Fungal Biology 123 (2019) 423–430

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

The effector SIX8 is required for virulence of *Fusarium oxysporum* f.sp. *cubense* tropical race 4 to Cavendish banana

Bang An [1], Xingrong Hou [1], Yunfeng Guo, Shixue Zhao, Hongli Luo, Chaozu He[**], Qiannan Wang[*]

Hainan Key Laboratory for Sustainable Utilization of Tropical Bioresources and College of Biology, Institute of Tropical Agriculture and Forestry, Hainan University, Haikou, 570228, China

ARTICLE INFO

Article history:
Received 2 November 2018
Received in revised form
3 March 2019
Accepted 5 March 2019
Available online 11 March 2019

Corresponding Editor: Nik Money

Keywords:
Banana wilt disease
Expression profiles
Secreted in xylem
Secretory protein

ABSTRACT

Plant pathogens employ effectors as molecular weapons to manipulate host immunity and facilitate colonization. *Fusarium oxysporum* f. sp. *cubense* is the agent of wilt disease in banana plantlets and four races of the pathogen have been identified based on the cultivar specificity. A total of 9 *SIX* genes have been detected in the genome of Foc TR4 and 6 genes detected in Foc1. Among these *SIX* genes, *SIX2* and *SIX8* are only detected in Foc TR4, not identified in Foc1. Expression profiles analysis revealed that *SIX* genes of Foc TR4 are highly induced after inoculation to Cavendish banana plantlets. Virulence analysis of the *SIX2* and *SIX8* knock-out mutants showed that *SIX8* is required for the virulence of Foc TR4 while *SIX2* has no obvious functions. Over expression of SIX8-FLAG proteins in the *SIX8* knock-out mutant partly restored the virulence. Western blot analysis suggested that SIX8 could be secreted into the extracellular space and a signal peptide resided the N-terminal polypeptide sequence. This study provides some clues for further research on mechanism of *SIX8* in regulating virulence of Foc TR4.

1. Introduction

Fusarium oxysporum species are major pathogens of many important crops worldwide. These soil-borne fungi could infect a wide range of plant hosts, cause wilt disease, and lead to huge economic loss. According to their host specificity, pathogenic isolates of *F. oxysporum* are grouped into formae speciales (f. spp.) (Laurence et al., 2012; Michielse and Rep, 2009). Formae speciales are further divided into 'races' based on the cultivar specificity of the same host species. *F. oxysporum* f. sp. *cubense* (Foc) was identified as the agent of banana (*Musa* spp.) wilt disease (also named as 'panama disease'). Four races of Foc have been so far: Race 1 (Foc1) causes disease in Gros Michel (AAA) and cultivars with the AAB genome; race 2 infects race 1-susceptible cultivars and cooking cultivars with ABB genome; race 3 affects *Heliconia* species; race 4 has a broad host range, including 'Dwarf Cavendish' (AAA genome) as well as all race 1- and race 2-susceptible cultivars. The isolates within Race 4 are further divided into 'tropical race 4' (TR4) and 'subtropical race 4' (SR4) groups (Stover, 1990; Ordonez et al., 2015; Ploetz, 2005). Earlier in the 20 century, Gros Michel was the main cultivar of the world, but the wilt disease caused by Foc1 nearly destroyed the banana industry. Then Cavendish cultivars, which are resistant to Foc1, replaced Gros Michel and became the main cultivar of banana industry. However, Foc race 4 is capable of attacking Cavendish cultivars, leading to the serious loss around the world (Grimm, 2008). Genome analysis revealed high similarity between Foc TR4 and Foc1, with 15 140 orthologues at 96.7 % of amino acid identity. However, Foc TR4 has evolved with some race specific gene families such as transporters and transcription factors, which might be required for the virulence to banana (Guo et al., 2015).

In the long periods of co-evolution, pathogenic fungi have evolved complex mechanisms to overcome plant immune systems; one of the ways is to secret virulence factors known as "effectors" into the host cells to suppress plant innate immunity. By analyzing the xylem sap proteome of tomato plantlets infected with *F. oxysporum* f. sp. *lycopersici* (Fol), SIX (secreted in xylem) proteins were first identified in *F. oxysporum* (Rep et al., 2002).

Abbreviations: secreted in xylem, SIX; *Fusarium oxysporum* f.sp. *cubense*, Foc; wild type, WT.

* Corresponding author.
** Corresponding author.
E-mail addresses: czhe@hainu.edu.cn (C. He), wangqiannan@hainu.edu.cn (Q. Wang).

[1] Those author contribute equally to the article.

https://doi.org/10.1016/j.funbio.2019.03.001
1878-6146/

Thus far, a total of 15 *SIX* genes have been identified in Fol and other *F. oxysporum* formae speciales (Czislowski et al., 2018; Houterman et al., 2007; Lievens et al., 2009; Ma et al., 2010; Rep et al., 2004; Rep and Kistler, 2010; Schmidt et al., 2013; Simbaqueba et al., 2018; Thatcher et al., 2012; van der Does and Rep, 2007; Williams et al., 2016). The protein sequences of SIX display little homology with other known proteins or to each other, and the biological functions of SIX largely remain unknown. Study on Fol has provided some clues for the function analysis of *SIX* genes. In Fol, *SIX1*, *SIX3* and *SIX4* act both as virulence factors to contribute to full virulence of the pathogen to susceptible tomato lines or *Arabidopsis*, and as an avirulence gene (Avr) to induce the R gene mediated tomato plant resistance in tomato lines harbor *I*-1, *I*-2 and *I*-3. Thus, the three *SIX* genes have also been assigned the names AVR1 (*SIX4*), AVR2 (*SIX3*), and AVR3 (*SIX1*). Further research revealed that SIX4 functions as suppressor of *I*-2 and *I*-3 mediated resistance (Houterman et al., 2008; Houterman et al., 2009; Rep et al., 2004, 2005). Fol *SIX5* and *SIX3* share the same promoter and the two genes are transcribed in opposite directions; furthermore, SIX5 and SIX3 physically interact with each other and are both required to activate *I*-2-mediated immunity in tomato (Ma et al., 2015). SIX6, with homologues found in *Colletotrichum* spp, is required for full virulence in a susceptible host; and transient expression in *Nicotiana benthamiana* showed the effector could suppress the *I*-2 mediated cell death (Gawehns et al., 2014).

