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Identification of *mimp*-associated effector genes in *Fusarium oxysporum* f. sp. *cubense* race 1 and race 4 and virulence confirmation of a candidate effector gene

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ABSTRACT

Effectors secreted by microbes contribute to pathogen virulence and/or avirulence on host plants in the interaction of plants and microbes. Also, the effector repertoire determines the host specificity of a pathogen. *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is the causal agent of banana wilt; however, knowledge about *Foc* effector genes is very limited. In this study, genome-wide effector gene identification was performed in *Foc* race 1 (*Foc* 1) and *Foc* race 4 (*Foc* 4) based on the context association between the effector genes and the transposable element *mimp*. A total of 20 candidate effector genes were identified, of which 3 were *Foc* 1-specific, 6 were *Foc* 4-specific, and 11 were present in both *Foc* 1 and *Foc* 4. Most genes (14 out of 20) showed a significant transcriptional burst *in planta* compared with in-culture conditions, from more than 10-fold to 1,617-fold, and at the highest 32,725-fold. In addition to *Foc* 1- and *Foc* 4-specific genes, the genes *Foc 283*, *Foc 495*, and *Foc 594* also exhibited transcriptional race specificity. Sixteen of the twenty genes were predicted to have a signal peptide, nine genes might encode real effectors predicted by EffectorP 2.0, and eight genes had predicted motifs. To validate the pathogenicity of the candidate effector genes, we generated knockout mutant and complementants of the gene *Foc 1324* and tested their virulence on banana plants. The results showed that *Foc 1324* was a virulent factor and required for the pathogenicity of *Foc* 4.

1. Introduction

In the arms race between plants and virulent microbes, plants have evolved two induced strategies to defeat pathogen infection. The first mechanism is called PAMP-triggered immunity (PTI), through which plants recognize microbial conserved pathogen-associated molecular patterns (PAMPs) and induce immune responses. However, successful microbes can secrete so-called "effector" proteins to suppress or evade plant PTI responses. Plants have thus developed a second strategy called effector-triggered immunity (ETI) to withstand pathogen invasion through the recognition of effectors (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Therefore, identification and characterization of pathogen effectors are helpful to elucidate the mechanisms underlying microbe invasion and plant defenses.

Effectors have been characterized in the *Fusarium oxysporum* species complex (FOSC). Typically, *F. oxysporum* f. sp. *lycopersici* (*Fol*), the causal agent of tomato wilt, secretes a suite of effector factors into host xylem sap when infecting tomato plants. These effectors are termed SIX (secreted in xylem), and 14 SIX proteins have been identified to date (Houterman et al., 2007; Schmidt et al., 2013). SIX1, SIX3, SIX5, and SIX6 are genuine effector factors and confer virulence to susceptible plants (Rep et al., 2004; Houterman et al., 2009; Gawehns et al., 2014; Ma et al., 2015). However, for resistant tomato plants, some of these effectors can serve as avirulence factors (Avr) and be recognized by host plants to elicit immune responses. Avr1 (SIX4) triggers defensive responses in tomato plants containing the resistance gene (*R*) *I* (Houterman et al., 2008); Avr2 (SIX3) induces resistance in *I-2*-containing tomato plants (Houterman et al., 2009); and Avr3 (SIX1) is recognized by *I-3* plants (Rep et al., 2004). Out of the tomato wilt *Fol*, homologs of *SIX* genes are also found in pathogens virulent to banana, soybean, *Brassica oleracea*, watermelon, cucumber, melon, cotton, and others (Guo et al., 2014; Chakrabarti et al., 2015; Lanubile et al., 2016; Niu et al., 2016; Pu et al., 2016; Williams et al., 2016; van Dam et al., 2017b). Furthermore, phylogenetic analysis has revealed that the host preference of *F. oxysporum* species is paralleled with their specific effector suites, which renders the effector repertoire a molecular marker

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to differentiate specific formae speciales (ff. spp.) (van Dam et al., 2016, 2017a). *SIX1*, *SIX2*, *SIX3,* and *SIX5* can be used to discriminate *Fol* from other *F. oxysporum* species. In addition, *SIX4* can be used for the identification of race 1 strains of *Fol*, while polymorphisms in *SIX3* can differentiate race 2 from race 3 strains (Lievens et al., 2009).

In *F. oxysporum*, pathogenicity-related effectors are always present in lineage-specific (LS) chromosomes that are characterized by a large content of transposable elements (TEs) (Ma et al., 2010). Frequently, effector genes reside in class II TE-enriched chromosomal subregions as single genes or mini-gene clusters (Schmidt et al., 2013). Miniature inverted-repeat transposable elements (MITEs) are short non-autonomous class II transposons containing 27–30 nucleotide terminal inverted repeats (TIRs) (Feschotte and Pritham, 2007; Lu et al., 2011). Through locus analysis towards effector genes, a class of MITEs called *mimp* (a miniature impala) has been found to be always present in the promoter regions of effector genes (Schmidt et al., 2013). The *mimp* association of effector genes provides a way to identify unknown candidate effector genes by scanning for *mimp* sequences.

