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Development of a molecular marker for specific detection of *Fusarium oxysporum* f. sp. *cubense* race 4

Ying-Hong Lin · Jing-Yi Chang · En-Tzu Liu · Chih-Ping Chao · Jenn-Wen Huang · Pi-Fang Linda Chang

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Abstract *Fusarium oxysporum* f. sp. *cubense* is the causal agent of Panama disease of banana. A rapid and reliable diagnosis is the foundation of integrated disease management practices in commodity crops. For this diagnostic purpose, we have developed a reliable molecular method to detect Foc race 4 isolates in Taiwan. By PCR amplification, the primer set Foc-1/Foc-2 derived from the sequence of a random primer OP-A02 amplified fragment produced a 242 bp size DNA fragment which was specific to Foc race 4. With the optimized PCR parameters, the molecular method was sensitive and could detect small quantities of Foc DNA as low as 10 pg in 50 to 2,000 ng host genomic DNA with high efficiency. We also demonstrated that by using our PCR assay with Foc-1/Foc-2 primer set, Foc race 4 could be easily distinguished from other Foc races 1 and 2, and separated other *formae speciales* of *F. oxysporum*.

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Y.-H. Lin · J.-Y. Chang · E.-T. Liu · J.-W. Huang · P.-F. L. Chang (✉)
Department of Plant Pathology,
National Chung Hsing University,
Taichung, Taiwan 402, Republic of China
e-mail: pfchang@nchu.edu.tw

C.-P. Chao
Taiwan Banana Research Institute,
Pingtung, Taiwan 904, Republic of China

Keywords Molecular detection · Panama disease · PCR · RAPD · Reliable diagnosis

Introduction

Fusarium wilt of banana (*Musa* spp.), commonly known as Panama disease caused by *Fusarium oxysporum* f. sp. *cubense* Snyder and Hanson (1940), is one of the most serious fungal diseases in banana, and reported to be one of the major limiting factors for banana production worldwide (Getha and Vikineswary 2002; O'Donnell et al. 1998). Foc in infected soil can survive as a saprophyte for numerous years, and start to infect banana pseudostems during plant cultivation (Beckman and Roberts 1995; O'Donnell et al. 1998). The disruption of water translocation in vascular tissues leads to typical wilt symptoms including foliage chlorosis, necrosis and ultimately drooping of the leaves (Beckman 1990).

Based on the specific pathogenicity toward banana cultivars, Foc can be divided into three races (Groenewald et al. 2006). Race 1 is pathogenic to 'Gros Michel (genome type = AAA)' and 'Silk (AAB)' (Stover and Malo 1972; Su et al. 1986), while race 2 infects 'Bluggoe' (AAB) and other closely related cooking bananas (Moore et al. 1995; Waite and Stover 1960). Race 4 mainly causes disease in Cavendish cultivars as well as those susceptible to race 1 and race 2 (Hwang and Ko 2004; Su et al. 1977). Race 4 is further divided into tropical (T4) and subtropical (S4). The Foc S4 attacks Cavendish cultivars in subtropical

areas such as Taiwan, Canary Islands, South Africa, and Australia (Brake et al. 1990; Gerlach et al. 2000; Ploetz 1990; Ploetz et al. 1990; Su et al. 1986). Conversely, the Foc T4 invades Cavendish cultivars in the tropical regions of Southeast Asia and Australia (Bentley et al. 1998; Ploetz 1994; Ploetz and Pegg 2000). To date no Foc T4 has been reported in Taiwan; however, in the recent survey in 2004, some Taiwan isolates of Foc were grouped as VCG01213/16 (Linda J Smith, personal communication) which was defined as T4 (tropical race 4) according to Bentley et al. (1998) and Ploetz and Pegg (2000).

To date, few effective and economically safe methods to protect banana from Fusarium wilt disease have been developed (Forsyth et al. 2006). The best way to manage Fusarium wilt disease at the present time is based on plant breeding for resistant lines (Diener and Ausubel 2005). It appears that a rapid and reliable diagnosis is the foundation of integrated disease management practices in commodity crops. Conventional field identification of the Panama disease pathogen is time-consuming and destructive (Daniells et al. 1995). Identification of Foc is usually based on morphological characteristics, which requires a great knowledge of *Fusarium* taxonomy (Jurado et al. 2006). Furthermore, *Fusarium* identification with microscopy is inefficient and difficult to detect in plant tissues, which may contain various disease complexes (Yergeau et al. 2005). A quick diagnostic assay for Foc would reduce the use of infected-symptomless plant materials for planting and propagation in plant breeding for resistant lines. Therefore, it is of vital importance that a sensitive and specific diagnostic method to identify Foc pathogen is available.

DNA fingerprinting with random amplification of polymorphic DNA (RAPD) is one of the powerful molecular tools for fungal pathogen identifications (Fungaro et al. 2004; Koike et al. 1995; Mes et al. 1999). The nucleotide amplification with polymerase chain reaction (PCR) is effective and specific, and its high sensitivity allows a direct identification of the pathogen in complex mixtures even when fungal mycelia are invisible under the microscope (Jurado et al. 2006). In this study, we have developed a novel primer set Foc-1/Foc-2 for specific molecular identification and detection of Foc race 4 isolates in Taiwan. Preliminary results have been presented (Chang et al. 2003).

