation ST4 from TR4 within R4 isolates

Our previous study reported a method for detection of TR4 isolates, through which 124 samples were tested to be positive (Zhang *et al.* 2013). Meanwhile, the method was described here for the detection of R4 isolates which contain TR4 and ST4 isolates. Thus, we compared test results simultaneously from both R4 and TR4-specific detection methods to determine the occurrence of ST4 isolate in this study. The comparative analysis demonstrated that 4 ST4 infested soil samples were found in this survey (Fig. S2; Table S1).

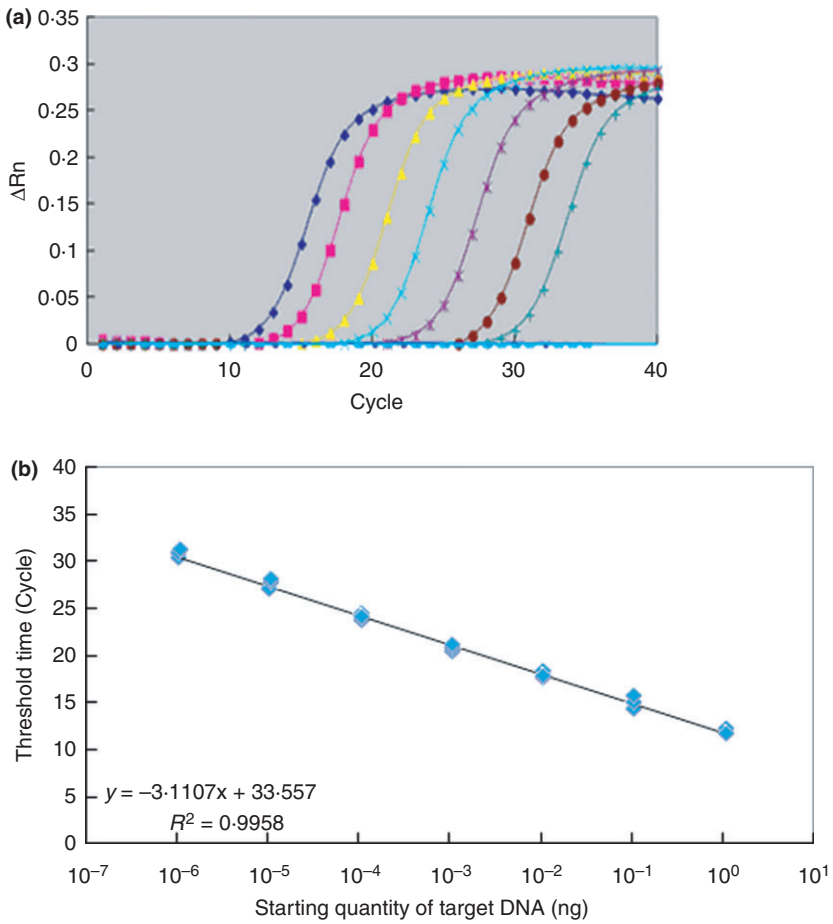

Figure 3 Determination of the detection limit of real-time PCR and standard curve. (a) Sensitivity test of real-time PCR. The real-time fluorescence units are plotted against concentration of initiate plasmid DNA ranging from 3·82 × 10^8 to 3·82 × 10^2 copies of target DNA using a Fast Real-Time PCR cycler. (b) Standard curve calculated from panel A. Standard curve generated using known concentration of 10-fold serially diluted pMD-18-T-R4 plasmid DNA against the threshold time (Tt). Every DNA concentration was measured 3 times. (—♦—) 3·82 × 10^8 copies; (—■—) 3·82 × 10^7 copies; (—▲—) 3·82 × 10^6 copies; (—×—) 3·82 × 10^5 copies; (—✳—) 3·82 × 10^4 copies; (—●—) 3·82 × 10^3 copies; (—+—) 3·82 × 10^2 copies; (—▼—) 3·82 × 10^1 copies and (—) Negative Control.