In Foc, homologues of *SIX1*, *SIX2*, *SIX6*, *SIX7*, *SIX8*, *SIX9* and *SIX13* have been identified; and the genomic investigation of different isolates provide strong evidence for the horizontal transmission of *SIX* genes in Foc (Czislowski et al., 2018). Among the *SIX* genes, *SIX2* and *SIX8* have only been detected in isolates of Foc race 4. *SIX8* have been proved to be an effective molecular marker, which allows race 4 to be distinguished from race 1 and 2 isolates; in addition, variations of SNP of SIX8 could further differentiates tropical and subtropical race 4 isolates (Fraser-Smith et al., 2014). It has been reported that knocking out of *SIX1*a in Foc TR4 severely reduced virulence to Cavendish banana (Widinugraheni et al., 2018). But functions of other *SIX* genes remain unknown. In the present study, the expression profiles of Foc TR4 *SIX* genes during infection process to Cavendish banana were analyzed. Furthermore, functions of *SIX2* and *SIX8* in regulating virulence of Foc TR4 were investigated.

2. Materials and methods

2.1. Fungal strains and culture conditions

F. oxysporum f.sp. *cubense* race 4 (Foc TR4, isolate B2) and race 1 (Foc1, isolate N2) were isolated respectively from diseased rhizomes of banana (*Musa* spp.) cultivars 'Brazilian' (AAA group) and 'Pisang Awak' (ABB group) in Hainan of China (Guo et al., 2015). All the strains were maintained on the potato dextrose agar (PDA) medium or Malt extract agar medium (Oxoid, Basingstoke, England) at 28 °C. For liquid culture, complete medium and modified Czapeck liquid medium in which glucose was replaced with apple pectin were employed.

2.2. Identification of SIX genes in the genomes of Foc TR4 and Foc1

To identify *SIX* genes in Foc TR4 and Foc1, protein sequences of SIX proteins of Fol 4287, *F. oxysporum* f. sp. *pisi* and *F. oxysporum* f. sp. *cepae* downloaded from public NCBI databases were used to BLAST search against the genome assemblies of Foc TR4 and Foc1.

2.3. Inoculation of banana plantlets and virulence assay

Banana plantlets (*Musa acuminata* L. AAA group, 'Brazilian') at five-leaf stage, about 90 d old, were obtained from the Tissue Culture Center of Chinese Academy of Tropical Agricultural Sciences. The matrix composed of vermiculite, sand, and coconut chaff, which could be easily cleaned from the plant roots, was used to grown the plantlets. The plantlets with a healthy root system were cultured in glasshouse and used for treatments. Foc TR4 strains were cultured in the liquid complete medium for 3 d, and conidia were collected by filtration, washed and resuspended with ddH_2O to a final concentration of 10^6 conidia mL^{-1}. Then 200 mL of the conidia suspension was irrigated into the root of one banana plantlet. A total of 20 banana plantlets were used for each treatment. The leaf disease symptoms were recorded at 3, 4 and 5-week post-inoculation. The disease scores were defined as follows: 0- the plantlet showed no disease symptoms; 1- less than 25 % of leaves showed disease symptoms; 2- more than 25 % but less than 50 % of leaves showed disease symptoms; 3- more than 50 % but less than 75 % of leaves showed disease symptoms; 4- more than 75 % of leaves showed disease symptoms. Internal symptoms and the darkening of the rhizome and pseudostem were evaluated at 5 week post-inoculation. Differences in the distributions of disease scores between treatments were tested for statistical significance by Mann–Whitney tests. The experiment was conducted twice.

2.4. RNA isolation, reverse transcript, and quantitative real-time PCR

For the gene expression analysis, the banana plantlets after inoculation for 1, 3, 5 and 7 d were sampled. At each time point, 5 banana plantlets were picked randomly as an independent sample. The root was washed clean, cut from the plants and used for RNA extraction through the CTAB method. Briefly, about 4 g of root tissue were ground to a fine powder in liquid nitrogen, placed into 65 °C extraction buffer (2 % CTAB, 4 % PVP, 100 mM Tris-HCl, 25 mM EDTA, 3 M NaCl, 2 % β-mercaptoethanol, and DEPC H_2O), vortexed and incubated at 65 °C for 5 min. After centrifugation at 4 °C, 11 000 g for 10 min, the supernatant was collected, added with the same volume of chloroform/isoamyl alcohol (24:1) and vortexed vigorously. After another centrifugation at 4 °C, 11 000 g for 20 min, the aqueous phase was collected and RNA was precipitated with an equal volume of 4 M LiCl at 4 °C overnight. After that, the RNA was collected by centrifugation for 20 min at 4 °C and 11 000 g, washed with 70 % ethanol twice and air-dried. The dried pellet was dissolved in the DEPC H_2O. The contaminating DNA was eliminated by using RNase-free DNase according to the manufacturer's instruction (NEB, USA). After quantification using NANODROP 2000 (Thermo Fisher Scientific, MA, USA), the first-strand cDNA was synthesized with Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA). The Quantitative real-time PCR was then performed using FastStart Universal SYBR Green Master (Roche, Switzerland) with a LightCycler 96 Real-Time PCR System (Roche, Switzerland) instrument. The relative expression levels of the target genes were assessed based on $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *Actin* (EMT65107.1) of Foc TR4 was employed as the internal control for gene expression analyses. The samples after inoculation for 1 d were employed as the reference to calculate the changes in expression of the genes.