Materials and methods

Growth of fungal and bacterial species

Fusarium wilt pathogens including 96 Foc race 4 isolates in Taiwan confirmed by pathogenicity tests on banana cv. Cavendish, seven reference Foc isolates (ATCC76247 and ATCC96285, race 1; ATCC76257 and ATCC96288, race 2; ATCC76262, ATCC96289, and ATCC96290, subtropical race 4), nine other *F. oxysporum formae speciales*, and one *Fusarium verticillioides* were used in this study (Table 1). The genomic DNA (gDNA) from two other fungal pathogens (*Colletotrichum* and *Phytophthora*) and one bacterium (*Xanthomonas*) were used for comparison (Table 1). A single-spore culture of each tested *Fusarium* isolate was grown on a Nash-PCNB plate (1.5% peptone, 2% agar, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% pentachloronitrobenzene, 0.03% streptomycin, and 0.1% neomycin; Nash and Snyder 1962). *Colletotrichum gloeosporioides* and *Phytophthora infestans* were grown on potato dextrose agar (PDA) plates (200 g l^{-1} of potato extracts, 1% glucose, and 2% agar). Single colonies of *Xanthomonas oryzae* pv. *oryzae* were grown on peptone sucrose agar (PSA) plate (1% peptone, 1% sucrose, 0.1% glutamic acid, pH 7.0, and 2% agar).

DNA isolation

Genomic DNA was extracted according to Dellaporta et al. (1983) with minor modifications. Dried fungal mycelium (1 g), overnight-grown bacterial culture (1.5 ml), and banana pseudostem tissues were snap frozen in liquid nitrogen and ground to fine powders using a mortar and pestle. DNA was extracted with 5 ml modified TNE buffer (100 mM Tris-HCl, pH 8.0; 50 mM Na_2EDTA, pH 8.0; 50 mM NaCl; 8 μM β-mercaptoethanol; 1% SDS and 10 μg ml^{-1} RNase) and incubated at 65°C for 30 min. A 0.33× volume of 5 M potassium acetate was added, mixed and centrifuged at 20,000× *g* for 5 min. The supernatant was transferred into a fresh tube, and mixed with an equal volume of isopropanol to precipitate crude DNA. The samples were incubated at −20°C for 20 min and centrifuged at 4°C, 20,000× *g* for 20 min. The DNA pellet was re-suspended in 200 μl dH_2O, and an equal volume of chloroform/isoamyl alcohol (24:1; *v*/*v*) was added and

Table 1 Isolates of *Fusarium* and other fungal and bacterial pathogens used for PCR amplification

Species	Races	Original hosts	Geographic locations	Providers
F. oxysporum f. sp. *cubense*		Banana (*Musa* sp.)		
ATCC76247 (VCG 0126)	1	Banana	Honduras	ATCC
ATCC96285 (VCG 0124)	1	Banana	SE. Queensland, Australia	ATCC
ATCC76257 (VCG 0124[a])	2	Banana	Honduras	ATCC
ATCC96288 (VCG 0128)	2	Banana	N. Queensland, Australia	ATCC
ATCC76262 (VCG 0121)	S4	Banana	Taiwan	ATCC
ATCC96289 (VCG 0120)	S4	Banana	SE. Queensland, Australia	ATCC
ATCC96290 (VCG 0129)	S4	Banana	SE. Queensland, Australia	ATCC
Foc-7-9	4	Banana	Chiyi, Taiwan	TBRI
Foc-6-2	4	Banana	Hualien, Taiwan	TBRI
Foc-6-3	4	Banana	Hualien, Taiwan	TBRI
Foc-6-5	4	Banana	Hualien, Taiwan	TBRI
Foc-6-6	4	Banana	Hualien, Taiwan	TBRI
Foc-4-1	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-2 (VCG 01213/16[b])	T4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-4	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-5	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-6	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-7	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-8	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-10	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-4-11	4	Banana	Kaohsiung, Taiwan	TBRI
Foc-TN3	4	Banana	Kaohsiung, Taiwan	ARI
Foc-TN5	4	Banana	Kaohsiung, Taiwan	ARI
Foc-7-13	4	Banana	Nantow, Taiwan	TBRI
Foc-7-14	4	Banana	Nantow, Taiwan	TBRI
Foc-7-16	4	Banana	Nantow, Taiwan	TBRI
Foc-7-17	4	Banana	Nantow, Taiwan	TBRI
Foc-7-18	4	Banana	Nantow, Taiwan	TBRI
Foc-7-19	4	Banana	Nantow, Taiwan	TBRI
Foc-7-20	4	Banana	Nantow, Taiwan	TBRI
Foc-7-21	4	Banana	Nantow, Taiwan	TBRI
Foc-7-22	4	Banana	Nantow, Taiwan	TBRI
Foc-7-23	4	Banana	Nantow, Taiwan	TBRI
Foc-T105	4	Banana	Nantow, Taiwan	J.-W. Huang
Foc-T202	4	Banana	Nantow, Taiwan	J.-W. Huang
Foc-3-1	4	Banana	Pingtung, Taiwan	TBRI
Foc-3-3	4	Banana	Pingtung, Taiwan	TBRI
Foc-3-15	4	Banana	Pingtung, Taiwan	TBRI
Foc-3-19	4	Banana	Pingtung, Taiwan	TBRI
Foc-5-1	4	Banana	Pingtung, Taiwan	TBRI
Foc-5-3	4	Banana	Pingtung, Taiwan	TBRI
Foc-5-5	4	Banana	Pingtung, Taiwan	TBRI
Foc-5-7	4	Banana	Pingtung, Taiwan	TBRI
Foc-5-13	4	Banana	Pingtung, Taiwan	TBRI
Foc-5-21	4	Banana	Pingtung, Taiwan	TBRI
Foc-5-25	4	Banana	Pingtung, Taiwan	TBRI
Foc-6-7	4	Banana	Taitung, Taiwan	TBRI
Foc-6-8	4	Banana	Taitung, Taiwan	TBRI
Foc-6-10	4	Banana	Taitung, Taiwan	TBRI
Foc-6-11	4	Banana	Taitung, Taiwan	TBRI
Foc-6-12	4	Banana	Taitung, Taiwan	TBRI

