Histological and Gene Expression Analyses in Banana Reveals the Pathogenic Differences between Races 1 and 4 of Banana Fusarium Wilt Pathogen

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ABSTRACT

Fusarium oxysporum f. sp. *cubense*, the causative agent of Panama disease, is classified into three races: *Foc1*, *Foc2*, and *Foc4*. However, the histological characteristics, the accumulation of fusaric acid (FA), and resistant gene expression in banana infected with different races remain unclear. In this study, we compared the infection processes, FA contents, and gene expression levels in a Cavendish banana cultivar (*Musa* AAA Brazilian) inoculated with *Foc1* and *Foc4*. Results showed that *Foc4* can rapidly extend from the roots to the leaves, whereas *Foc1* expands slowly from the roots to the rhizomes but cannot expand further upward. In addition, the colonization of plants by *Foc4* was significantly higher compared with *Foc1*, as was the content of FA in those infected plant tissues. We observed that a large amount of starch granules was produced in the rhizomes and the number of starch granules was significantly higher after infection with *Foc1* than after infection with *Foc4*. We further found that starch has an important inhibitory effect on the phytotoxicity

induced by FA, thus leading to more resistance to the pathogens in the plants with high amounts of starch accumulation than in those with a low amount of starch accumulation. Moreover, the expression levels of 10 defense-related genes were analyzed and the results showed that the induction levels of those genes were higher after infection with *Foc1* than after infection with *Foc4*. These results suggest that the observed differences in the invasion of host tissues and FA accumulation, and the number of starch granules and expression of defense-related genes, may contribute to a difference in virulence between the two races and the resulting difference in host resistance response, respectively.

Keywords: analytical and theoretical plant pathology, banana, fusaric acid, *Fusarium oxysporum* f. sp. *cubense*, gene expression, histological observation, mycology, pathogenic differences, starch granules.

Banana (*Musa* spp. AAA) is a principal fruit crop in tropical and subtropical regions worldwide. However, banana production is threatened by Fusarium wilt, a highly destructive fungal disease (Stover 1962). Fusarium wilt of banana, also called Panama disease, is caused by the soilborne fungus *Fusarium oxysporum* f. sp. *cubense* (Ploetz 2006). The disease was first reported in Australia in 1876, and it caused significant damage in export plantations in the Western tropics before 1960. The pathogen invades banana roots or root injury sites, colonizes and occludes the vascular tissues, and then induces a reddish-brown discoloration of the rhizomes and pseudostems, leading to leaf collapse and, finally, the death of banana plants (Stover 1962). In addition, *F. oxysporum* f. sp. *cubense* can survive in the soil for decades (Ploetz 1994). Soils infected with *F. oxysporum* f. sp. *cubense* cannot be replanted with susceptible cultivars.

F. oxysporum f. sp. *cubense* can be classified into three physiological races, according to their degree of pathogenicity against different host cultivars (Dita et al. 2010; Ploetz 2006). Race 1 (*Foc1*) attacks the cultivar Gros Michel (*Musa* spp., AAA) and has caused epidemics in the past century. Race 2 (*Foc2*) affects Bluggoe

(*Musa* spp., ABB), as well as other cooking banana cultivars, and race 4 (*Foc4*) causes diseases to Cavendish banana cultivars (*Musa* spp., AAA) and all other banana cultivars susceptible to *Foc1* and *Foc2* (Ploetz 2006). Fusarium wilt caused by *Foc1* destroyed the Gros Michel banana and was responsible for significant yield losses earlier in the last century (Ploetz et al. 1990; Stover 1962). Eventually, *Foc1*-susceptible Gros Michel banana was replaced by resistant Cavendish cultivars. However, *Foc4*, which can attack Cavendish cultivars, was reported in Taiwan in 1967 (Ploetz 2006). To date, the present production of banana is largely restricted by Fusarium wilt caused by *Foc4* because most banana plants are Cavendish cultivars (Dita et al. 2010; Ploetz 2006; Stover and Malo 1972).

Currently, various strategies have been employed to control this destructive pathogen; however, the use of resistant cultivars is the most acceptable and economical to deal with Fusarium wilt (Michielse and Rep 2009). Although researchers have been striving to improve the resistance of banana, commercially available varieties to resistant Fusarium wilt disease are limited thus far. Investigation of the interaction between banana and *F. oxysporum* f. sp. *cubense* will provide new insights into the mechanism by which the host plant responds to the disease and may lead to the discovery of efficient ways to control banana Fusarium wilt disease.

Most previous studies focused on the development of resistant Cavendish cultivars (Getha and Vikineswary 2002; Li et al. 2015; Nel et al. 2006), molecular detection (B. Li et al. 2013; Peng et al. 2014; Zhang et al. 2013), biological control by using beneficial microorganisms that colonize the banana rhizosphere (Fu et al. 2017; Naik et al. 2008), pathogenesis-related fungal genes (Fraser-Smith et al. 2014; Sutherland et al. 2013), resistance-related genes

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of plants (Peraza-Echeverria et al. 2008), and disease control (Akila et al. 2011; Zacky and Ting 2015). Studies on the infection process of *F. oxysporum* f. sp. *cubense* mainly focused on susceptible banana varieties inoculated with *Foc1*, *Foc4* (Beckman et al. 1961; Li et al. 2011; Waite 1977; Wardlaw 1931), or both (Guo et al. 2015). These studies suggest that *F. oxysporum* f. sp. *cubense* can penetrate the susceptible roots of banana cultivars, expand through the vascular organization, and, finally, cause wilt in banana plants. Xiao et al. (2013) suggested that effective retardation of the *Foc4* hyphal movement from the rhizomes to the pseudostems may delay banana wilt disease. Li et al. (2017) indicated that direct root penetration and rhizome vascular colonization are the key steps of successful *F. oxysporum* f. sp. *cubense* infection. However, insufficient information is available about the differences in the infection processes between *Foc1* and *Foc4*, especially how both races expand from the roots to the upper parts of banana plants and the different effects of these races on the histological characteristics and defense-related gene expression patterns in each part of banana plants.

In addition, fusaric acid (FA), a nonspecific toxin produced by *Fusarium* spp., is known for its high phytotoxicity (Gapillout et al. 1996). Although several studies on the mode of action of FA have been conducted (Niehaus et al. 2014; Stipanovic et al. 2011), the role of FA in pathogenicity differences between *Foc1* and *Foc4* to banana is poorly understood.