2.5. Vector constructions

Nucleotide of *SIX2* and *SIX8* were knocked out by homologous recombination and the strategies were showed in Fig. 2. Vector pBS-NEO was used as backbone to construct replacement vectors,

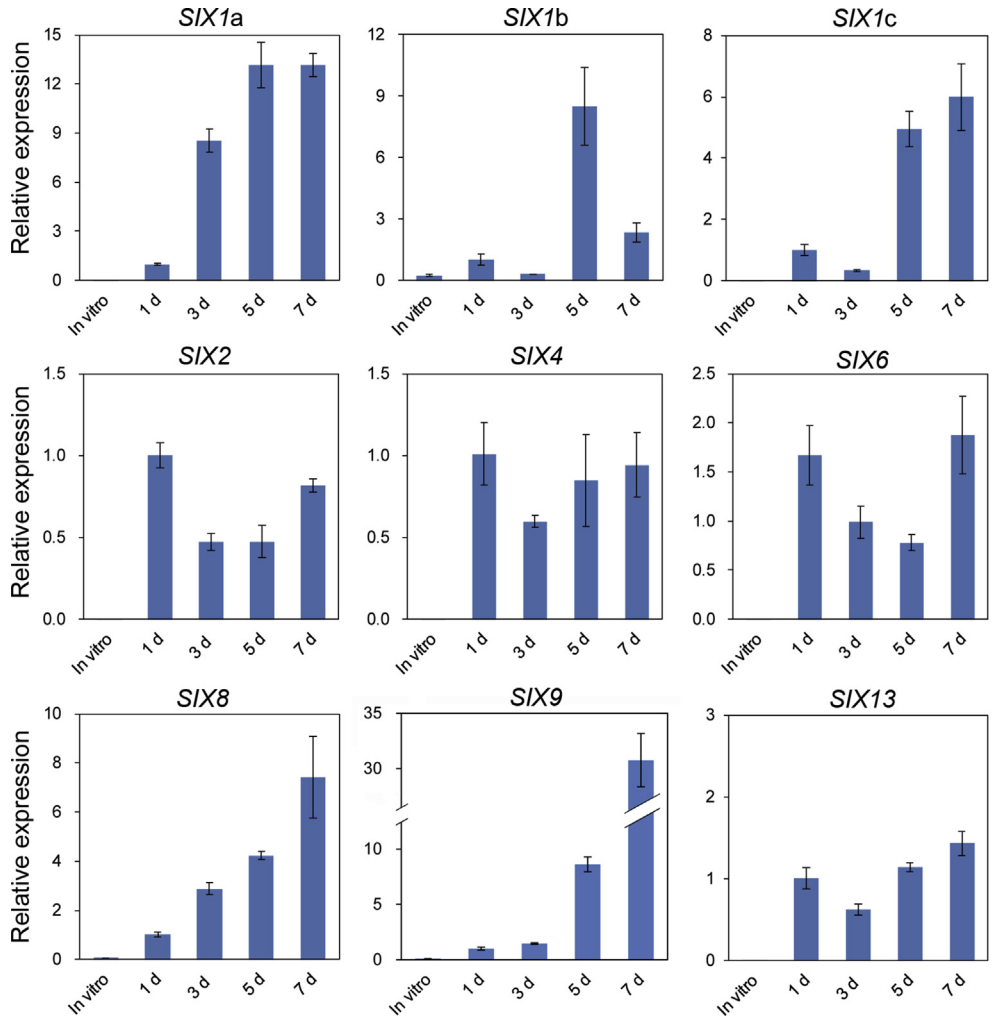

Fig. 1. Quantitative real-time PCR analysis of the expression of *SIX* after inoculation to banana plantlets for 1, 3, 5, and 7 d. The relative expression levels of the target genes are assessed based on $2^{-\Delta\Delta Ct}$ method through normalization against the expression level of 1 d. Foc TR4 *Actin* has been used as the internal control. Bars represent standard deviations (SD).

and the Neomycin phosphotransferase gene (*NPT*II) conferring resistance to Geneticin (G418) was used as the selection marker. Briefly, the flanking regions of the target gene were amplified and ligated into the vector pBS-NEO. The replacement vectors were linearized with *Sac*I before the protoplast transformation.

The vector pFoNDHTN was constructed to build an efficient expression system for Foc TR4, which uses the locus of nitrate reductase (*niaD*) as the targeted integration of reporter gene constructs (Schumacher, 2012). The strategy for the vector construction was showed in Fig. S1A: the 5′ and the 3' flanking regions of the *niaD* of Foc TR4, the *HPH* cassette, the promoter of ToxA and the terminator of nos from the vector pCT74 (Lorang et al., 2001) were amplified and ligated into the vector pBlueScript SK (+) to generate pFoNDHTN. For overexpression of *SIX8*, the ORF of *SIX8* was merged with the coding sequence of 3 X FLAG, and ligated into the vector pFoNDHTN (Fig. S1B). The overexpression vector was linearized with *Sac*I before the protoplast transformation.

2.6. Transformation of Foc TR4, PCR diagnosis, and single conidia purification

For generation of knock-out mutants, protoplast preparation and transformation were conducted as description in our previous work (Hou et al., 2018). For construction of *ToxA*::*SIX8-FLAG* overexpression mutant, the *SIX8* knock-out mutant was used as recipient strain, and regeneration medium containing 300 μg mL^{-1} Hygromycin B (Sigma–Aldrich, St Louis, MO, USA) and 100 μg mL^{-1} G418 was used to select the transformants. Transformants were analyzed by PCR with the primer pairs as shown in Table S2. Single conidial isolates of the correct transformants were obtained by spreading 100 μL of conidial suspension (10^4 conidia mL^{-1}) on Malt extract agar medium containing G418 or G418/Hygromycin respectively. The Single conidial isolated strains were also confirmed by PCR.