Table 1 (continued)

Species	Races	Original hosts	Geographic locations	Providers
Foc-6-13	4	Banana	Taitung, Taiwan	TBRI
Foc-6-14	4	Banana	Taitung, Taiwan	TBRI
Foc-6-15	4	Banana	Taitung, Taiwan	TBRI
Foc-6-16	4	Banana	Taitung, Taiwan	TBRI
Foc-6-17	4	Banana	Taitung, Taiwan	TBRI
Foc-6-18	4	Banana	Taitung, Taiwan	TBRI
Foc-6-19	4	Banana	Taitung, Taiwan	TBRI
Foc-6-20	4	Banana	Taitung, Taiwan	TBRI
Foc-6-21	4	Banana	Taitung, Taiwan	TBRI
Foc-6-22	4	Banana	Taitung, Taiwan	TBRI
Foc-6-23	4	Banana	Taitung, Taiwan	TBRI
Foc-6-24	4	Banana	Taitung, Taiwan	TBRI
Foc-6-25	4	Banana	Taitung, Taiwan	TBRI
Foc-6-26	4	Banana	Taitung, Taiwan	TBRI
Foc-7-1	4	Banana	Tainan, Taiwan	TBRI
Foc-7-3	4	Banana	Taitung, Taiwan	TBRI
Foc-7-4	4	Banana	Taitung, Taiwan	TBRI
Foc-7-5	4	Banana	Taitung, Taiwan	TBRI
Foc-7-6	4	Banana	Taitung, Taiwan	TBRI
Foc-7-7	4	Banana	Taitung, Taiwan	TBRI
Foc-7-8	4	Banana	Taitung, Taiwan	TBRI
Foc-T12	4	Banana	Taitung, Taiwan	TDARES
Foc-T13	4	Banana	Taitung, Taiwan	TDARES
Foc-T14	4	Banana	Taitung, Taiwan	TDARES
Foc-T31	4	Banana	Taitung, Taiwan	TDARES
Foc-T33	4	Banana	Taitung, Taiwan	TDARES
Foc-T34	4	Banana	Taitung, Taiwan	TDARES
Foc-T35	4	Banana	Taitung, Taiwan	TDARES
Foc-T36	4	Banana	Taitung, Taiwan	TDARES
Foc-T37	4	Banana	Taitung, Taiwan	TDARES
Foc-T38	4	Banana	Taitung, Taiwan	TDARES
Foc-T41	4	Banana	Taitung, Taiwan	TDARES
Foc-T43	4	Banana	Taitung, Taiwan	TDARES
Foc-T44	4	Banana	Taitung, Taiwan	TDARES
Foc-1	4	Banana	Taiwan	TBRI
Foc-3	4	Banana	Taiwan	TBRI
Foc-5	4	Banana	Taiwan	TBRI
Foc-7	4	Banana	Taiwan	TBRI
Foc-8	4	Banana	Taiwan	TBRI
Foc-9	4	Banana	Taiwan	TBRI
Foc-14	4	Banana	Taiwan	TBRI
Foc-15	4	Banana	Taiwan	TBRI
Foc-21	4	Banana	Taiwan	TBRI
Foc-22	4	Banana	Taiwan	TBRI
Foc-23	4	Banana	Taiwan	TBRI
Foc-24	4	Banana	Taiwan	TBRI
Foc-25	4	Banana	Taiwan	TBRI
Foc-26	4	Banana	Taiwan	TBRI
Foc-27	4	Banana	Taiwan	TBRI
Foc-28	4	Banana	Taiwan	TBRI
Foc-29	4	Banana	Taiwan	TBRI
Foc-30	4	Banana	Taiwan	TBRI

Table 1 (continued)

Species	Races	Original hosts	Geographic locations	Providers
F. oxysporum f. sp. *chrysanthemi*				
Foch-11-28		Garland chrysanthemum (*C. coronarium*)	Changhua, Taiwan	J.-W. Huang
F. oxysporum f. sp. *Lactucum*				
Fola-11-13		Lettuce (*L. sativa*)	Yunlin, Taiwan	J.-W. Huang
Fola-32-14		Lettuce	Yunlin, Taiwan	J.-W. Huang
F. oxysporum f. sp. *lilii*				
Foli-F16		Lily (*Lilium* Oriental hybrid 'Casa Blanca')	Changhua, Taiwan	J.-W. Huang
Foli-G16		Lily	Changhua, Taiwan	J.-W. Huang
F. oxysporum f. sp. *Luffae*				
Folu-S167		Loofah (*L. cylindrica*)	Nantow, Taiwan	Y.-S. Lin
Folu-114		Loofah	Nantow, Taiwan	Y.-S. Lin
F. oxysporum f. sp. *lycopersici*				
Foly-4		Tomato (*S. lycopersicum*)	–	WVC/AVRDC
Foly-8		Tomato	–	WVC/AVRDC
F. oxysporum f. sp. *Momordicae*				
Fom-101		Balsam pear (*M. charantia*)	Changhua, Taiwan	Y.-S. Lin
F. oxysporum f. sp. *Niveum*				
Fon-H0103		Watermelon (*C. lanatus*)	Miaoli, Taiwan	J.-W. Huang
F. oxysporum f. sp. *raphani*				
For-4566		Radish (*R. sativus*)	Nantow, Taiwan	J.-W. Huang
F. oxysporum				
Fo-F66		Anoectochilus (*A. formosanus*)	Nantow, Taiwan	S.-P.-Y. Hsieh
F. verticillioides				
Fv		Rice (*O. sativa*)	Tantiz, Taiwan	J.-W. Huang
C. gloeosporioides				
Coll		Banana	Taichung, Taiwan	P.-F. L. Chang
P. infestans				
Phyi109		Tomato	Changhua, Taiwan	J.-W. Huang
X. oryzae pv. *Oryzae*				
Xoo		Rice	–	ARI