In the current investigation, two green fluorescent protein (GFP)-tagged isolates of *Foc1* and *Foc4* were developed and utilized to study the infection process on a Cavendish cultivar, Brazilian, susceptible to *Foc4* but resistant to *Foc1*, by using confocal laser-scanning microscopy (LSM). The root, rhizome, pseudostem, leaf sheath, and leaf tissues of this cultivar at different time points after inoculation with *Foc1* and *Foc4* were investigated. Histological differences, FA contents, and defense-related gene expression patterns in the different tissues of Brazilian plants infected by *Foc1* and *Foc4* were compared to understand the pathogenicity differences between races *Foc1* and *Foc4*.

MATERIALS AND METHODS

Plant materials. Cavendish banana cultivar Brazilian (*Musa* spp. AAA) was used in this study. Each plantlet with five leaves and approximately 15 cm in height was transplanted into a plastic pot (10 cm in diameter) containing sterile coconut chop medium. All banana plantlets were kept in a constant-temperature incubator at 28°C with a photoperiod of 16 h of light and 8 h of darkness and a light intensity of 5,000 Lux.

***Fusarium* strains and GFP-tagged modification.** Strains of *Foc1* FJZ3 (VCG 01221) and tropical *Foc4* XJZ2 (VCG 01216) were modified with the *GFP* gene by using the sGFP expression cassette from a vector gGFP (Lorang et al. 2001) provided by Professor Amir Sharon (Tel Aviv University, Israel). *Agrobacterium tumefaciens* strain AGL-1 and binary vector pBHT1 were provided by the South China Agricultural University fungal laboratory. Plasmid construction and *A. tumefaciens*-mediated transformation (ATMT) were described by Mullins et al. (2001). The growth characteristics and pathogenicity of GFP-tagged *F. oxysporum* f. sp. *cubense* were examined using the procedures described by Li et al. (2007).

Plant inoculation. The GFP-tagged isolates of *Foc1* and *Foc4* were used to observe the infection process. Before the research, we performed inoculation experiments with *F. oxysporum* f. sp. *cubense* and confirmed that Brazilian was susceptible to *Foc4* and resistant to *Foc1*. Plants with healthy root systems were selected for inoculation, and plant inoculation was performed as described by Van Den Berg et al. (2007), with some modifications. Plant roots were soaked in 800 ml of GFP-tagged *Foc4* or GFP-tagged *Foc1* spore suspension at 10^6 conidia/ml for 30 min, then planted back to the pots. The mock-inoculated control plants were treated with

sterile distilled water (mock control). Afterward, the infected plantlet tissues were harvested at 13 different time points: 24, 48, 72, 96, 120, 144, 168, 216, 264, 312, 360, 480, and 720 h after inoculation. In total, 10 plants were prepared for each time point, and each experiment was repeated thrice.

Microscopic observation. The roots, rhizomes, pseudostems, leaf sheaths, and leaves of the plants inoculated with either *Foc1* or *Foc4* were examined for infection process and colonization at 13 different time points after inoculation. For microscopic observation, banana tissues were washed in sterile distilled water, then cut into thin sections along the vertical and longitudinal axes by hand sectioning. Then, the tissue sections were placed on a microscope slide, submerged in a water droplet, and covered with a glass cover slip. Microscopic observation was carried out under a confocal laser-scanning microscope (LSM 780; Zeiss) equipped with filter blocks with spectral properties matching the fluorescence of GFP (excitation wavelength at 488 nm and emission wavelength at 520 nm) and the autofluorescence of plant tissues (excitation wavelength at 543 nm and emission wavelength at 590 nm). Five plants were prepared for each time point, and each experiment was repeated thrice.

Starch granule staining observation. The starch granule staining observation was carried out according to the method described by Hovenkamp-Hermelink et al. (1987), with some minor modification. Briefly, 1 g of iodine was dissolved in 100 ml of 5% potassium iodide (vol/wt) to prepare a solution of 1% iodine-potassium iodide (1% I₂-KI). The rhizomes were sectioned, immersed in 1% I₂-KI solution for 1 min, then washed with sterile distilled water and observed by a light transmission microscopy (UC103i; UOP).

Real-time polymerase chain reaction amplification and quantification for *F. oxysporum* f. sp. *cubense* abundance. The roots, rhizomes, pseudostems, leaf sheaths, and leaves of plants inoculated with either *Foc1* or *Foc4* were collected at 13 different time points after inoculation, immediately frozen in liquid nitrogen, then stored in a freezer at -80°C until use. Genomic DNA was extracted using the Magen HiPure Plant DNA Mini Kit, in accordance with the manufacturer's instructions. To detect *Foc1* and *Foc4* abundance, we used two specific primer sets, *Foc-1/Foc-2* for *Foc1* (M.-H. Li et al. 2012) and W1805F/W1805R for *Foc4* (Lin et al. 2009). The primer sequences are listed in Supplementary Table S1. Standard curves were prepared according to the method described by Fan et al. (2017). A 242-bp fragment was produced by polymerase chain reaction (PCR) from *Foc4* genomic DNA (gDNA) and a 354-bp fragment from *Foc1* gDNA. The *F. oxysporum* f. sp. *cubense* fragments were gel purified, cloned into pMD™18-T vector (Takara), and sequenced. After plasmid purification, the plasmid DNA concentration was quantified using spectrophotometer (Nanodrop). The copy number of standard plasmids was calculated according to plasmid (2,692 bp) plus insert lengths (242 or 354 bp) and assuming a molecular mass of 660 Da for a base pair. Standard DNA stock solutions of plasmid at 10^9 copies μL^{-1} were prepared. For standard curve plotting and melting-curve analysis of real-time PCR amplification, 10-fold serial dilutions of the target DNA, ranging from 10^7 to 10^2 copies/reaction, were performed and yielded linear and reliable results (efficiency, 90 to 110%; correlation coefficient, $R^2 > 0.99$).

Finally, a real-time PCR assay was conducted to detect the abundance of *F. oxysporum* f. sp. *cubense* in *F. oxysporum* f. sp. *cubense*-infected banana. When testing DNA samples, banana ribosomal protein S2 gene (*RPS2*) was employed as the reference gene to make samples to be normalized (Chen et al. 2011). Ten plants were prepared for each time point, and each experiment was repeated thrice.