2.7. Western blot analysis

Intracellular and extracellular proteins of Foc TR4 were extracted as description in our previous work (Hou et al., 2018). Conidia were inoculated into 200 mL modified Czapeck liquid medium in which glucose was replaced with apple pectin (Sigma–Aldrich, St Louis, MO, USA) to the initial concentration of 10^3 conidia mL^{-1}. Then conidia were cultured for 3 d. For intracellular protein extraction, mycelium was collected by centrifugation and used for intracellular proteins extraction. Briefly, mycelium was disrupted in liquid nitrogen by grinding in a mortar with a pestle.

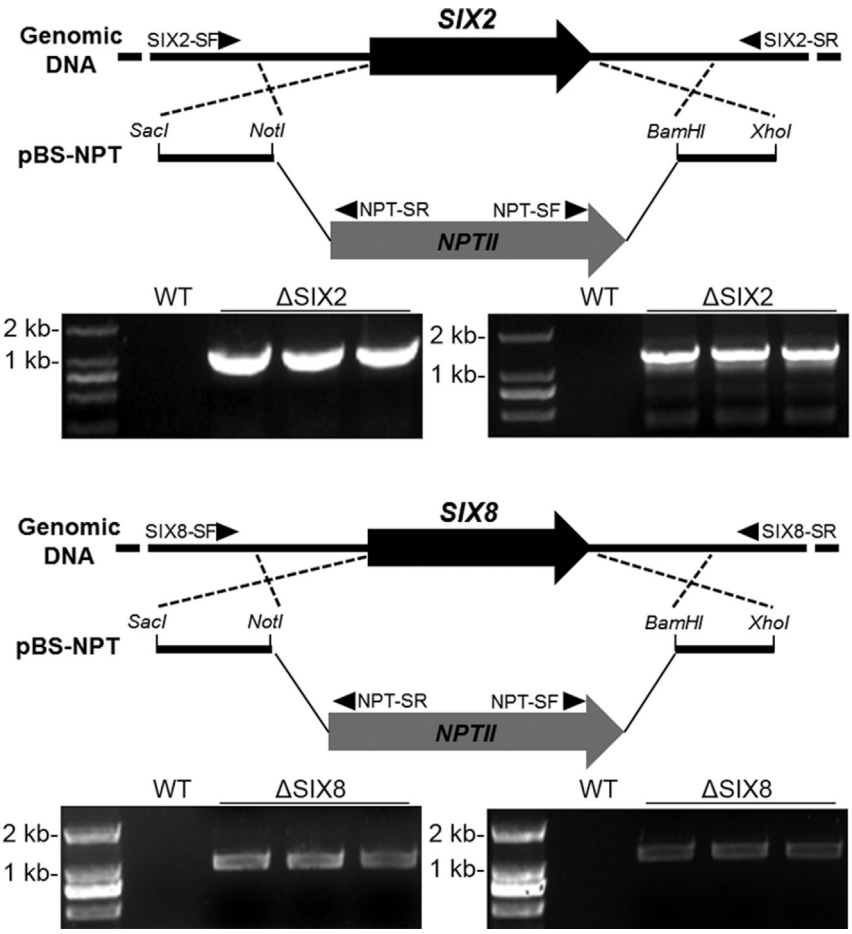

Fig. 2. The gene knock-out strategy and PCR diagnosis of the *SIX2* and *SIX8* knock-out mutants. The knock-out mutants has been created by replacing the *SIX* genes with Neomycin phosphotransferase gene (*NPTII*). Putative mutants have been screened with diagnostic primers indicated with black triangles. Electrophoresis show the PCR analysis of upstream and downstream diagnostic fragments of the target gene loci.

Total proteins were extracted with the extraction buffer containing 0.5 M Tris-HCl, pH 8.3, 2 % (v/v) NP-40, 20 mM $MgCl_2$, 2 % (v/v) β-mercaptoethanol, and 1 mM PMSF. After removing the cell debris by centrifugation, the supernatant was added with sodium deoxycholate (Sigma–Aldrich, St Louis, MO, USA) at the final concentration of 0.03 % (wt/vol), followed by incubation on ice for 30 min; subsequently, trichloric acetic acid (Sigma–Aldrich, St Louis, MO, USA) was added into the mixture to the final concentration of 10 % (wt/vol), followed by incubation on ice for another 30 min. After that, the proteins were collected by centrifugation at 16 000 ×*g* at 4 °C for 30 min and were washed three times with cold acetone. For extracellular protein extraction, mycelium was removed from the culture, then the supernatant was centrifuged and filtrated to remove impurities. After that, proteins were precipitated from the supernatant with sodium deoxycholate and trichloric acetic acid as mentioned above. A total of 10 μg of protein for each sample was separated by 12 % SDS-PAGE, transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, USA), hybridized with monoclonal ANTI-FLAG antibody produced in mouse and HRP-conjugated goat Anti-Mouse IgG successively (Sigma–Aldrich, St Louis, MO, USA). After detection with Amersham ECL Prime reagent (GE Healthcare) according to the manufacturer's instructions, the chemiluminescence of protein samples were observed under Image-Quant LAS 4000 Mini (GE Healthcare). Molecular weights of protein bands were calibrated with PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, MA, USA).