S4 Subtropical race 4, *T4* tropical race 4, *ATCC* American-Type Culture Collection (Manassas, VA, USA), *TBRI* Taiwan Banana Research Institute (Pingtung, Taiwan), *WVC/AVRDC* The World Vegetable Centre (AVRDC, Tainan, Taiwan), *ARI* Agricultural Research Institute (Taichung, Taiwan), *VCG* vegetative compatibility group

[a] VCG of ATCC76257 was according to Ploetz and Correll (1988)

[b] VCG of Foc-4-2 was identified by Miss Linda J. Smith from the Plant Protection Unit, Queensland Department of Primary Industries, QDPI, Australia.

mixed thoroughly. After centrifugation at 4°C, 20,000× *g* for 5 min, the upper aqueous phase was transferred to a fresh tube, and 0.1× volume of 3 M sodium acetate (pH 5.2) and 2.5× volume of absolute ethanol were added. After centrifugation at 4°C, 20,000× *g* for 5 min, the supernatant was decanted and the DNA pellet was washed with 300 μl 75% ethanol, allowed to air dry, and finally dissolved in 1× TE buffer (10 mM Tris–HCl, pH 8; 0.1 mM EDTA) for further analysis.

Primer design and RAPD analysis

In order to obtain nucleotide markers that could specifically differentiate Foc race 4 from Foc races 1 and 2, *F. oxysporum formae speciales* infecting other plants and *Fusarium* species, more than 100 random decamers were used for PCR amplification. For RAPD analysis, a 25 μl PCR mixture contained 50 ng gDNA, 1× reaction buffer (10 mM Tris–HCl, pH9.0; 50 mM KC1; 2.5 mM $MgCl_2$), 0.1 mM of each

dNTP, 0.8 μM random decamer primer (Operon Technologies Inc., Alameda, CA, USA), and 2.5 unit *Taq* DNA polymerase (MDBio, Inc., Taipei, Taiwan). The parameters for PCR were denatured at 94°C for 90 s, followed by 30 cycles of denaturing at 94°C for 30 s, touchdown annealing temperatures (30 s) at 36°C, 34°C, and 32°C for 5, 5, and 20 cycles, respectively, and polymerising at 72°C for 60 s, and then a final extension at 72°C for 10 min. RAPD products were subjected to electrophoresis in 2.0% agarose gels.

Specific PCR and sensitivity experiment of the assay

For the sensitivity experiment, a 50 μl PCR mixture contained 200 to 10^{-5} ng Foc-24 gDNA, 1× reaction buffer (10 mM Tris–HCl, pH 9.0; 50 mM KC1; 2.5 mM $MgCl_2$), 0.1 mM of each dNTP, 0.4 μM of each specific primer, and 2.5 unit *Taq* DNA polymerase (MDBio, Inc., Taipei, Taiwan). The parameters for PCR were denatured at 94°C for 60 s, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 68°C for 30 s, and polymerising at 72°C for 90 s, and with a final extension at 72°C for 10 min. PCR products were subjected to electrophoresis in 1.5% agarose gels.

Southern hybridisation

DNA gel blots of RAPD and PCR products were subjected to Southern hybridisation using the Foc race 4-specific DNA fragment of RAPD as a probe. After