Determination of starch contents in banana rhizomes. Fresh rhizome tissues of plants inoculated with either *Foc1* or *Foc4* were collected at 0, 168, 216, 264, 312, 360, 480, and 720 h after inoculation. Afterward, the samples were immediately frozen in

liquid nitrogen, ground to powder, and weighed. The starch contents in 0.1 g of the rhizome powder were determined using a plant starch content assay kit (Comin Biotechnology Co., Ltd.) in accordance with the manufacturer's instructions. Ten plants were prepared for each time point, and the experiment was repeated thrice.

FA production of *F. oxysporum* f. sp. *cubense* in vitro culture. FA production of *F. oxysporum* f. sp. *cubense* in vitro culture was performed according to the method described by Morpurgo et al. (1994), with some modification. Briefly, nine mycelial discs of *Foc1* or *Foc4*, cultured for 7 days on potato dextrose agar (PDA) plates, were inoculated into 300 ml of the modified sterilized Richard culture medium (10 g of KNO₃, 5 g of KH₂PO₄, 2.5 g of MgSO₄·7H₂O, 0.02 g of Fe₂(SO₄)₃, 50 g of sucrose, and 0.1 g of ZnSO₄·7H₂O), and incubated for 9 days at 30°C and a rotation speed of 140 rpm with the continuous illumination of a light intensity of 5,000 Lux. The crude culture solution was sterilized at 121°C for 18 min, treated by ultrasonication for 10 min (Ruisheng Instrument & Meter Co., Ltd.), then filtered with double layers of gauze. The filtrate was centrifuged at 6,000 rpm at normal temperature for 30 min, and the supernatant was concentrated under reduced pressure at 45°C to 1/10 of the original volume. After centrifugation at 5,000 rpm for 20 min, the supernatant was filtered through a sterile filter paper, and the filtrate was stored at 4°C for FA extraction.

FA extraction and determination assay. FA was extracted according to the method described by Chakrabarti and Basu Chaudhary (1980) and Curir et al. (2000), with some modification. Briefly, banana roots, rhizomes, and pseudostems at 168, 360, and 480 h postinoculation with either *Foc1* or *Foc4* were weighed (50 g) and homogenized in a homogenizer with 80 ml of double-distilled H₂O. An equal volume of ethyl acetate was added and the mixture was mixed, then placed in a 28°C constant-temperature shaker at 170 rpm overnight. The mixture was allowed to stand for 20 min to allow the ethyl acetate and water phases to separate. Then, the upper ethyl acetate fraction was removed and added to a round-bottom flask for evaporation at 50°C using a rotary evaporator. The residue was dissolved by adding 10 ml of ethanol, and the precipitate was discarded by centrifugation. The supernatant was steamed to dryness at 50°C in a desiccator under vacuum condition. The dried sample was dissolved by adding 10 ml of methanol to prepare a sample solution.

FA contents were determined by the method of ultraviolet absorption using a Multiscan GO high-quality UV/VIS full-wavelength reader (Thermo Fisher Scientific) at 268 nm. A calibration curve was made with pure FA (Sigma) dissolved in methanol.

Evaluation of starch effects in phytotoxicity induced with FA. Starch powder (Yuanye Biotechnology Co. Ltd.) was dissolved in sterile distilled water to prepare starch powder solutions of different concentrations (2, 6, 10, and 20 mg/ml [wt/vol]). These starch powder solutions were added to treatment solutions containing FA at 100 µg/ml (wt/vol), and then the roots of a banana plantlet were immersed in the different treatment solutions. All treated banana plantlets were kept in a constant-temperature incubator at 28°C with a photoperiod of 16 h of light and 8 h of darkness and a light intensity of 5,000 Lux. The plant symptoms at different time points were observed.

Quantitative real-time PCR analysis on the expressions of key genes in the starch synthetic pathway and defense-related genes. We measured the expression levels of 6 key genes in the starch synthetic pathway (ADP-glucose pyrophosphorylase [*AGPase*], granule-binding starch synthase [*GBSS*], starch branching enzyme [*SBE*], soluble starch synthases [*SSS*], starch phosphorylase [*SP*], and glucose-1-phosphate adenylyltransferase [*GPA*]) in banana roots and rhizomes at 168 h postinoculation with either *Foc1* or *Foc4*, and 10 defense-related genes (chitin elicitor receptor kinase 1 [*CERK1*], flagellin sensitive 2 [*FLS2*], serine/threonine-protein kinase [*PBS1*], WRKY transcription factor 22

[*WRKY22*], pathogenesis-related proteins 1 [*PR-1*], chitinase, lipoxygenases [*LOX*], jasmonate ZIM-domain protein [*JAZ*], glutathione-S-transferase [*GST*], and cellulose synthase) in banana roots, rhizomes, pseudostems, leaf sheaths, and leaves at 0, 48, and 168 h postinoculation with either *Foc1* or *Foc4*. Moreover, *RPS2* was used as the reference gene (Chen et al. 2011). Total RNA was extracted using the TaKaRa MiniBEST Plant RNA Extraction Kit and treated with RNase free DNase I (TaKaRa), and cDNA was synthesized in 20 µl of reaction system using the PrimeScript RT Master Mix Kit (TaKaRa). In addition, a no-RT control reaction was performed to ensure there did not have any DNA contamination. Reverse-transcription quantitative (RT-q)PCR was performed using the SYBR Premix Ex Taq Kit (TaKaRa) in accordance with the manufacturer's protocols. Relative changes in gene expression levels were calculated using the 2^{-ΔΔCT} method, as described by Zhao et al. (2013). The primer sequences used for RT-qPCR analysis are listed in Supplementary Table S2. Ten plants were prepared for each time point, and the experiment was repeated thrice.

Statistical analysis. Data were analyzed with analysis of variance by using SPSS 16.0 for Windows (SPSS Inc.) and were graphed using Graph Pad Prism 7 (Graph Pad Software). All values were presented as mean values ± standard deviations. Statistically significant differences were determined using Duncan's multiple range tests (*P* < 0.05) or Student's *t* test (* and ** indicate *P* < 0.05 and 0.01, respectively).