Fusarium oxysporum f. sp. *cubense* (*Foc*) is the causal agent of banana wilt, and race 1 (*Foc* 1) and race 4 (*Foc* 4) are the predominant agents threatening global banana production (Ghag et al., 2015). Race 1 infects Gros Michel (*Musa* sp. AAA group), Pome, Silk, and Pisang Awak (*Musa* sp. AAB group) varieties. Race 4 infects almost all banana cultivars, including Cavendish (*Musa* sp. AAA group), the most widely commercially grown cultivar, as well as both race 1 and race 2 susceptible cultivars (Meldrum et al., 2012; Mostert et al., 2017). *Foc* race 4 is divided into 'tropical race 4' (TR4) and 'subtropical race 4' (ST4). ST4 isolates cause disease on Cavendish in the presence of disease-predisposing cold temperatures that occur in the subtropics, while TR4 isolates cause disease on Cavendish in both tropical and subtropical conditions, making TR4 the major threat to banana production at present (Fraser-Smith et al., 2014). In *Foc*, to date, homologs of *SIX1, SIX2, SIX6, SIX7*, *SIX8, SIX9, SIX10,* and *SIX13* have been identified, and different races of *Foc* carry unique combination of *SIX* genes (Meldrum et al., 2012; Guo et al., 2014; Czislowski et al., 2018). In race 1, *SIX1*, *SIX6*, *SIX9*, and *SIX13* have been detected, while in race 4, all eight *SIX* genes have been detected (Guo et al., 2014; Czislowski et al., 2018). For *Foc* 4, ST4 carries *SIX1*, *SIX2*, *SIX7*, *SIX8*, and *SIX9*, while TR4 carries *SIX1*, *SIX2*, *SIX6*, *SIX8*, *SIX9*, and *SIX13* (Czislowski et al., 2018). With the exception of the homologous *SIX* genes, the investigation of effector genes in *Foc* 1 and *Foc* 4, however, has been very limited (Fraser-Smith et al., 2014; Widinugraheni et al., 2018; An et al., 2019). The identification and comparison of the effector content in *Foc* 1 and *Foc* 4 would provide candidates for functional studies as well as clues for the elucidation of host specificity of *Foc* 1 and *Foc* 4.

In this study, we used 13 *mimp* sequences reported in *Foc* strains II5 and B2 as query sequences to search local *Foc* 1 and *Foc* 4 genome databases and predicted the effector genes located downstream of the *mimp* sequences. The specific presence and the expression patterns of these candidate genes during infection were also investigated. Furthermore, the virulence of a candidate gene, *Foc 1324*, was confirmed in this study, implying that these predicted genes might play key roles in banana invasion by *Foc*.

2. Materials and methods

2.1. Plant and strain materials and banana inoculation

Banana cultivars FJ (susceptible to *Foc* 1) and BX (susceptible to *Foc* 4) were bought from the Tissue Culture Centre of the Chinese Academy of Tropical Agricultural Sciences, Danzhou, China. *Foc* 1 and *Foc* 4 isolates were isolated from the Hainan island of China and stored by our laboratory. For spore suspension, a fresh *Foc* agar block was inoculated into potato dextrose broth (PDB) and grown for 3 days with shaking at 150 rpm at 28 °C. Spores were isolated through filtration with four layers of sterilized lens paper, collected by centrifugation, and resuspended in deionized water to a final concentration of 1×10^7/ml. Three-month-old banana plantlets were inoculated with freshly prepared spore suspension according to the root dip method. Briefly, the plantlets were carefully removed from the soil, and the roots were cleaned with tap water, scratched with a sterilized blade, and dipped into the spore suspension for 30 min. Cultivars FJ and BX were inoculated with *Foc* 1 and *Foc* 4, respectively.

2.2. Identification of mimp-associated candidate effector genes

To extensively identify the *mimp* sequences in *Foc* 1 and *Foc* 4 genomes, we performed a local BLASTn search against the *Foc* 1 and *Foc* 4 genome databases using 13 full *mimp* sequences obtained from *Foc* II5, B2, and N2 as queries (van Dam et al., 2016; van Dam and Rep, 2017). Sequences with similarities higher than 90 % were extracted, and their upstream and downstream sequences of 2500 bp were subjected for gene prediction using the software AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/submission.php) and ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). After removing short sequences encoding less than 30 aa, those with an ORF located downstream of the *mimp* were collected as candidate effector genes. The transcription and protein sequences of each candidate effector were subjected to BLASTn and BLASTp searches, respectively. Signal peptides were predicted with SignaIP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), effectors were predicted using the EffectorP 2.0 service (http://effectorp.csiro.au/), and protein domains were inspected using InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search).