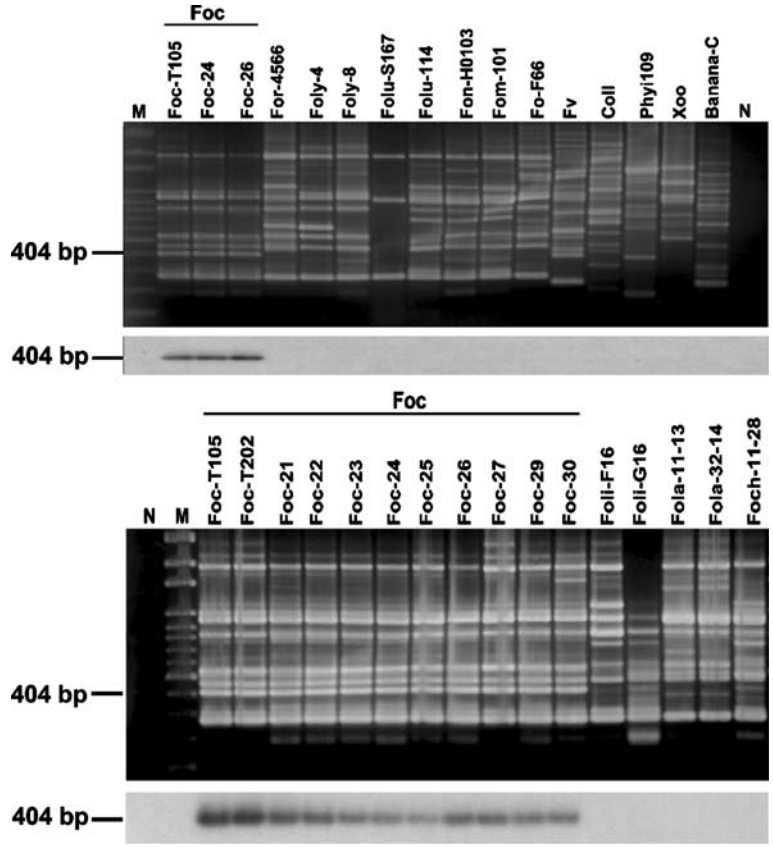

Fig. 1 Fingerprinting of random amplified polymorphic DNA and their Southern blot hybridisation in *Fusarium oxysporum* species. Genomic DNA of 11 *F. oxysporum* f. sp. *cubense* race 4 isolates, 13 isolates of other *F. oxysporum formae speciales*, and one *F. verticillioides* (Fv), one *Colletotrichum* (Coll), one *Phytophthora* (Phyi109), one bacterium (*Xanthomonas oryzae* pv. *oryzae*, Xoo), and the disease-free banana pseudostem (Banana-C) were used as templates (see Table 1) for amplification by using random primer OP-A02. The RAPD products were subjected to Southern blot hybridisation (shown as the *panel* below each ethidium bromide-stained DNA gel pattern with *light background*) using the Biotin-labelled OP-A02 amplified fragment $OPA02_{404}$ as a probe. The 404-bp size DNA band specific to Foc race 4 is indicated on the left. *N* = negative control using sterile dH_2O as the PCR template. *M* = molecular markers of Gen-100 DNA ladder (GeneMark Technology Co., Ltd., Tainan, Taiwan)

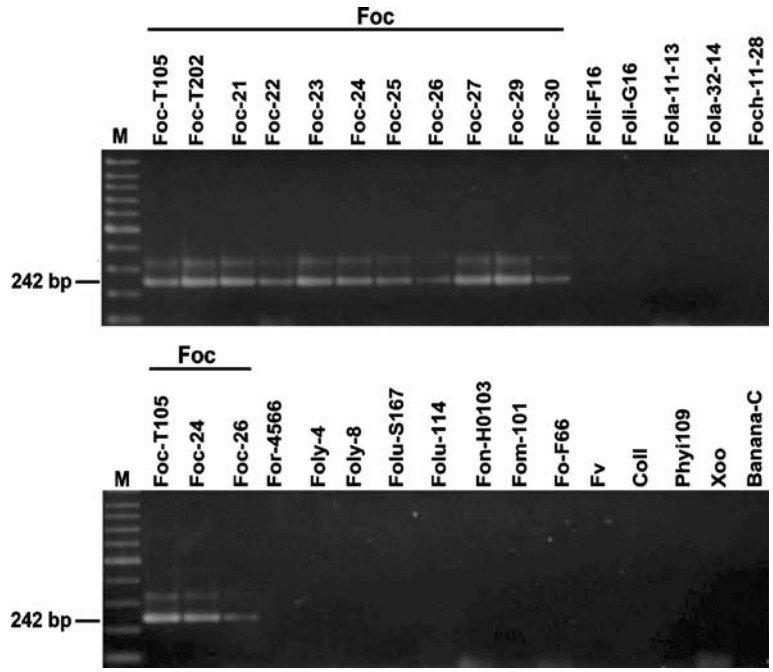

Fig. 2 Amplification of PCR products using the primer set Foc-1/Foc-2 specific to *Fusarium oxysporum* f. sp. *cubense* race 4 isolates. The fungal isolates, bacterial pathogen, and plant material used for extracting genomic DNA as PCR templates (50 ng) are listed in Fig. 1 and Table 1. The location of a 242-bp DNA band specific to *F. oxysporum* f. sp. *cubense* race 4 isolates (labelled as Foc) is indicated on the left. Banana-C = disease-free banana pseudostem gDNA as the PCR template. *M* = molecular markers of Gen-100 DNA ladder

gel electrophoresis, DNA was transferred to nylon membranes (GeneScreen Hybridisation Transfer Membrane, PerkinElmer Life Sciences, Inc., Boston, MA, USA). Probe randomly biotinylated labelling, DNA hybridisation (at 68°C), and subsequent chemiluminescent detection were carried out using NEBlot® Phototope® Kit and Phototope®-Star Detection Kit (New England BioLabs, Inc., Ipswich, MA, USA) following the instruction manuals.

Results

Screening of a RAPD marker specific to *F. oxysporum* f. sp. *cubense* race 4

A RAPD fragment specific to Foc race 4 isolates from Taiwan was amplified by the random primer OP-A02, 5′-TGCCGAGCTG (Fig. 1). This PCR fragment (called $OPA02_{404}$) was purified, cloned into pGEM®-T Easy vector (Promega Co, Madison, WI, USA), and sequenced. The nucleotide sequence (accession number: EU379562) and Southern hybridisation data confirmed that this RAPD fragment was 404 bp long and had the original primer sequence (OP-A02) at both ends. This sequence has been published by Liu (2003) in our group, and it matched the sequence of a *F. oxysporum* f. sp. *cubense* isolate FOC-FT marker for molecular detection and quantitation genomic sequence (accession number: EF155535) deposited to GenBank in December of 2006.

Specificity and the PCR amplification

In order to develop a molecular detection system for Foc race 4 isolates in Taiwan, a specific primer set Foc-1/Foc-2 (5′-CAGGGGATGTATGAGGAGGCT/5′-GTGACAGCGTCGTCTAGTTCC) was designed from the $OPA02_{404}$ nucleotide sequence (accession number EU379562, nt79-nt99/nt300-nt320) for PCR amplification. A 242-bp size fragment was produced by PCR from Foc race 4 gDNA. This primer set was able to amplify the corresponding DNA fragment of 242 bp (called Foc_{242}) only from gDNA of 11 Foc race 4 isolates in Taiwan but not from gDNA of any other tested isolates (Fig. 2). The specificity is the

most important premise for developing a molecular diagnosis protocol. Therefore, we confirmed that the Foc_{242} marker is present in all 99 tested isolates of Foc race 4 (96 Taiwanese and three reference isolates as listed in Table 1; see supplement data for the other Taiwanese isolates not shown in Figs. 2 and 3). Moreover, using the gDNA of seven Foc reference isolates (race 1, 2, and S4) from the American-Type Culture Collection and one T4 isolate (Foc-4-2), both PCR products, $OPA02_{404}$ (Fig. 3a) and Foc_{242} (Fig. 3b), were present in all the tested Foc race 4 isolates (both T4 and S4) but not in the tested race 1 and 2 isolates.