RESULTS

GFP transformation and observation of GFP-tagged *F. oxysporum* f. sp. *cubense*. Two transformed isolates, *Foc1* and *Foc4* expressing GFP, were obtained by ATMT. The expression of GFP in both transformed *F. oxysporum* f. sp. *cubense* isolates remained stable after successively transferring 40 generations on PDA medium with or without hygromycin B (data not shown). Biological experiments proved that neither the pathogenicity nor morphological features of the isolates were changed by the integration of the *GFP* gene to the genome of each *F. oxysporum* f. sp. *cubense* isolate by ATMT compared with the wild-type isolates (data not shown). Both *GFP*-tagged *Foc1* and *Foc4* emitted bright fluorescence under green light excitation of 488 nm and were readily visible via confocal LSM (Fig. 1). Therefore, these isolates can be used for further research on the expansion and colonization of banana plants.

Histopathological observation on the different tissues of plants inoculated with GFP-tagged *Foc1* and *Foc4*. To study the infection differences between *Foc1* and *Foc4*, we monitored the pathogen infection process using two *GFP*-expressing *F. oxysporum* f. sp. *cubense* isolates. The roots, rhizomes, pseudostems, leaf sheaths, and leaves of the Brazilian plants inoculated with the two

Fig. 1. Spores and mycelia morphological characteristics of green fluorescent protein-tagged *Fusarium oxysporum* f. sp. *cubense* causing Fusarium wilt of banana observed with a confocal microscopy. Scale bar = 50 µm. A, Race *Foc4* and B, race *Foc1*.

racers of *GFP*-tagged *F. oxysporum* f. sp. *cubense* were sampled at different time points and observed via confocal LSM. The results were shown as follows. In addition, observations of the mock-inoculated control are shown in Supplementary Figure S1.

Roots. Both *GFP*-tagged *Foc1* and *Foc4* could penetrate the vascular bundle tissues of the roots (Fig. 2). However, some differences in their infection process were observed. The conidia and hyphae of both *Foc1* and *Foc4* attached to the epidermal cells of the banana roots 24 h after infection (Fig. 2A, A1 and H, H1, respectively) and penetrated the cortex parenchyma of the roots at 48 h (Fig. 2B, B1 and I, I1, respectively). At 72 h, the hyphae of *Foc4* began to grow longitudinally along the intercellular spaces (Fig. 2J and J1) and, at 96 h, the hyphae entered the xylem catheters (Fig. 2K and K1). At 360 h, plant tissue slightly collapsed, and a massive amount of hyphae and spores of *Foc4* were observed on the vascular bundle tissues. However, the hyphae of *Foc1* were not observed in the xylem catheters of banana roots until 144 h after infection (Fig. 2E and E1). Moreover, the amount of colonization was obviously limited from 48 to 216 h after infection. These results indicate that

Fig. 2. Expansion and colonization in the roots of banana variety Brazilian (*Musa* spp. AAA) infected with either green fluorescent protein-tagged isolates *Foc1* or *Foc4* of *Fusarium oxysporum* f. sp. *cubense* at different time points (24, 48, 72, 96, 144, 216, and 360 h) after inoculation. **A** to **G**, Infected by *Foc1*. **H** to **N**, Infected by *Foc4*. **A** to **G** and **H** to **N** = transverse section, and **A1** to **G1** and **H1** to **N1** = longitudinal section. Scale bar = 100 μ m.

the expansion of *Foc4* is apparently faster than that of *Foc1*, and the amount of *Foc4* colonization is distinctly higher than that of *Foc1* colonization at the same time points in the infected roots.

Rhizomes. Banana rhizomes showed slightly brownish discoloration 168 h after inoculation with *Foc4* (Supplementary Fig. S2). However, no symptom was found after inoculation with *Foc1* and

Fig. 3. Expansion and colonization in the rhizomes of banana variety Brazilian (*Musa* spp. AAA) infected with either green fluorescent protein-tagged isolates *Foc1* or *Foc4* of *Fusarium oxysporum* f. sp. *cubense* at different time points (168, 216, 264, 312, 360, 480, and 720 h) after inoculation. **A** to **G**, Infected by *Foc1*. **H** to **N**, Infected by *Foc4*. Scale bar = 100 μ m.

the mock control. Therefore, we selected the rhizome tissues each at 168, 216, 264, 312, 360, 480, and 720 h postinoculation for observation. Results showed that the hyphae of *Foc4* were observed in the rhizomes 168 h after root inoculation (Fig. 3H). Subsequently, *Foc4* expanded its hyphal network by growing in the intercellular spaces along the junctions of the rhizome epidermal cells at

312 h (Fig. 3K). Between 360 and 720 h, the rhizome tissues became infected with soft rot, and the plantlet gradually withered and eventually died. At this stage, vigorous hyphae were still observed in the rotten tissues (Fig. 3L to N). However, *Foc1* was not observed in the rhizome until 264 h (Fig. 3C). At the end of the observation, although some hyphae of *Foc1* could be observed, the amount of *Foc1* colonization was much lower than that of *Foc4* colonization. Moreover, the tissue structures of the rhizomes might be intact during the different time points after infection with *Foc1*. Thus, no evident disease symptom was observed in the rhizomes.

Pseudostems. To further understand the expansion and colonization of pathogens in banana plants, we examined the status of *F. oxysporum* f. sp. *cubense* in the pseudostems from 168 to 720 h after root inoculation. Small amounts of *Foc4* hyphae were observed

Fig. 4. Expansion and colonization in the pseudostems of banana variety Brazilian (*Musa* spp. AAA) infected with either green fluorescent protein-tagged isolates *Foc1* or *Foc4* of *Fusarium oxysporum* f. sp. *cubense* at different time points (168, 216, 264, 312, 360, 480, and 720 h) after inoculation. **A to G,** Infected by *Foc1*. **H to N,** Infected by *Foc4*. Scale bar = 100 μ m.