2.3. Expression analysis of predicted effector genes in vitro and in planta

For *in vitro Foc* RNA, 1 ml of 10^6 spores/ml were added into 50 ml of PDB medium and incubated at room temperature for three days in a stationary condition. Mycelia were isolated by lens paper filtration, and RNA was extracted using a Fungal RNA Extraction Kit (Omega Bio-Tec, Norcross, Georgia). For *in planta* expression, total RNA of infected root samples at 3 dpi was extracted using a modified SDS method. Briefly, 300 mg of roots were ground in liquid nitrogen and incubated in 5 ml of extraction solution (2 % SDS, 50 mM EDTA, 100 mM Tris − HCl pH 7.4, 1.4 M NaCl, 2 % β-mercaptoethanol) for 15 min at 65 °C. Extracts were given 1/3 vol of 5 M KAc (pH 4.8) and incubated at − 20 °C for 10 min. Following centrifugation for 10 min at 13,000 × *g* at 4 °C, the supernatant was mixed with two volumes of ethanol and placed at − 20 °C for at least 2 h for RNA precipitation. The precipitate was dissolved in RNase-free water and treated with water-saturated phenol:-chloroform:iso-amylalcohol (24:24:1, pH 4.2) twice. RNA was isolated at − 20 °C for 4 h by adding 1/3 vol of 12 M LiCl. After washing with 75 % ethanol repeatedly, the RNA pellet was suspended in DEPC-treated H_2O.

RNA samples were digested with DNase I to remove gDNA and reverse-transcribed using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, California, USA). qRT-PCR was conducted using the UltraSYBR Mixture (CW Biotech, Beijing, China) on an Mx3000 P QPCR System (Stratagene, San Diego, CA). The *Foc* 4 *ACTIN* gene was used as an internal control to normalize the transcriptional levels of all samples. Amplification was hot-started at 95 °C for 10 min and followed by 42 cycles of denaturation at 94 °C for 10 s, annealing at 57 °C for 30 s, and extension at 72 °C for 15 s. Melting curves were generated to confirm the specificity of PCR products. The relative expression level was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three biological replicates were conducted in the qRT-PCR assay. qRT-PCR primers for each gene are listed in Table S1.

2.4. Creation of knockout construct of Foc 1324

The construct was built through fusion PCR. The schematic diagram

is shown in Fig. S1. In brief, the 1,664-bp selection marker hygromycin resistance cassette region obtained from vector pCT74 was flanked by a 1,110-bp upstream and a 997-bp downstream region of the *Foc 1324* ORF. To generate the construct, the 3'-terminus of the upstream region and the 5'-terminus of the downstream region of *Foc 1324* were introduced into 19-20 bp oligonucleotides overlapped with the hygromycin marker through primers K-1324-R1 and K-1324-F2. A total of 1,000 ng of three fragments were added into a 50-μl pfu PCR reaction system with a 1:1:1 molar ratio to carry out a fusion PCR without the addition of any primers. The reaction was conducted for 30 cycles of 95 ˚C for 30 s, 58 ˚C for 30 s, and 72 ˚C for 2.6 min. Following the fusion PCR, 1 μl of the reaction product was used in a pfu PCR reaction to amplify the complete knockout construct with primer pairs K-1324-F1 and K-1324-R2. Primers used are listed in Table S2.

2.5. Generation of a Foc 1324 knockout in Foc race 4

For the *Foc 1324* knockout mutant, protoplasts of *Foc* 4 were prepared and transformed with the knockout construct of *Foc 1324* using a PEG/$CaCl_2$-mediated method (Xiao et al., 2009). The transformants were selected from a PDA plate containing 100 μg/ml of hygromycin and confirmed by PCR with primer pairs 1324-up/Hyg-R and Hyg-F/1324-down for *in-locus* recombination and with primer pairs 1324-F/1324-R for *1324* deletion confirmation. Primers used are shown in Table S2.

2.6. Complementation of the Foc 1324 knockout mutant

Modified vector pCT74 (MpCT74) was used to build the construct for *Foc 1324* complementation. In MpCT74, the hygromycin resistance cassette was replaced with the G418 resistance cassette for a selection marker. To generate MpCT74, pCT74 was first linearized by PCR using primers pCT74-D-Hyg-F and pCT74-D-Hyg-R to remove the hygromycin resistance cassette, and then the linearized vector was ligated with the G418 resistance cassette obtained from a PCR amplification with primer pairs G418-to-pCT74-F and G418-to-pCT74-R through 15–20 overlapped nucleotides using the In-Fusion® HD Cloning Kit (Takara Bio USA, Inc.). For *Foc 1324* complementation, the SGFP ORF was removed from vector MpCT74 by PCR with primer pairs pCT74-D-SGFP-F and pCT74-D-SGFP-F and replaced with *Foc 1324* ORF obtained with primers 1324-to-pCT74-F and 1324-to-pCT74-R through overlapped nucleotides using the In-Fusion® HD Cloning Kit. The complementary construct was transformed into *the Foc 1324* knockout mutant through protoplasts. The complementary transformants were selected from a PDA plate containing 100 μg/ml of G418 and confirmed by PCR with primer pairs 1324-F and 1324-R. Primers for modification of pCT74 and complementary constructs are listed in Table S2.