3. Results

3.1. Genome wide identification of SIX genes in Foc

Through BLAST search against the genomes of Foc TR4 and Foc1 using SIX protein sequences of Fol 4287, *F. oxysporum* f. sp. *pisi* and *F. oxysporum* f. sp. *cepae*, a total of 9 *SIX* genes were identified in Foc TR4, including *SIX1*a, *SIX1*b, *SIX1*c, *SIX2*, *SIX4*, *SIX6*, *SIX8*, *SIX 9* and *SIX13*; and 6 genes were found in Foc1, including *SIX1*, an interrupted *SIX1*, *SIX4*, *SIX6*, *SIX 9* and *SIX13* (Table 1). The predicted coding sequences and the protein sequences are listed in Table S1. Among these genes, *SIX2* and *SIX8* were only identified in Foc TR4. PCR analysis confirmed that genome of Foc1 does not contain *SIX2* and *SIX8* genes. Alignment of amino acid sequences revealed that Foc TR4 SIX2 shows 62 % identities with that of Fol, and Foc TR4 SIX8 shows 89 % identities with that of Fol. Besides, RT-PCR and nucleotide sequencing showed that the nucleotide of Foc TR4 *SIX8* transcribes to a 564 nt mRNA, which can be translated to a protein with molecular weight of 20.34 kDa.

3.2. Expression profiles of SIX in Foc TR4

To explore the roles of *SIX* genes in infection of Foc TR4 to Cavendish banana plantlets, the expression profiles of the 9 Foc TR4 *SIX* genes were analyzed. After inoculation to banana roots for 1, 3, 5 and 7 d, the relative expression levels of *SIX* genes were assessed using quantitative RT-PCR. As shown in Fig. 1, all of the Foc TR4 *SIX*

Table 1
Homologues of *Secreted In Xylem* (*SIX*) genes detected in isolates of *Fusarium oxysporum* f. sp. *cubense* race 1 (Foc1, N2) and tropical race 4 (Foc TR4, B2). A '—' has been used to denote the absence of a *SIX* gene in the corresponding isolate.

Strain	Race	GenBank assembly accession	SIX genes														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Foc TR4 (B2)	4	GCA_000350365.1	*SIX1*a *SIX1*b *SIX1*c	*SIX2*	—	*SIX4* (truncated)	—	*SIX6*	—	*SIX8*	*SIX9*	—	—	—	*SIX13*	—	—
Foc1 (N2)	1	GCA_000350345.1	*SIX1* *SIX1* (interrupted)	—	—	*SIX4* (truncated)	—	*SIX6*	—	—	*SIX9*	—	—	—	*SIX13*	—	—

genes were significantly up-regulated after inoculation into planta compared with the samples cultured *in vitro*. Furthermore, *SIX1*a, *SIX1*b, *SIX1*c, *SIX8* and *SIX9* exhibited a pattern of increased expression over the course of the infection post inoculation: all three *SIX1* homologies showed an increase in transcript level of 6–10 fold up after inoculation for 5 or 7 d; transcript level of *SIX8* increased about 8 fold after 7 d; and level of *SIX9* increased about 30 fold at 7 d compared with that at 1 d. The expression levels of other *SIX* genes did not vary significantly at different time post inoculation.

3.3. Generation of the knock-out mutants

To explore the functions of Foc TR4 *SIX2* and *SIX8*, these two genes were knocked out of the genome using a replacement strategy with *NPTII* conferring resistance to G418 as the selection marker. The linearized knock-out vectors were used to transform protoplast. The G418 resistant colonies were analyzed by two round PCR; primer pairs with one primer being located out of the recombinant fragment, and the other in the recombinant fragment were used. For diagnosis of the *SIX2* knock-out mutants, primer pairs SIX2-SF/NPT-SR and NPT-SF/SIX2-SR were used to confirm the correct integration of the recombinant fragment into the *SIX2* locus; for diagnosis of the *SIX8* knock-out mutants, primer pairs SIX8-SF/NPT-SR and NPT-SF/SIX8-SR were used. The results of the two round PCR analysis showed that both upstream and downstream diagnostic fragments of the target gene loci were detected in at least three mutants, indicating the successful integration of the transforming DNA to *SIX2* or *SIX8* loci. After purification by single conidia isolation,