Fig. 5. Expansion and colonization in the leaf sheaths of banana variety Brazilian (*Musa* spp. AAA) infected with either green fluorescent protein-tagged isolates *Foc1* or *Foc4* of *Fusarium oxysporum* f. sp. *cubense* at different time points (312, 360, 480, and 720 h) after inoculation. **A to D,** Infected by *Foc1*. **E to H,** Infected by *Foc4*. Scale bar = 100 μ m.

in the vascular tissues of the pseudostems at 168 and 216 h (Fig. 4H and I) and then expanded to the interval tissues between the parenchyma and aerenchyma of the pseudostems at 264 h (Fig. 4J). At subsequent time points, *Foc4* hyphae covered large sections of the pseudostems (Fig. 4K to M), and the plant tissues gradually collapsed (Fig. 4N). However, *Foc1* hyphae were not observed in the pseudostems at all time points from 168 to 720 h after the infection (Fig. 4A to G). These results indicate that *Foc4*, not *Foc1*, can expand from the rhizomes to the pseudostems after pathogen infection from the roots.

Leaf sheaths. At 312 h after *Foc4* infection from the roots, the plant leaves showed yellowing, and the leaf sheaths exhibited brown patches near the rhizome site. Meanwhile, the plants looked relatively healthy at 312 h after *Foc1* infection (Supplementary Fig. S3). Also, at this stage, the leaf sheaths were observed. *Foc4* hyphae were distributed to whole tissues of the leaf sheaths (Fig. 5E). From 312 to 480 h, increasing amounts of hyphae in the tissues were found (Fig. 5F and G). At 720 h, the tissues collapsed, and few hyphae were still observed (Fig. 5H). However, as expected, no *Foc1* hyphae were found at each time point (Fig. 5A to D).

Leaves. The leaves of infected plants became bright yellow after *Foc4* inoculation at 312 h. However, the plants were normal after *Foc1* inoculation. The leaves of plants infected with *Foc1* or *Foc4* were observed from 360 to 720 h after inoculation. The results showed that no fungal material was observed at any time point after

Foc1 infection (Fig. 6A to C). In contrast, apparent fungal hyphae were found on the leaf stomata and on the guard cells of yellowing and wilted leaves 360 to 720 h after *Foc4* infection (Fig. 6D to F). This result indicates that *Foc4* hyphae can expand from the infected roots to the leaves.

Quantification of pathogen abundance in different parts of banana plant. Combining the results obtained from histopathological observations, we analyzed the pathogen abundance by RT-qPCR in different parts of the plants at different time points after the roots were inoculated with *F. oxysporum* f. sp. *cubense* (Fig. 7). Our results showed that the abundance of *Foc4* was significantly higher than that of *Foc1* in banana roots and rhizomes at the same time points from 24 to 720 h (Fig. 7A and B). Moreover, *Foc1* in the pseudostems, leaf sheaths, and leaves was not detected at each time point from 168 to 720 h. However, *Foc4* was detected in the pseudostems at all time points from 168 to 720 h, and the abundance gradually increased with time (Fig. 7C). Similarly, *Foc4* could be detected in the leaf sheaths at all time points from 168 to 720 h (Fig. 7D) and in the leaves from 360 to 720 h (Fig. 7E). However, the abundance of *Foc4* first increased from 168 to 312 h and then slightly decreased at 360 h in the leaf sheaths (Fig. 7D). Meanwhile, *Foc4* was only detected in the leaves starting at 360 h, and the abundance gradually increased from 360 to 720 h (Fig. 7E). These results are consistent with our histopathological observation.

Starch granule observation and determination of starch contents in rhizomes. Examination of the infection process of GFP-tagged *F. oxysporum* f. sp. *cubense* in banana rhizomes showed that the starch granules filled the rhizome cells at 168 h after inoculation. However, their size was similar and the number was significantly higher after infection with *Foc1* (Fig. 8A and B) than after infection with *Foc4* (Fig. 8C and D) and the mock control (Fig. 8E and F). Moreover, at subsequent time points from 216 to 720 h, the number of starch granules further increased after infection with *Foc1*, thereby resulting in a higher number differences compared with infection with *Foc4*. To further confirm the abovementioned results, we stained the sliced rhizome tissues at 168 h after treatment with 1% I₂-KI and observed them under a light transmission microscope. The number of the starch granules remarkably increased in the tissues infected with *Foc1* (Fig. 8G and H) compared with those infected with *Foc4* (Fig. 8I and J) and the mock control (Fig. 8K and L). In addition, similar results were observed at 216 to 720 h (data not shown).

To verify the abovementioned results, we determined the total starch contents in the rhizomes at different time points after inoculation with *F. oxysporum* f. sp. *cubense*. Results showed that the starch contents in the plants infected with *Foc1* were significantly higher ($P < 0.05$) than those in the plants infected with *Foc4* at all time points. The starch contents gradually increased from 0 to 480 h and then slightly decreased at 720 h after inoculation with *Foc1* (Fig. 9). By contrast, the starch contents remained constant and did not show statistically significant differences ($P < 0.05$) from 0 to 720 h after the plants were inoculated with *Foc4*. The results were similar to those observed in the mock control. These results indicate that *Foc1* infection can significantly induce an increase in the starch granules in banana rhizomes.

Expression analysis of key genes in the starch synthetic pathway in rhizomes and roots. To explore the change in starch contents during the interaction between *F. oxysporum* f. sp. *cubense* and plants, we selected six key genes related to the pathway of starch synthesis that were retrieved from our transcriptome data (Bai et al. 2013). These genes included *AGPase*, *GBSS*, *SBE*, *SSS*, *SP*, and *GPA*. The RNA expression levels of these key genes in the rhizomes were analyzed using RT-qPCR. Results showed that all of six genes were induced by *F. oxysporum* f. sp. *cubense* inoculation, and the expression levels of these genes were significantly upregulated. Moreover, the levels of upregulation at all time points were much higher after infection with *Foc1* than after infection with *Foc4* (Fig. 10).

Fig. 6. Expansion and colonization in the leaves of banana variety Brazilian (*Musa* spp. AAA) infected with either green fluorescent protein-tagged isolates *Foc1* or *Foc4* of *Fusarium oxysporum* f. sp. *cubense* at different time points (360, 480, and 720 h) after inoculation. **A to C,** Infected by *Foc1*. **D to F,** Infected by *Foc4*. Scale bar = 100 μ m.

In addition, expression analysis of these six genes was carried out in banana roots at different time points after *F. oxysporum* f. sp. *cubense* inoculation. Results showed that the induction patterns of six genes were significantly different between *Foc1* and *Foc4* (Supplementary Fig. S4). Moreover, the expression levels of these genes were considerably higher at most time points after infection with *Foc1* than after infection with *Foc4*. These results further suggest that the starch content differences in the plant tissues infected with *Foc1* and *Foc4* are consistent with the expression levels of the starch synthesis-related genes.