Discussion

In this study, a RealAmp assay was developed for the rapid and quantitative detection of R4 in soil. Compared to conventional real-time PCR, no expensive reagents and equipments are required in the assay. A portable fluorescent reader (ESE-Quant Tube Scanner) is sufficient to run a RealAmp assay (Lucchi *et al.* 2010; Peng *et al.* 2013; Zhang *et al.* 2013). Besides the quantitative detection using SYTO-9 fluorescent dye, an improved close-tube visual inspection was achieved by addition of 1 μl SYBR Green I to the inner of the lid of the amplification tube prior to start of the reaction, which facilitates rapid screening of samples and minimizes the risk of cross-contamination.

The established RealAmp assay has been proved to be highly specific to Foc R4 isolates, including TR4 and ST4 isolates. Lin *et al.* (2009) firstly assessed the genetic variation between various *Fusarium* species and then screened for a RAPD marker specific to R4 (Lin *et al.* 2009). The specificity test showed that only R4 isolates produced DNA ladder-like patterns. The PCR assay with R4-specific primers and fluorescence signals plotted from ESE-Quant Tube Scanner further confirmed the designed primer set was specific to R4 and available for subsequent application in soil samples.

The RealAmp assay showed high tolerance to inhibitory substances in soil in this study. As Foc is a soil-borne pathogen, artificial inoculated soil samples were used to evaluate the tolerance of RealAmp assay. The sensitivity of the RealAmp assay for the detection of pure spores was 100 times lower than that of real-time PCR (Fig. 4). However, the detection limit of the RealAmp assay for DNA from artificially infested soil was nearly