Sensitivity of the Foc race 4-specific PCR

Serial dilutions of gDNA of Foc-24 isolate, ranging from 200 to 10^{-5} ng, were prepared to test the sensitivity of Foc race 4-specific PCR assay. It appeared that primer set Foc-1/Foc-2 was able to amplify the 242 bp-size fragment as low as 10 pg (10^{-2} ng) of gDNA in a 25 μl reaction mixture (Fig. 4a). Using $OPA02_{404}$ fragment (which includes the whole region of Foc_{242}) as a probe for Southern hybridisation, the sensitivity to detect Foc_{242} PCR product was enhanced to 0.1 pg (10^{-4} ng; Fig. 4b). In addition, the sensitivity of PCR assays with primer set Foc-1/Foc-2 was not affected with different amounts of banana gDNA (50 ng to 2 μg) added to the reaction mixture containing 25 ng of Foc-24 gDNA (Fig. 5).

In order to determine the sensitivity of our PCR detection system using primer set Foc-1/Foc-2 against fungal mycelia present in banana tissues, gDNAs were extracted from the mixtures of dried Foc-24 mycelia and fresh diseased-free banana pseudostem at various ratios. Fifty nanogram of gDNA mixture was subjected to PCR amplification. The results indicated that as low a ratio of 0.005 (dry weight of fungal

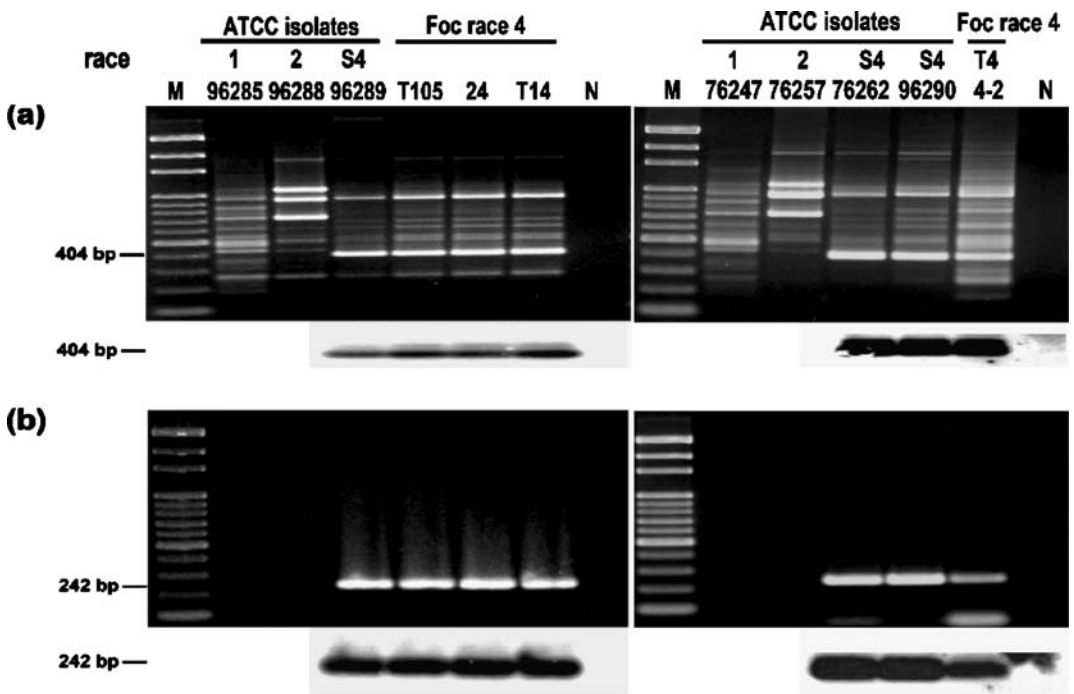

Fig. 3 Ethidium bromide-stained and DNA gel blot hybridisation patterns of (**a**) RAPDs and (**b**) PCR products using genomic DNA samples of *Fusarium oxysporum* f. sp. *cubense* subtropical (S4) and tropical (T4) race 4 isolates as templates. The primers used for RAPDs and PCR were random primer OP-A02 and the specific primer set Foc-1/Foc-2, respectively. All tested gDNA samples of the seven reference isolates (labelled as 'ATCC isolates') and four Foc race 4 isolates from Taiwan (labelled as 'Foc race 4'), including the Foc-4-2 isolate which belongs to T4, are as listed in Table 1. The DNA gels were subjected to Southern hybridisation (shown as the panel below each ethidium bromide-stained DNA gel pattern with light background) using the Biotin-labelled $OPA02_{404}$ as a probe. The locations of 404- and 242-bp DNA bands specific to Foc race 4 isolates are indicated on the left. *N* = negative control using sterile dH_2O as the PCR template. *M* = molecular markers of Gen-100 DNA ladder