Detection of FA contents in production in vitro culture and infected banana tissues. To further investigate the pathogenicity differences between *Foc1* and *Foc4*, first, a standard curve ($y = 21.496x + 0.0882$) was established by measuring the optical density at 268 nm values of different concentrations of pure FA, and the correlation coefficient was calculated to be 0.9954 (Fig. 11A). Then, the FA amounts were determined in *F. oxysporum* f. sp. *cubense* in vitro culture and infected banana tissues (roots, rhizomes, and pseudostems) at 168, 360, and 480 h after *F. oxysporum* f. sp. *cubense* inoculation. The results showed that the amounts of FA production in vitro culture were not significantly different between *Foc1* and *Foc4* (data not shown). However, the amounts of FA were significantly higher in the infected banana roots, rhizomes, and pseudostems at each of three time points after infection with *Foc4* than after infection with *Foc1* (Fig. 11B). A certain concentration of FA can lead to blight symptoms, and an overconcentration of FA from *Foc4* may be an important cause of the plant disease.

Evaluation of starch effects in phytotoxicity induced with FA. Before evaluating the effect of starch on the phytotoxicity induced with FA, an experiment of phytotoxicity induced with FA was performed. The Brazilian plant roots were immersed in different contents of FA (0, 50, 100, 200, 300, and 500 µg/ml

[wt/vol]) and the results are shown in Supplementary Figure S5. As the contents of FA increased, the phytotoxicity gradually increased. When FA was used at 100 µg/ml, the rhizomes became apparently brown at 96 h after treatment. Therefore, FA at 100 µg/ml was chosen to evaluate the effect of starch on phytotoxicity induced with FA, and the results are shown in Figure 12. Although the plants were treated in the solution containing only FA, the leaves and rhizomes of the plants began to wilt and become brown, respectively, at 48 h (Fig. 12H); then, whole plants died at 120 h (Fig. 12T). However, the plants were treated in the mixed solution containing FA and starch powder at 20 mg/ml, leaf wilting at 120 h and rhizome browning at 48 h were apparently observed, and the plants still survived at 120 h (Fig. 12L to X). By analysis of different treatments, we conclude that the resistance of banana plants increases gradually against FA with the increasing of the starch powder contents in the treatment solutions. This suggests that starch has an important inhibitory effect on the phytotoxicity induced by FA.

Expression analysis of the defense-associated genes in banana plants. We selected 10 defense-related genes (namely, *CERK1*, *FLS2*, *PBS1*, *WRKY22*, *PR-1*, *chitinase*, *LOX*, *JAZ*, *GST*, and *cellulose synthase*) and then analyzed their expression levels in the roots, rhizomes, pseudostems, leaf sheaths, and leaves of the banana plants infected by both *F. oxysporum* f. sp. *cubense* races after inoculation at 48 and 168 h. All 10 genes were induced by infection with *Foc1* and *Foc4* (Fig. 13). However, the induction patterns were significantly different. For example, all genes except *cellulose synthase* were upregulated in the banana roots at 48 h after inoculation with *Foc1* (Fig. 13A). Moreover, eight genes, including *CERK1*, *PBS*, *WRKY22*, *PR-1*, *chitinase*, *LOX*, *JAZ*, and *GST*, were upregulated by infection with *Foc1*. However, these genes were downregulated or slightly changed at 48 h after infection with *Foc4* (Fig. 13A). Statistical analysis indicated that the expression levels of four genes (*WRKY22*, *PR-1*, *chitinase*, and *LOX*) were

Fig. 7. Abundance of races *Foc1* and *Foc4* of *Fusarium oxysporum* f. sp. *cubense* in different parts of banana (*Musa* spp. AAA) at different time points after *F. oxysporum* f. sp. *cubense* inoculation. **A**, Root; **B**, rhizome; **C**, pseudostem; **D**, leaf sheath; and **E**, leaf. Data represent mean value of three independent replicates. Significant differences were determined according to Duncan's multiple range test at $P < 0.05$. Error bars represent + standard deviation.

significantly different ($P < 0.05$) in the banana roots infected with *Foc1* and *Foc4* at 48 h (Fig. 13A).

At 168 h after inoculation, although the expression levels of these defense-related genes in the roots, rhizomes, pseudostems, leaf sheaths, and leaves showed different dynamic variations, the expression levels of these genes after infection with *Foc1* were generally higher than those after infection with *Foc4* (Fig. 13B to F). Statistical analysis showed that the expression levels of different genes in different tissues of plants infected with *Foc1* and *Foc4* were significantly different. For example, five genes (*FLS2*, *PBS*, *LOX*, *GST*, and *cellulose synthase*) in the roots (Fig. 13B), three genes (*CERK1*, *GST*, and *cellulose synthase*) in the rhizomes (Fig. 13C), five genes (*CERK1*, *WRKY 22*, *PR-1*, *chitinase*, and *LOX*) in the pseudostems (Fig. 13D), nine genes (*CERK1*, *FLS2*, *PBS*, *PR-1*, *chitinase*, *LOX*, *JAZ*, *GST*, and *cellulose synthase*) in the leaf sheaths (Fig. 13E), and six genes (*CERK1*, *FLS2*, *WRKY22*, *PR-1*, *JAZ*, and *cellulose synthase*) in the leaves (Fig. 13F) showed significantly different expression levels ($P < 0.05$) after infection with *Foc1* and *Foc4*. Based on these data, we conclude that the higher expression levels of these defense-related genes in the different parts of plants after infection with *Foc1* than after infection with *Foc4* may have contributed to the stronger resistance of banana variety Brazilian against *Foc1* than *Foc4*.

DISCUSSION

In the past, *Foc1* was responsible for the widespread epidemics of Fusarium wilt disease (Panama disease), which caused significant losses to banana production. Since then, Cavendish varieties have

been the most widely used varieties in the majority of banana plantations because of their resistance to *Foc1* (O'Donnell et al. 1998). However, the appearance of *Foc4* has posed a serious threat to the Cavendish banana varieties.