2.7. Virulence assay

Three-month-old BX plants were obtained from the Tissue Culture Center of the Chinese Academy of Tropical Agricultural Sciences and used for virulence assays. 1×10^7/ml of spore suspension of wild *Foc* 4, *Foc 1324* knockout, and three complementants were freshly prepared and used for the inoculation of banana plantlets using the root dip method described above. The control plants were treated with deionized water. Ten plantlets were used for each treatment, and three independent biological repeats were carried out. The plantlets were replanted in culture pots, and external and internal (pseudostem) symptoms were detected three weeks after inoculation. For the disease scoring system, the scale of symptoms proposed by Dita et al. (2014) was used (see supplementary Fig. S2).

3. Results

3.1. Genome-wide identification of mimp-associated effector genes in Foc 1 and Foc 4

Mimps have been investigated in 3 *Foc* strains in previous studies, but unlike the II5 (*Foc* tropical race 4) strain that contains 11 *mimps*, only 2 *mimps* were found in strain B2 (*Foc* race 4) and 0 in strain N2 (*Foc* race1) (van Dam et al., 2016; van Dam and Rep, 2017). To extensively exploit the *mimp* sequences, we searched our local genome databases of *Foc* 1 and *Foc* 4 using 13 *mimp* sequences collected from *Foc* strains II5 and B2 as queries. As a result, a total of 41 *mimps* (20 in *Foc* 1 and 21 in *Foc* 4) with > 90 % similarity were obtained from *Foc* 1 and *Foc* 4 genomes. Next, the *mimp*-associated genes were predicted, and finally 20 candidate effector genes downstream of *mimp* were obtained, among which 3 were from *Foc* 1, 6 from *Foc* 4, and 11 were found both in *Foc* 1 and *Foc* 4. These predicted genes were named according to their DNA lengths. All the candidate effector genes are listed in Supplementary Table S3.

3.2. Specific presence of effector genes in Foc 1, Foc 4, and F. oxysporum ff. spp

Given that the effector repertoire can determine the specificity of the host, it is necessary to determine the effector factors specific to *Foc* 1 or *Foc* 4. For each predicted gene, specific PCR bands were obtained from *Foc* 1 and/or *Foc* 4 genomic DNA using primers listed in Table S1. The sequence and size of the PCR products were confirmed by sequencing, and the resulting DNA size for each gene is listed in Table S1. As shown in Fig. 1, genes *339*, *810*, and *1053* were *Foc* 1-specific; genes *177*, *201*, *330*, *844*, *1324*, and *2421* were *Foc* 4-specific; and the other 11 genes were present both in *Foc* 1 and *Foc* 4. Furthermore, BLASTn

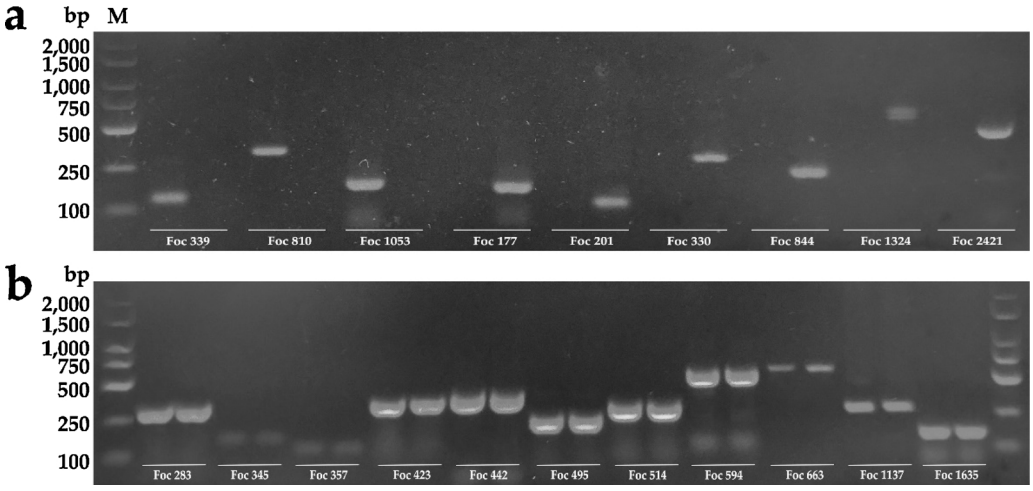

Fig. 1. Specific presence of candidate effector genes in *Foc* 1 and *Foc* 4. PCR products were amplified from *Foc* 1 and *Foc* 4 genomic DNA using primers listed in Table S1. Lane M indicates a DNA marker. For each gene, the left lane shows the band from *Foc* 1, and the right lane shows the band from *Foc* 4. (a) shows genes that are present specifically in *Foc* 1 or *Foc* 4, and (b) shows genes that are present in both *Foc* 1 and *Foc* 4.

Table 1
Expression of all effector genes *in vitro* and *in planta* at 3 dpi.