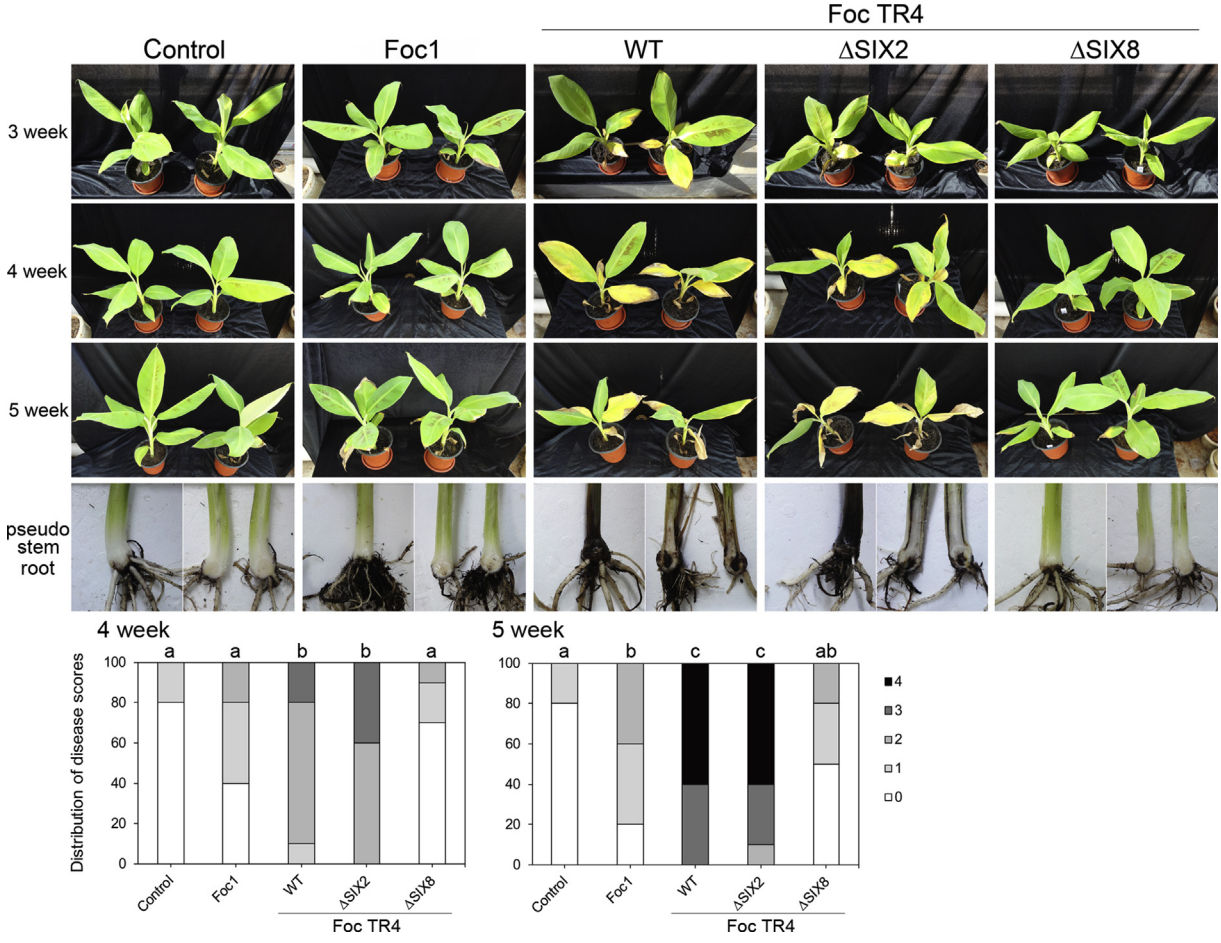

Fig. 3. Virulence analysis of *SIX2* and *SIX8* knock-out mutants. A total of 20 banana plantlets have been used for each treatment. (A) Leaf disease symptoms of banana plantlets after inoculation for 3, 4, and 5 week and internal symptoms of rhizome and pseudostem of banana plantlets after inoculation for 5 week. (B) Distribution of leaf disease scores after inoculation for 4 and 5 week. Treatments with different letters are significantly different at $P = 0.05$.

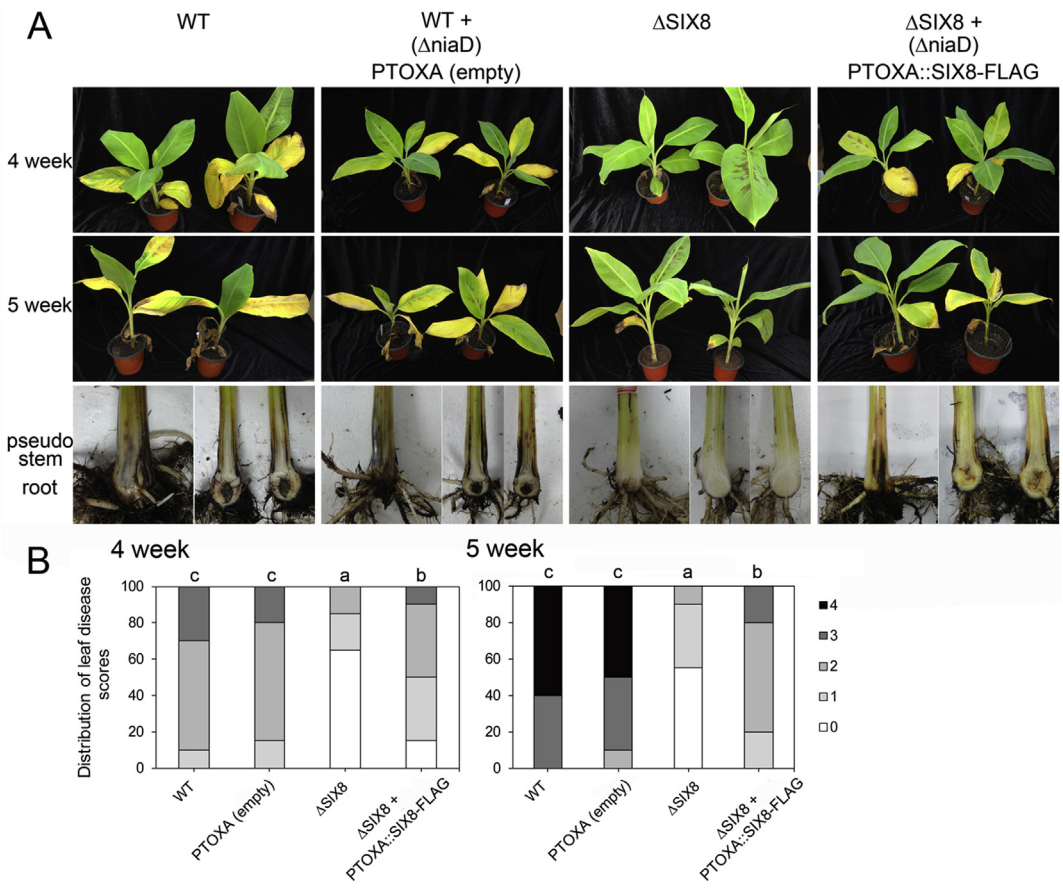

Fig. 4. Virulence analysis of ΔSIX8 mutant and SIX8-FLAG over expression mutant constructed based on *niaD* knock-out mutant. Foc TR4 was used as WT strain. A total of 20 banana plantlets have been used for each treatment. (A) Leaf disease symptoms of banana plantlets after inoculation for 4 and 5 week and internal symptoms of rhizome and pseudostem of banana plantlets after inoculation for 5 week. (B) Distribution of leaf disease scores after inoculation for 4 and 5 week. Treatments with different letters are significantly different at $P = 0.05$.