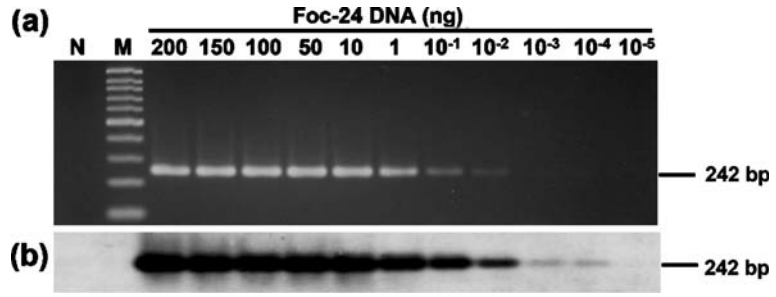

Fig. 4 Detection sensitivity of the primer set Foc-1/Foc-2 amplified fragment in genomic DNA of *Fusarium oxysporum* f. sp. *cubense* race 4 isolate Foc-24. A serial of dilutions of Foc-24 gDNA ranging from 200 to 10^{-5} ng were used as templates. The PCR products (**a**) were subjected to Southern blot hybridisation (**b**) using the Biotin-labelled amplified fragment $OPA02_{404}$ as a probe. The location of the 242-bp size DNA band specific to Foc race 4 is indicated on the right. *N* = negative control using sterile dH_2O as the PCR template. *M* = molecular markers of Gen-100 DNA ladder

mycelia to fresh weight of banana pseudostem, 1 g of fresh weight equals to 0.06 g of dry weight of banana pseudostem) could generate the Foc_{242} DNA fragment by PCR (Fig. 6).

Foc-1/Foc-2, the primer set specific to Foc race 4 isolates, was also used to test naturally infected banana tissues collected from the field. It appeared that the Foc_{242} DNA fragment could easily be amplified in gDNA of symptomatic banana pseudostems, collected from two different fields, by PCR (Fig. 7, lanes 1–6). In addition, in the gDNA of symptomless banana leaves collected from the same two fields, Foc_{242} DNA was also amplified even though the amplicons were very faint (Fig. 7 lanes 7–12).

Discussion

PCR assays have been implemented successfully for identification and detection of economically important *Fusarium* species such as *Fusarium avenaceum* (Schilling et al. 1996; Turner et al. 1998), *Fusarium culmorum* (Klemsdal and Elen 2006; Nicholson et al. 1998; Schilling et al. 1996), *Fusarium graminearum* (Nicholson et al. 1998; Schilling et al. 1996; Yoder and Christianson 1998), *Fusarium langsethiae* (Wilson et al. 2004), *Fusarium moniliforme* (the official name is now *F. verticillioides*; Möller et al. 1999), *Fusarium subglutinans* (Möller et al. 1999), *Fusarium poae* (Parry and Nicholson 1996), *Fusarium sambucinum* (Yoder and Christianson 1998), *Fusarium sporotrichioides* (Wilson et al. 2004) and *Fusarium venenatum* (Yoder and Christianson 1998). Most of these molecular techniques are based on the development of species-specific primers.

The $OPA02_{404}$ DNA marker was not amplified from gDNA of nine tested *F. oxysporum formae speciales*, which were not banana pathogens, one non-pathogenic *F. oxysporum* isolated from banana field soil (Fo-DK1; Chang 2005), one other *Fusarium* sp., two other fungal species (*C. gloeosporioides* and *P. infestans*), and one bacterium strain (*X. oryzae* pv. *oryzae*). Furthermore,

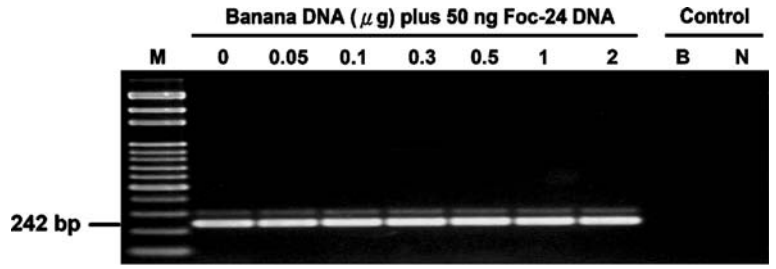

Fig. 5 Effect of plant genomic DNA on the detection sensitivity of the primer set Foc-1/Foc-2 amplified DNA fragment in *Fusarium oxysporum* f. sp. *cubense* race 4 isolate Foc-24. In 50 ng gDNA of Foc-24 isolate, 0.05 to 2 μg gDNA of disease-free banana pseudostem was added to each PCR. The location of a 242-bp DNA band specific to Foc race 4 is indicated on the left. *0* = positive control using only 50 ng Foc-24 gDNA without any banana gDNA as the PCR template. B = PCR control using 2 μg gDNA of disease-free banana pseudostem only as the template. *N* = negative control using sterile dH_2O as the PCR template. *M* = molecular markers of Gen-100 DNA ladder

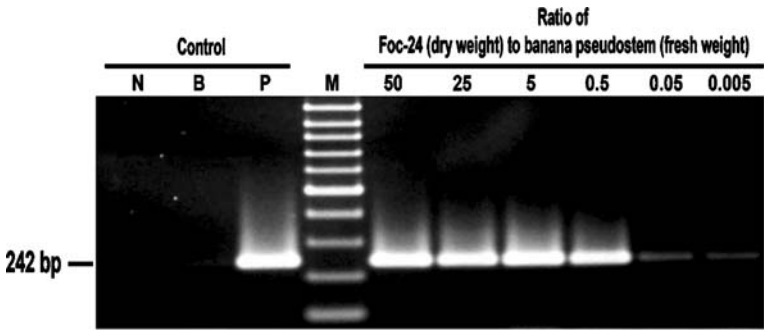

Fig. 6 Detection sensitivity of the primer set Foc-1/Foc-2 amplified fragment in dried mycelia of *Fusarium oxysporum* f. sp. *cubense* race 4 isolate Foc-24 in the presence of disease-free banana tissues. Genomic DNA was extracted from dried fungal mycelia plus fresh plant tissues with a different ratio (0.005 to 50) of fungal dry weight to plant fresh weight. For each PCR, 50 ng of total gDNA was used as the template. The location of a 242-bp DNA band specific to Foc race 4 is indicated on the left. *B* = PCR control using 1 μg gDNA of disease-free banana pseudostem only as the template. *N* = negative control using sterile dH_2O as the PCR template. *P* = positive control using 50 ng Foc-24 gDNA as the PCR template. *M* = molecular markers of Gen-100 DNA ladder