Gene	*Foc* 1-PDB	*Foc* 1-infection	*Foc* 4-PDB	*Foc* 4-infection
Foc 514	×	✓	×	✓
Foc 357	✓	✓ 32,725.41	✓	✓ 1,616.93
Foc 423	✓	✓ 59.19	✓	✓ 269.27
Foc 663	✓	✓ 68.31	✓	✓ 164.83
Foc 442	✓	✓ 127.97	✓	✓ 71.97
Foc 495	×	×	✓	✓ 106.84
Foc 283	✓	✓ 16.43	✓	✓ 1.81
Foc 1635	✓	✓ 0.23	✓	✓ 1.06
Foc 345	✓	✓ 1.14	✓	✓ 0.67
Foc 594	✓	✓ 308.11	×	×
Foc 1137	×	×	×	×
●*Foc 810*	✓	✓ 373.81	——	——
●*Foc 339*	×	×	——	——
●*Foc 1053*	✓	✓ 18.45	——	——
▲*Foc 1324*	——	——	✓	✓ 108.96
▲*Foc 177*	——	——	✓	✓ 15.95
▲*Foc 844*	——	——	✓	✓ 12.74
▲*Foc 2421*	——	——	✓	✓ 5.85
▲*Foc 201*	——	——	✓	✓ 2.36
▲*Foc 330*	——	——	✓	✓ 1.13

× indicates no expression detected; ✓ indicates expression detected; numbers following ✓ indicate multiples of transcriptional change; ● indicates *Foc* 1-specific genes; ▲ indicates *Foc* 4-specific genes.

searches against the FOSC WGS database indicated that, unlike most candidate genes that were widespread in *F. oxysporum* species, gene *339* only existed in *Foc* 1, gene *663* only existed in *Foc*, and genes *201*, *2421*, and *514* were present in *Foc* and a few *F. oxysporum* ff. spp. (Table S4).

3.3. Expression patterns of effector genes during banana infection of Foc 1 and Foc 4

To reveal the roles of the predicted genes in the occurrence of banana Fusarium wilt, the expression patterns of all the identified genes were investigated during *Foc* 1 and *Foc* 4 infection. As listed in Table 1, with the exception of genes *339* and *1137*, which were neither detected *in vitro* nor *in planta*, most genes (15 out of 20) were evidently upregulated during *Foc* 1 or *Foc* 4 infection. Especially for gene *357*, its transcription level increased dramatically by 32,725 and 1,617 times during *Foc* 1 and *Foc* 4 infection, respectively. Gene *514* expression was stimulated by infection, though it was undetectable in PDB. Similarly, the *Foc* 1-specific gene *810*; the *Foc* 4-specific gene *1324*; and genes *423*, *442*, *495*, *594*, and *663* had an expression level increase greater than 50- to 300-fold. Among the strongly expressed genes, genes *283*, *495*, and *594* showed different expression patterns between *Foc* 1 and *Foc* 4. Gene *283* was highly expressed in *Foc* 1 but not significantly in *Foc* 4, and genes *495* and *594* were highly expressed only in either *Foc* 4 or *Foc* 1, although they were present both in *Foc* 1 and *Foc* 4. In contrast, genes *345*, *1635*, *201*, and *330* displayed normal or downregulated expression during *Foc* 1 and *Foc* 4 colonization of banana.

3.4. Function prediction of effector genes

To annotate the candidate effectors, Software SignaIP 4.1 was used to predict the signal peptide, EffectorP 2.0 was used to perform effector prediction, and InterProScan was applied to find the protein motifs. As shown in Table 2, most *mimp*-associated effectors possessed an N-terminal signal peptide. Nine genes, including *Foc 283, 357, 177, 339, 423, 442, 514, 594*, and *810*, encoded possible fungal effectors predicted by EffectorP 2.0. All candidate effectors predicted by EffectorP 2.0 had a small protein size less than 300 aa, and most of them had a relatively high cysteine content. Candidate effector 357 was found to be homologous to SIX 9, having 87 % amino acid identity with Foc SIX 9 (ARR28612.1), and 53 % identity with Fol SIX 9 (XP_018253918.1). Foc 283, 345, 357, 495, 1137,1324, 1053, and 1635 contained distinct motifs in their protein sequences; however, there were not any motifs found in the other 12 candidate effectors (Table 2).

3.5. Knockout of Foc 1324 impaired the virulence of Foc 4

To confirm the pathogenicity of the candidate effector genes, a knockout mutant of the gene *Foc 1324* was generated and subjected to the pathogenic test. Three weeks after inoculation, plants inoculated with wild *Foc* 4 showed obvious banana wilt symptoms with inhibited growth, such as withering leaves, turning completely yellow, and suffering severe pseudostem browning compared with water-treated control plants (Fig. 2a). Also, the disease indices for the external and internal symptoms of *Foc* 4-treated plants were significantly higher than that of the water-treated plants (Fig. 2b). In contrast, plants inoculated with the knockout mutant Foc Δ1324 showed a significant reduction of disease symptoms with significantly lower disease indices for both external and internal symptoms compared with Foc 4-inoculated plants (Fig. 2).