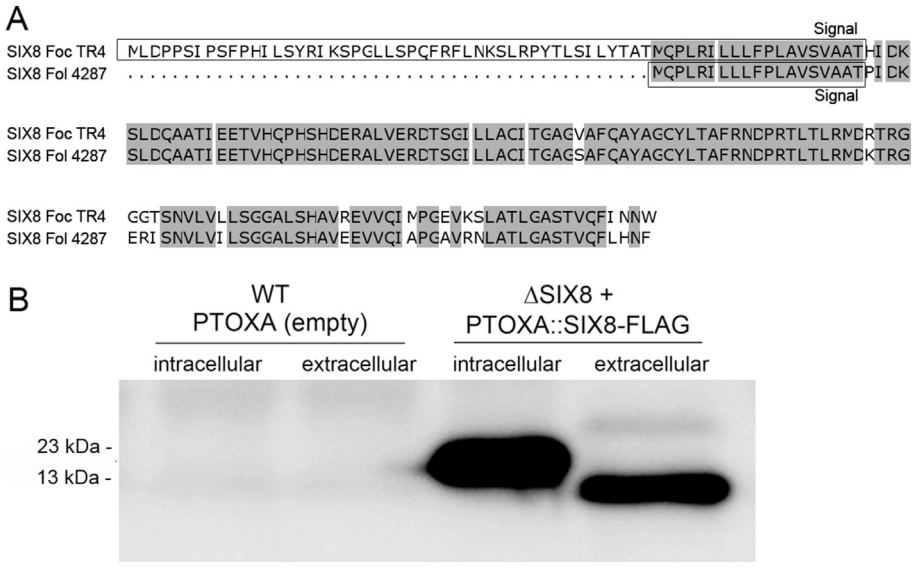

Fig. 5. (A) Alignment of amino acid sequences of Foc TR4 SIX8 and Fol SIX8. The predicted signal peptides are marked with a bounding box. (B) Western blot analysis of expression of SIX8-FLAG in intracellular and extracellular proteome of the SIX8-FLAG over expression mutant.

the transformants were further analyzed by PCR using the primer pairs of nucleotide sequences of *SIX2* and *SIX8* to exclude heterokaryon. Then the correct knock-out mutants were named as ΔSIX2 and ΔSIX8.

3.4. SIX8 *is required for virulence*

Virulence of the mutants were analyzed by inoculation to Cavendish banana plantlets at five-leaf stage. As shown in Fig. 3, the

plantlets inoculated with water (Control) or Foc1 showed no disease symptoms during the observation period of 5 week. For the plantlets inoculated with WT strain of Foc TR4, leaf disease symptoms began to appear after 3 week, with some leaves turning yellow and withered; and the leaf disease symptoms aggravated with the inoculation time. After inoculation for 5 week, all the WT strain treated plantlets showed serious symptoms with some leaves completely withered. Investigation of the root systems showed significant browning of rhizome and pseudostem. The plantlets inoculated with ΔSIX2 showed the similar disease progress as the WT strain treated ones. While the plantlets inoculated with ΔSIX8 showed few disease symptoms after treatment for 5 week, with healthy leaves, rhizome and pseudostem, suggesting significant impairment in virulence of ΔSIX8.

3.5. *SIX8 is an extracellular protein*

For generation of the SIX8-FLAG over-expression mutants, the pFoNDHTN-SIX8-FLAG (Fig. S1B) was transformed into protoplast of ΔSIX8, and *HPH* was used as the selection marker. After PCR analysis, the correct transformants were named as ΔSIX8 + PTOXA::SIX8-FLAG. Virulence analysis revealed that over expression of SIX8-FLAG partly restored virulence of ΔSIX8 mutant (Fig. 4). Prediction with the software DNAMAN suggested that SIX8 is a secreted protein, with a 66 amino acid signal peptide located at the N-terminal of SIX8 protein; and the mature SIX8 polypeptide contained 121 amino acid residues of 13 kDa (Fig. 5A). To verify whether SIX8 could be secreted to the extracellular space, SDS-PAGE and western blot analysis were conducted. As shown in Fig. 5B, SIX8-FLAG could be both detected in intracellular and extracellular proteome. Furthermore, the molecular weight of the intra- and extracellular SIX8-FLAG are consistent with the expectation.

4. Discussion

Effectors of plant pathogens are mainly expressed during the *in vivo* colonization and show different expression profiles during stages of infection (O'Connell et al., 2012). In addition, effector genes are often up-regulated in a coordinated way. Fol employs lineage-specific (LS) genomic regions, also named as “virulence” chromosomes, which are rich in genes related to virulence, such as *SIX* genes. These chromosomes could be exchanged between Fol strains and convert a non-pathogenic strain into a pathogen (Ma et al., 2010; van Dam et al., 2016). Genome analysis showed that Foc does not contain the LS regions like that of Fol (Guo et al., 2015), suggesting that there might be some different regulatory mechanism of effector genes in Foc. Here we found that when cultured *in vitro*, the transcription levels of the Foc TR4 *SIX* genes were extremely low; while after inoculation to the plants, all the *SIX* genes were significantly induced. Furthermore, the expression of *SIX1*a, *SIX1*b, *SIX1*c, *SIX8* and *SIX9* increased over the course of the infection, suggesting that these 5 genes play important roles in infection and colonization of Foc TR4 to banana plants (Fig. 1). It is interesting that *SIX2* and *SIX8* transcribed in correlation with the infection process of the pathogen, suggesting that they are involved in the virulence of Foc TR4 to banana.