Fig. 9. Contents of starch in banana (*Musa* spp. AAA) rhizomes at different time points (168, 216, 264, 312, 360, 480, and 720 h) after infection by *Fusarium oxysporum* f. sp. *cubense* races *Foc1* and *Foc4*. Data represent an average of three biological replicates and were normalized to the mock control. Error bars are standard deviation (+SD). Values followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$.

Fig. 8. Microscopic observation of starch granules in banana rhizomes at 168 h after *Fusarium oxysporum* f. sp. *cubense* and mock inoculation. Confocal **A** to **F**, Banana rhizomes were sectioned and observed with a confocal microscopy; iodine-potassium iodide (I_2 -KI) **G** to **L**, banana rhizomes were sectioned, stained with 1% I_2 -KI, and observed by light transmission microscopy. **A**, **B**, **G**, and **H** = Mock-inoculation control; **C**, **D**, **I**, and **J** = infected by *Foc1*; and **E**, **F**, **K**, and **L** = infected by *Foc4*. Scale bars represent 100 μ m in **A** to **F** and 200 μ m in **G** to **L**. **S** = starch granules and **Hy** = hyphae.

Currently, breakthroughs have been made in the study of the interaction between host plants and pathogens using GFP-tagged pathogens. GFP-transformed plant pathogens can be visualized in plant tissues without any manipulation of samples, thereby making the technology extremely useful in observing the pathogen infection process (Lorang et al. 2001). In the current study, to provide assistance in monitoring the *F. oxysporum* f. sp. *cubense* infection process in banana, we successfully transformed the *GFP* gene into the isolates of both *F. oxysporum* f. sp. *cubense* races by ATMT. Similar to the results obtained by previous studies (Freitag et al. 2001; Lorang et al. 2001; Visser et al. 2004), transformed

GFP-tagged *F. oxysporum* f. sp. *cubense* races were able to emit bright, uniform fluorescence. This result laid the foundation for follow-up experiments.

In this study, we used confocal LSM to observe the pathogen infection process of different parts of Brazilian plants at different time points after inoculation with *GFP*-tagged *Foc1* and *Foc4*. Our results indicated that both *Foc1* and *Foc4* could penetrate the epidermis of the roots and invade the xylem vessels. This finding was consistent with the results of previous studies (Li et al. 2011; C.-Q. Li et al. 2013; Guo et al. 2014, 2015). Li et al. (2017) pointed out that *Foc4* could penetrate the roots from the wounds and

Fig. 10. Relative expression levels of key genes in the pathway of starch synthesis in banana (*Musa* spp. AAA) rhizomes at different time points (168, 216, 264, 312, 360, 480, and 720 h) after infection by *Foc1* and *Foc4*. **A**, ADP-glucose pyrophosphorylase (*AGPase*); **B**, granule-binding starch synthase (*GBSS*); **C**, starch branching enzyme (*SBE*); **D**, soluble starch synthases (*SSS*); **E**, starch phosphorylase (*SP*); and **F**, glucose-1-phosphate adenylyltransferase (*GPA*). The banana gene *ribosomal protein S2* (*RPS2*) was used as the internal control. Data represent an average of three biological replicates. Error bars are standard deviation (+SD). Values followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$.

intercellular space of the epidermis whereas *Foc1* mainly penetrated the wounds. In the current research, we further observed that pathogens were more likely to invade the wounds and the lateral roots. In addition, *Foc1* required 144 h to expand into the xylem catheters after infection, whereas *Foc4* did so by 96 h. Furthermore, the fungal biomass of *Foc4* was significantly higher than that of *Foc1* at each time point from 24 to 360 h after infection. These results showed that the infection rates and the hyphae enrichment of the two *F. oxysporum* f. sp. *cubense* races significantly differed after invasion. Kuang et al. (2013) pointed out that the plants of banana cultivars with different resistance to *F. oxysporum* f. sp. *cubense* altered the cell structure when they were infected with *F. oxysporum* f. sp. *cubense*, and one of the alterations was that the spaces between vessel perforation plates of the plants became smaller in resistant varieties than in susceptible varieties. Thus, the migration of fungal mycelium through the vascular bundles in the resistant cultivars was somewhat inhibited, thereby minimizing the pathogen infection rate (Beckman et al. 1961). It also seemed that the activity of the pectin methylesterase (PME) induced by the infection with *Foc1* and *Foc4* was different, and the higher increase of PME activity induced by infection with *Foc4* than infection with *Foc1* resulted in the direct damage of cell wall (Fan et al. 2017; Ma et al. 2013). Therefore, *Foc4* is more invasive than *Foc1* during the early stage of the infection process in Brazilian banana plants.

Most previous reports focused on banana root infection with *F. oxysporum* f. sp. *cubense* (Beckman et al. 1961; Guo et al. 2015; Li et al. 2011; Li et al. 2017; Wardlaw 1931; Xiao et al. 2013). In the present study, we systematically analyzed the differences of two races in the expansion and localization of the rhizomes, pseudostems, leaf sheaths, and leaves of Brazilian plants at different time points after inoculation. An interesting finding was that the expansion ranges of both *F. oxysporum* f. sp. *cubense* races were remarkably different in these parts after *F. oxysporum* f. sp. *cubense* infection. Li et al. (2017) observed the presence of *Foc1* hyphae only in the roots but we observed the presence of *Foc1* in root and rhizome tissues after infection with *Foc1*. According to previous reports, the expansion range of *Foc4* was mostly from root to rhizome (Li et al. 2017) or from root to rhizome and pseudostem (Xiao et al. 2013); however, no fungal conidia or hyphae of *F. oxysporum* f. sp. *cubense* were observed in the yellowing and wilted leaves. Surprisingly, we found that *Foc4* could spread beyond the rhizome, into the pseudostem and up to the leaves. It might be explained that sporulation and germination of secondary mycelium was crucial for rapid upward colonization (Beckman and Roberts

1995). By comparing the difference of expansion ranges between *Foc1* and *Foc4*, we conclude that the difference of the two races in invasiveness to extend further upward from the rhizomes to the upper parts of plants defines their difference in aggressiveness.