3.6. Foc 1324 complementation restored the pathogenicity of Foc Δ1324

To verify the virulence of the *Foc 1324*, we generated complementary mutants of *Foc Δ1324* and performed the pathogenicity test. As shown in Fig. 2, the introduction of the *Foc 1324* restored the virulence of *Foc Δ1324* to levels comparable to the wild *Foc* 4, implying that *Foc 1324* is required for the virulence of *Foc* 4.

4. Discussion

The effector factors of pathogens play key roles in the process of plant aggression. However, due to the insufficient number of effector genes identified in *Foc*, systematic functional disclosure of *Foc* effector factors is greatly limited. In this study, we identified 20 candidate effector genes from the *Foc* 1 and *Foc* 4 genomes based on the locus association of the transposable element *mimp* and effector factors on chromosomes. This expands our knowledge of effector factors in *Foc* 1 and *Foc* 4, two important pathogenic races of banana fusarium wilt that include the strain most destructive to Cavendish, TR4. Since TR4 carries homologs of specific candidate effector genes identified in *Foc* 4, these genes might function similarly in TR4. We therefore predict that Foc 1324, identified as a pathogenic factor in this study, contributes to TR4 pathogenicity.

Of the 20 predicted genes, some however had gene annotations discrepant with those available in the *Foc* 1 and *Foc* 4 genomes. To confirm the sequence and to check for the presence of the cDNA of the candidate genes, we sequenced the PCR products from cDNA templates using primers listed in Table S1. The results indicated that some of the predicted genes were not annotated or were incorrectly annotated in the *Foc* 1 and *Foc* 4 genomes. The complete cDNA sequence of each gene needs to be further confirmed in studies targeting each specific gene.

As key pathogenic factors, it is reasonable to hypothesize that effector factors have a high expression level during plant infection. The results in this study agree with this inference, given that most candidate genes showed significantly increased transcriptional levels in the course of fungal infection. Particular attention should be paid to genes *357* and *514*, whose expression levels increased dramatically, suggesting that they play important roles during *Foc* infection. *357* was identified as a homolog of the *SIX9* gene, which is one of *SIX* genes often showing dramatic transcriptional improvement during infection in many *F. oxysporum* species (Gawehns et al., 2014; Thatcher et al., 2016; van Dam et al., 2016). The high expression levels for genes that exist both in *Foc* 1 and *Foc* 4 implies that they are functionally conserved and involved in the course of infection of each *Foc* race. Since *Foc* 1 and *Foc* 4 infect different banana cultivars, it is reasonable to think that *Foc* 1 and *Foc* 4 employ specific effector suites to infect their respective hosts.

Table 2
Function prediction of effector candidates.

Effector genes	Function annotation	Signal peptide	Effector prediction	Predicted protein size (aa)	Cysteine number/ content (%) (excluding signal peptide)
Foc 283	RALF-like 33	Yes	✓	75	4/7 %
Foc 345	Kex1 protease	Yes		847/826	7/1 %
Foc 357	SIX9 homolog	Yes	✓	118	6/6 %
Foc 495	GPI anchor	Yes		164	7/5 %
Foc 1137	ABC transporter	Yes		364/363	0/0 %
Foc 1324	Zinc-metalloproteinase	Yes		401	12/3 %
Foc 1053	Transposase, Tc1-like	No		350	7/2 %
Foc 1635	Zinc finger, CCHC-type	No		544	11/2 %
Foc 177	Unknown	Yes	✓	58	1/3 %
Foc 201	Unknown	Yes		66	0/0 %
Foc 339	Unknown	Yes	✓	112	7/7 %
Foc 423	Unknown	Yes	✓	104/96	5/6 % (Foc1); 4/5 % (Foc4)
Foc 442	Unknown	Yes	✓	111	7/7 %
Foc 514	Unknown	Yes	✓	134	6/5 %
Foc 594	Unknown	Yes	✓	197	8/4 %
Foc 810	Unknown	Yes	✓	269	8/3 %
Foc 844	Unknown	Yes		263	8/3 %
Foc 330	Unknown	No	✓	109	0/0 %
Foc 663	Unknown	No	✓	169	2/1 %
Foc 2421	Unknown	No		736	12/2 %

"✓" indicates that the candidate genes were predicted by EffectorP 2.0 as possible effectors, and "✓ "indicates possible effectors predicted by EffectorP 1.0. The individual numbers in the "predicted protein size" column represent the same size of encoded peptides in *Foc* 1 and *Foc* 4, and the format "847/826″ indicates the respective size of encoded peptides in *Foc* 1 and *Foc* 4 (*Foc* 1/*Foc* 4).