different race isolates from two different geographic regions (ATCC76247 and ATCC96285 for race 1; ATCC76257 and ATCC96288 for race 2; ATCC76262, ATCC96289, and ATCC96290 for subtropical race 4) showed dissimilar RAPD patterns with the random primer OP-A02 amplification. Moreover, the OP-A02 RAPD patterns of the three subtropical race 4 (S4) isolates tested were similar but different from that of the tropical race 4 (T4) isolate (Foc-4-2). In addition, Foc_{242} was present in all Foc race 4 (both S4 and T4) isolates tested including 96 Taiwanese and three reference S4 (ATCC76262, ATCC96289, and ATCC96290) isolates. Therefore, the $OPA02_{404}$ and Foc_{242} amplified fragments of Foc isolates could be used as molecular markers for identification and detection of Foc race 4 in Taiwan. In the future, more Foc isolates from various geographic origins, races differentiated by pathogenicity tests, will be used to confirm the specificity of our PCR assay method to the tropical race 4 (T4) isolates worldwide.

The Foc-1/Foc-2 primer set occasionally amplified an unexpected amplicon of approximately 300 bp in our PCR assays. This DNA fragment, named Foc_{303} according to Chang (2005), was sequenced and compared with that of the Foc_{242} (Chang 2005). This Foc_{303} fragment was only amplified by the Foc-2 primer. However, the DNA sequences of Foc_{242} and Foc_{303} are dissimilar.

The set of Foc-1/Foc-2 primers presented in this work allowed us to process small amounts of DNA

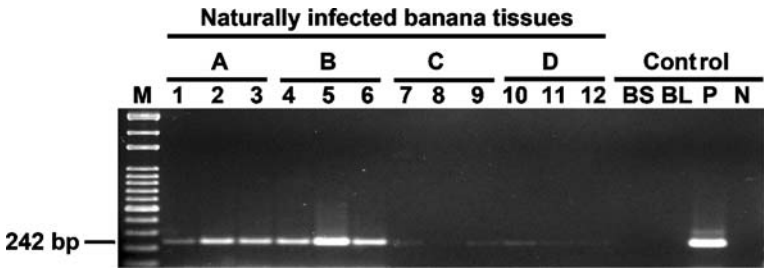

Fig. 7 Molecular detection of *Fusarium oxysporum* f. sp. *cubense* race 4 in naturally infected banana tissues. Genomic DNA samples extracted from two symptomatic pseudostems (*A* and *B*) and two symptomless leaves (*C* and *D*) of bananas collected from two different fields were used for PCR by primer set Foc-1/Foc-2. Three regions (10 cm^2 each) of each pseudostem (*lanes 1–3* for *A*, and *lanes 4–6* for *B*) and leaf (*lanes 7–9* for *C*, and *lanes 10–12* for *D*) were sampled for gDNA extraction for PCR amplification. For each PCR, 50 ng of total gDNA was used as the template. The location of a 242-bp DNA band specific to Foc race 4 is indicated on the left. *BS* = PCR control using 50 ng gDNA of disease-free tissue-cultured banana pseudostem as the template. *BL* = PCR control using 50 ng gDNA of disease-free tissue-cultured banana leaf as the template. *N* = negative control using sterile dH_2O as the PCR template. *P* = positive control using 50 ng Foc-24 gDNA as the PCR template. *M* = molecular markers of Gen-100 DNA ladder

samples and obtain the detection results within hours, in comparison with the time-consuming traditional agar plating or pathogenicity tests which may take days or weeks. Agar plating to differentiate *Fusarium* species requires the knowledge of morphological characters whereas pathogenicity tests to identify *formae speciales* of *F. oxysporum* are labour-intensive.

The sensitivity of our PCR assay was comparable to those reported for *F. culmorum*, one of the causal agents of wheat head blight disease (Nicholson et al. 1998; Schilling et al. 1996). However, with a nested PCR method, the detection sensitivity was increased up to 5–50 fg for the detection of *F. culmorum* in cereal samples (Klemsdal and Elen 2006). For PCR, usage of more than one primer pair in a reaction could result in a higher probability of cross-annealing with non-selective templates, such as plant gDNA, to generate non-specific PCR products, which might interfere with the result interpretation. In our study, amplification of the Foc race 4-specific marker, Foc_{242}, was not affected by the presence of banana gDNA. Therefore Foc_{242} is suitable for detecting pathogen Foc in infected banana.

It appears that our PCR diagnosis protocol with the primer set Foc-1/Foc-2 was applicable to screen naturally Foc-infected banana samples. Even though positive results were obtained by PCR, however, we were able to recover Foc only from the diseased banana pseudostems but not the symptomless banana leaves by a plate-out assay. These results suggest that the PCR assay we developed here is more sensitive than the plate-out assay. Nevertheless, we could not rule out the possibility that the leaf samples (0.5×2 cm^2 of leaf area) for the plate-out assay were free of Foc, but the nearby leaf area (at least 10 times larger than those for the plate-out assay) picked for PCR contained Foc. On the other hand, in combination with Southern blot hybridisation, our PCR assay with the Foc-1/Foc-2 primer set could increase sensitivity 100-fold. Therefore, the developed molecular detection method with the primer set Foc-1/Foc-2 may lead to efficient disease management practices for banana production due to quick and accurate diagnosis of Fusarium wilt disease, be beneficial to rapidly test the presence of Foc race 4 in breeding materials of banana for resistance to Foc race 4, and be useful for basic research in epidemiology and fungal population genetics (Schilling et al. 1996).

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