Another intriguing finding was that, when the pathogens expanded to the banana rhizome tissues, large amounts of starch granules were produced in the rhizome cells, and the starch contents were much higher after infection with *Foc1* than after infection with *Foc4*. Consistent with this observation, the expression of the key genes in the starch synthetic pathway was significantly upregulated, and the upregulation levels were much higher after infection with *Foc1* than after infection with *Foc4*. In other studies, it has been noted that a large amount of starch accumulation occurs in the stem tissues of diseased orange (Exteberria et al. 2009), diseased citrus leaves (Schneider, 1968), and the protoplasts of the leaves of the symptomatic sweet orange (Kim et al. 2009) when these plants were infected with huanglongbing pathogens (*Candidatus Liberibacter* spp.). Moreover, average content of starch in the diseased citrus leaves was six times higher than that in the healthy citrus leaves (Takushi et al. 2007). However, little is known regarding the relationship between starch contents and the plant resistance. Our previous microscopic observation results indicated that *Foc1* spread slowly and was colonized less in the rhizomes, whereas *Foc4* showed opposite results. Thus, we speculate that the amount of starch accumulation may be significantly related to the resistance levels of banana to two races of pathogens. Starch is an important energy source in plant cells and may be involved in the synthesis of many resistance-related physical and biochemical substances. Moreover, a substantial increase in the starch contents in the cells may increase the density of cellular tissues (Kuang et al. 2013), thereby directly slowing down the expansion rate of *Foc1* in the banana rhizome and root tissues. However, these hypotheses need to be confirmed in future studies.

Matsumoto et al. (1995) and Dong et al. (2012) suggested that FA could act as a virulence factor in Fusarium wilt of banana. Harborne et al. (1993) and Tan et al. (2011) indicated that FA played a direct role in pathogenesis, and the contents of FA in diseased plants is positively correlated with the virulence of *F. oxysporum* f. sp. *cubense*. In the present study, we found that a certain concentration of FA could induce the same symptoms in the banana plants as the disease. Moreover, we observed that the contents of FA were significantly higher in the roots, rhizomes, and pseudostems after infection with *Foc4* than after infection with *Foc1*. However, the amounts of FA produced by *Foc1* and *Foc4* in vitro culture were not

Fig. 11. Determination of fusaric acid (FA) contents. **A**, Standards curve of detection for FA by UV (OD₂₆₈ = optical density at 268 nm) and **B**, FA contents in banana different tissues at different time points (168, 360, and 480 h) after infection by *Fusarium oxysporum* f. sp. *cubense* races *Foc1* and *Foc4*. Data represent an average of three biological replicates. Error bars are standard deviation (+SD). Values followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$.

Fig. 12. Effects of different amounts of starch powder on phytotoxicity induced by fusaric acid (FA) at different time points (24, 48, 72, and 96 h). **A, G, M,** and **S,** Treated with sterile water; **B, H, N,** and **T,** treated with FA at 100 µg/ml; **C, I, O,** and **U,** treated with FA at 100 µg/ml and starch powder at 2 mg/ml; **D, J, P,** and **V,** treated with FA at 100 µg/ml and starch powder at 6 mg/ml; **E, K, Q,** and **W,** treated with FA at 100 µg/ml and starch powder at 10 mg/ml; and **F, L, R,** and **X,** treated with FA at 100 µg/ml and starch powder at 20 mg/ml.

significantly different. This indicates that the amounts of FA production in banana plants between *Foc1* and *Foc4* reflects the difference of virulence between the two *F. oxysporum* f. sp. *cubense* races. High concentrations of FA in plants can lead to the destruction of the plant cell membrane (Bacon et al. 1996; D'Alton and Etherton 1984; López-Díaz et al. 2018; Ruiz et al. 2015) and cause plant tissues to lose water, resulting in plant withering (Dong et al. 2014). However, low concentrations of FA may induce the synthesis of phytoalexins and stimulate plant defense response signals (Bouizgarne et al. 2006). Consistent with the previous studies, our results showed that the lower amounts of FA in banana plants after *Foc1* infection could increase the expression level of defense-related genes (Fig. 13) and, ultimately, cause plants to

exhibit resistance to *Foc1*, whereas the production of a large amount of FA in banana plants after *Foc4* infection could destroy the cell membrane function of plant tissues and further weaken plant tissues to promote virulence of *Foc4*.

Because the starch contents in infected plant tissues might contribute to the difference in Brazilian resistance to *Foc1* and *Foc4*, we further conducted an evaluation assay of the starch effect on the phytotoxicity induced with FA. Our results showed that starch has an important inhibitory effect on the phytotoxicity induced by FA, thereby leading to more resistance to the pathogens in the banana plants with high amounts of starch accumulation than with low amounts of starch accumulation. Starch consists of 10% amylose and 70% amylopectin. The water-soluble

Fig. 13. Relative expression levels of 10 defense-related genes in different plant parts at 48 and 168 h after inoculation by *Fusarium oxysporum* f. sp. *cubense* races *Foc1* and *Foc4*. **A**, Roots at 48 h; **B**, roots at 168 h; **C**, rhizomes at 168 h; **D**, pseudostems at 168 h; **E**, leaf sheaths at 168 h; and **F**, leaves at 168 h. Relative expression levels of the defense-related genes (chitin elicitor receptor kinase 1 [*CERK1*], flagellin sensitive 2 [*FLS2*], serine/threonine-protein kinase [*PBS1*], WRKY transcription factor 22 [*WRKY22*], pathogenesis-related proteins 1 [*PR-1*], chitinase, lipoxigenases [*LOX*], jasmonate ZIM-domain protein [*JAZ*], glutathione-S-transferase [*GST*], and cellulose synthase) were analyzed through quantitative real-time polymerase chain reaction. Note that the relative expression level of each gene at the sample of mock-inoculation control was set as 1.0. Data represent means of three replicates $\pm$ standard deviation. Significant differences were determined according to Student's *t* test (* and ** indicate $P < 0.05$ and 0.01, respectively).

amylose is condensed by α -glucose molecules to form long spirals, in which hydroxyl groups are still exposed to the helix for each glucose unit. Previous experimental reports showed that some small molecular compounds can interact with the hydroxyl group in glucose, so that the small molecular compounds can be embedded into the axis of the starch helix to form inclusion body complexes (Biais et al. 2006; Godet et al. 1993; Kong and Ziegler 2014; Kong et al. 2018). Therefore, we speculate that the effect of starch to counteract the activity of FA may be due to the formation of a complex of starch-FA, thus reducing the phytotoxicity of FA to banana plants. However, this specific mode of action requires further research.

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