Therefore, those genes that are exclusively present in *Foc* 1 or *Foc* 4 should be paid close attention. Of the *Foc* 4-specific genes, four (*1324*, *177*, *844*, and *2421*) were strongly induced during banana infection, especially *1324*, which displayed a 109-fold expression enhancement. Similarly, in *Foc* 1, *810* and *1053* demonstrated an evident transcription increase, reaching 374-fold and 18-fold, respectively, while the transcription of gene *339* was not detected *in vitro* or *in planta*. The specific presence in *Foc* 1 or *Foc* 4 and strong transcriptional changes suggest that these genes might play roles in host selection. Although genes *283*, *495,* and *594* existed in both *Foc* 1 and *Foc* 4, they had different expression patterns in the two races, suggesting that their expressions are race-specific and play different roles in each race. In addition to race-specific genes in *Foc*, genes that are found only in a few *F. oxysporum* spp. or even only in *cubense* should be of particular concern, because they might be evolutionarily related to their banana host selection. Since we only took samples at 3 dpi, which is not representative of the entire infection process, the low or even zero expressions of some candidate genes may not mean that they were ineffective during *Foc* infection.

Effectors can be secreted by microorganisms to the host to facilitate infection and/or trigger host defense responses (Kamoun, 2006). Fungal effectors share no significant sequence similarity with each other due to their rapid divergence and host specialization (Sheppard et al., 2015). Given the lack of conserved sequence features, fungal effector prediction approaches have been based on relatively broad criteria, such as the presence of a secretion signal, a small protein size, a high cysteine content (Sheppard et al., 2015). Among our 20 candidate genes, 12 had both a predicted signal peptide and a protein size less than 300 aa, implying that they were possibly effectors. However, not all secreted proteins with a small size will have an effector function, and conversely, not all fungal effectors will be small (Sheppard et al., 2015). EffectorP is the first machine-learning method trained to predict fungal effectors from secretomes (Sperschneider et al., 2016, 2018). Of the 12 possible effectors predicted by signal peptide and protein size, 9 were predicted by EffectorP 2.0 as possible effectors. The presence of multiple cysteine residues is considered to be one of the common features of extracellular effectors (Stergiopoulos and de Wit, 2009). In our results, most of the candidate effector genes predicted by EffectorP 2.0 had a relatively high cysteine content, suggesting that they were likely to be real effectors. In particular, Foc 330 and Foc 663 were predicted by EffectorP 1.0 as possible effectors, although they had no signal peptides predicted by SignalP 4.1. In fact, Foc 330 was predicted to be non-classically secreted with NN scores above the threshold of 0.5 by SecretomeP 2.0 (for the prediction of non-classical protein secretion, http://www.cbs.dtu.dk/services/SecretomeP), suggesting that Foc 330 might be secreted by *Foc* though an unconventional secretion pathway (Liu et al., 2014). Foc 663, however, was predicted to be neither a signal peptide-containing protein nor a non-classical secretion protein, implying that it might either contain an unrecognized signal peptide separated by introns or be part of a larger protein. Accordingly, experimental evidence is needed to confirm the complete sequence of these two proteins and whether they are secreted and are real effectors. Given its accuracy, effector prediction through bioinformatic methods should be the first step in identifying real effectors, with further confirmation being essential in subsequent studies, including for proteins predicted as non-effectors in this study.

Gene *Foc 357* shares amino acid homology with the known SIX9 protein, which is among a class of typical effector factors widely reported in *F. oxysporum* species (Thatcher et al., 2012; Guo et al., 2014; Chakrabarti et al., 2015; de Sain and Rep, 2015; Laurence et al., 2015; Lanubile et al., 2016; Niu et al., 2016; Pu et al., 2016; Thatcher et al., 2016; Williams et al., 2016; van Dam et al., 2017b). Rapid alkalinization factor (RALF) was initially defined as a plant peptide able to trigger a pH increase in cell cultures (Pearce et al., 2001). *Fusarium RALFs* were thought to be acquired by horizontal gene transfer from plants. *Fol* RALF-B can induce plant responses typical of endogenous plant RALF, implying that pathogenic fungi might hijack endogenous plant physiological mechanisms to enhance their virulence (Thynne et al., 2017). *Foc 283* is homologous to FOIG_11494, which has been identified as a RALF in *Foc* TR4 II5, implying that *Foc* might stimulate its RALF activity similar to that of banana to facilitate infection. Most effectors obtained in this study, however, have unknown functions, and how these candidate genes work in *Foc* pathogenicity will be the focus of future research. According to the available evidence, it is reasonable to speculate that effector factors may act synergistically to complete the infection of the host. As the functions of more and more effector factors are revealed, it will help us understand the infection mechanisms of pathogens more clearly and comprehensively.

Foc 1324 was *Foc* 4 specific and showed a significant expression increase (109-fold) during *Foc* 4 invasion. To validate the pathogenicity of *Foc 1324*, we developed a knockout mutant and complementants of *Foc 1324* and tested their virulence on banana plants. Results showed