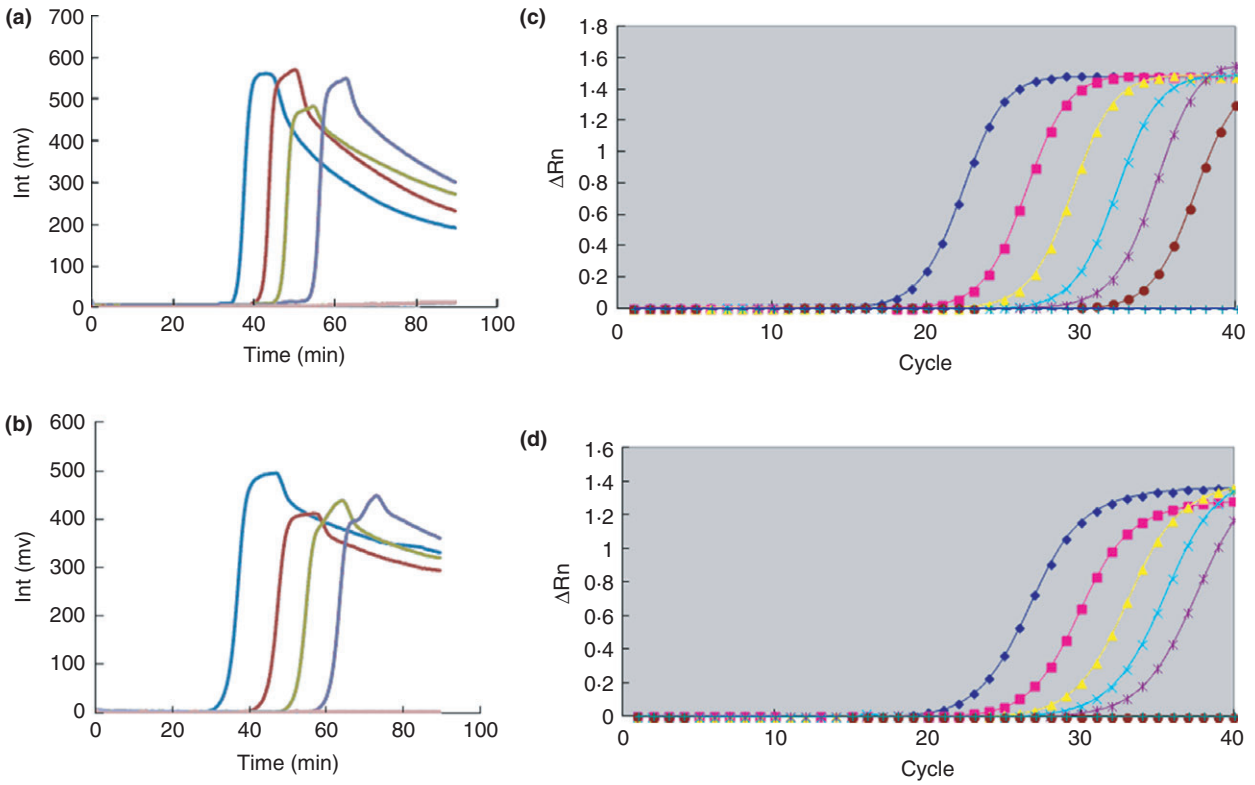

Figure 4 Comparative sensitivity of the RealAmp assay and real-time PCR for the detection of pure spores and spores in artificially infested soil. (a) and (b) are detection of R4 from pure spores and artificially infested soil samples, respectively, using the RealAmp assay. (c) and (d) are detection of R4 from pure spores and artificially infested soil sample, respectively, using real-time PCR. (a,b) (—) 10^6; (—) 10^5; (—) 10^4; (—) 10^3; (—) 10^2; (—) 10^1; (—)10^0 and (—) Negative Control; (c,d) (—♦—) 10^6; (—■—) 10^5; (—▲—) 10^4; (—×—) 10^3; (—✳—) 10^2; (—●—) 10^1; (—+—) 10^0 and (—●—) Negative Control

equal to that of real-time PCR, indicating that the LAMP-based assay has a higher tolerance to soil-borne inhibitory substances. In consistent with our results, LAMP assay for the detection of micro-organisms showed an increased tolerance to inhibitory substances and had a higher detection limit, as compared with PCR method (Kaneko *et al.* 2007).

No molecular methods for detecting ST4 isolates were reported so far due to no DNA marker sequence available. R4 could be further divided into TR4 and ST4, and thus, an indirect approach for the detection and differentiation of ST4 was developed through the comparative analysis of the detection results of TR4 and R4 isolates simultaneously. Referring to the previously reported detection results of TR4 (Zhang *et al.* 2013), 4 field-collected soil samples were tested positive by R4 detection but were negative in TR4 detection. Therefore, these samples were indirectly identified as ST4 isolates in this study. Recently, a survey of population diversity and distribution of Foc isolates in China showed the existence of VCG 0120/15 (Foc ST4) in the provinces of China (Li *et al.* 2013b). Our results are in consistent with the previously published results and further verify that ST4 isolates exist in China indeed (Table S1).

The detection approach developed in this study is clearly different from that in the previously published paper by Zhang *et al.* (2013), in which the method to detect tropical race 4 (TR4) isolates was established and the primers were designed according to the intergenic spacer (IGS) region of the nuclear ribosomal operon with the high SNP diversity (Dita *et al.* 2010). However, the method described here is to detect R4 isolates that include both TR4 and ST4 isolates. The primers were designed based on the RAPD marker sequence, which has been proved to be unique to Foc R4 isolate (Lin *et al.* 2009). Therefore, the two detection methods have different targets and different primers and can be used for the specific detection of Foc TR4 and R4 isolates, respectively.

In summary, the RealAmp assay was successfully developed for detection of R4 in soil, which exhibited a high specificity, sensitivity and tolerance to detection-inhibitory substance in soil. Combining with previously reported TR4-detecting method, we could further identify

and differentiate the ST4 after comparing the detection results of R4 and TR4. And thus, it would be a simple and effective approach for the quantitative detection and monitoring of Foc R4 in soil and be used to evaluate the occurrence and distribution of Foc race's in South China.

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Conflict of Interest

There is no conflict of interest in the submission of this manuscript, and all authors of this manuscript agreed with publication.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Nucleic acid sequence based on a RAPD molecular marker (GenBank accession number EF155535.1) used for designing inner and outer primers.

Figure S2. Partial quantitative results of Foc R4 in field soil samples using the RealAmp assay and real-time PCR method, respectively.

Table S1. Quantification of 136 soil samples by RealAmp assay and real-time PCR method, respectively. Data are the mean (±SD) of three replicates.