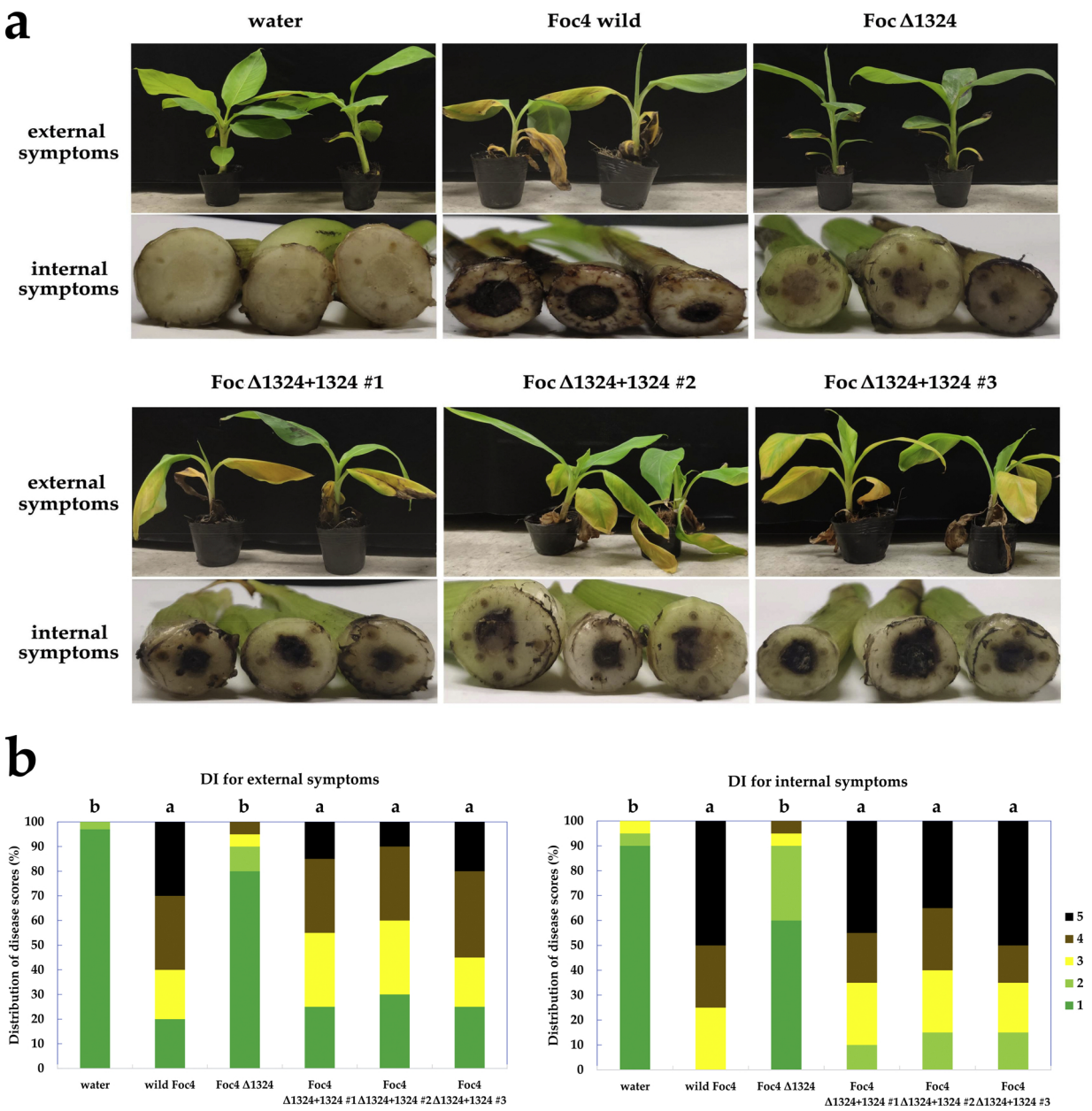

Fig. 2. *Foc 1324* is required for the virulence of *Foc* 4. Three-month-old BX banana plantlets were inoculated with water, wild *Foc* 4, *Foc 1324* knockout mutant (Foc Δ1324), and three complementants (Foc Δ1324 + 1324 #1-3). (a) External and internal (pseudostem) symptoms three weeks after inoculation. (b) Disease indices for external and internal symptoms. Disease grades (1–5) were based on the disease scoring system proposed by Dita et al. (2014, see Supplementary Fig. S2). Difference significances for external and internal symptoms (shown with lowercase letters) were analyzed by Kruskal-Wallis one-way ANOVA (k samples, $p = 0.05$).

that the disruption of *Foc 1324* significantly reduced the virulence of *Foc* 4, and the reintroduction of *Foc 1324* restored the pathogenicity of the *Foc 1324*-knockout mutant. These results suggest that *Foc 1324* is a virulent factor required for the pathogenicity of *Foc* 4. Since *Foc 1324* was *Foc* 4 specific and not present in *Foc* 1; whether it is a factor that results in the host differences between the two *Foc* races is an interesting topic to investigate. Hypothesis verification could be done through the integration of *Foc 1324* into *Foc* 1 DNA and testing the pathogenicity of *Foc* 1 on banana cultivar BX. If *Foc 1324*-introduced *Foc* 1 exhibits virulence on its resistant host BX, this will provide proof that *Foc 1324* is a factor that determines *Foc* host specificity.

In conclusion, the identification and functional verification of the promising candidate effector genes in this study will help to comprehensively reveal the roles of effector factors in *Foc*, enabling us to understand the mechanisms underlying *Foc* infection and plant defense.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126375.

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