It has been reported that variation in numbers and amino acid sequences of effectors could lead to difference in virulence of plant pathogens (Stergiopoulos et al., 2007; Lievens et al., 2009; Bhadauria et al., 2015). *SIX2* and *SIX8*, two effector genes, are only present in Foc TR4 and not detected in Foc1. However, little is known about the biological functions of *SIX2* and *SIX8* so far. Through construction of knock-out mutants, it was found that *SIX2* play little roles in virulence, while *SIX8* is required for virulence of Foc TR4 to Cavendish banana (Fig. 3). Furthermore, investigation of the rhizome and pseudostem of the plantlets revealed that ΔSIX8 lost the ability to colonize the banana roots as WT strain, suggesting that *SIX8* play important roles in regulating infection and early stage of biotrophic growth of Foc TR4. Genomic screening of international Foc isolates showed that two *SIX8* homologues, *SIX8a* and *SIX8b*, are present in the genomes of Foc4 strains and not detected in any race 1or 2 isolates. Besides, *SIX8a* is present in all race 4 isolates, whereas *SIX8b* is present in all subtropical race 4 isolates and not detected in tropical race 4 isolates (Fraser-Smith et al., 2014). Furthermore, two copies of *SIX8a*, with single nucleotide polymorphisms (SNPs), are detected in some isolates of Foc TR4 (Czislowski et al., 2018). The isolates of Foc TR4 used in the present study contains only one copy of *SIX8*. To confer the function of *SIX8* in virulence, the complemented mutants were constructed by over expressing SIX8-FLAG in the ΔSIX8 mutant. And over expression of *SIX8* at the *niaD* locus partly restored the virulence of ΔSIX8 (Fig. 4), suggesting that *SIX8* is dependent for virulence of Foc TR4 to Cavendish banana.

SIX proteins are expected to be secreted to xylem of plant host to ensure the virulence of *F. oxysporum* (Rep et al., 2002; Houterman et al., 2007). To verify the secretional characteristic of SIX8, western blot analysis of the intracellular and extracellular proteomes of the SIX8-FLAG mutants were analyzed. The results showed SIX8-FLAG could be secreted into the extracellular space of Foc TR4 (Fig. 5B); furthermore, the extracellular SIX8-FLAG was smaller than the intracellular SIX-FLAG, suggesting the excise of the signal peptide of SIX8 during the secretion process. Molecular weights of intracellular- and extracellular SIX8-FLAG were consistent with the expectation, which proved that N-terminal 66 amino acid polypeptide of SIX8 function as the signal peptide. Meanwhile, protein sequence alignment showed that the Foc TR4 SIX8 has a long N-terminal extension relative to Fol SIX8 (Fig. 5A). And the RT-PCR and nucleotide sequencing results showed that Foc TR4 *SIX8* transcribes to a 564 nt mRNA, which could be translated to a protein with a longer N-terminal than Fol SIX8.

5. Conclusions

In this study, genome screening of Foc TR4 and Foc1 showed that two effectors, *SIX2* and *SIX8*, are only existed in Foc TR4 isolate B2. Expression profiles analysis indicated that transcriptions of the *SIX* genes were correlated to the infection processes of Foc TR4 to Cavendish banana. Knocking out of *SIX8* lead to significant impairment on virulence of the pathogen. In addition, western blot analysis revealed that SIX8 could be secreted to extracellular matrix of Foc TR4. Further identification of the targets of SIX8 in host plant cells should be conducted to explain the mechanism of *SIX8* in regulating virulence of Foc TR4.

Conflicts of interest

The authors declared that they have no conflicts of interest to this work.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 31760037), the National Natural Science Foundation of China (No. 31571967), the National Natural Science Foundation of China (No. 31560044).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.03.001.

References

Bhadauria, V., MacLachlan, R., Pozniak, C., Banniza, S., 2015. Candidate effectors contribute to race differentiation and virulence of the lentil anthracnose pathogen *Colletotrichum lentis*. BMC Genomics 16, 628.

Czislowski, E., Fraser-Smith, S., Zander, M., O'Neill, W.T., Meldrum, R.A., Tran-Nguyen, L.T.T., Batley, J., Aitken, E.A.B., 2018. Investigation of the diversity of effector genes in the banana pathogen, *Fusarium oxysporum* f. sp. *cubense*, reveals evidence of horizontal gene transfer. Mol. Plant Pathol. 19, 1155–1171.

Fraser-Smith, S., Czislowski, E., Meldrum, R.A., Zander, M., O'Neill, W., Balali, G.R., Aitken, E.A.B., 2014. Sequence variation in the putative effector gene SIX8 facilitates molecular differentiation of *Fusarium oxysporum* f. sp. *cubense*. Plant Pathol. 63, 1044–1052.

Gawehns, F., Houterman, P.M., Ichou, F.A., Michielse, C.B., Hijdra, M., Cornelissen, B.J.C., Rep, M., Takken, F.L., 2014. The *Fusarium oxysporum* effector Six 6 contributes to virulence and suppresses *I*-2-mediated cell death. Mol. Plant Microbe Interact. 27, 336–348.

Grimm, D., 2008. Plant genomics. A bunch of trouble. Science 322, 1046–1047.

Guo, L., Han, L., Yang, L., Zeng, H., Fan, D., Zhu, Y., Feng, Y., Wang, G., Peng, C., Jiang, X., Zhou, D., Ni, P., Liang, C., Liu, L., Wang, J., Mao, C., Fang, X., Peng, M., Huang, J., 2015. Genome and transcriptome analysis of the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* causing banana vascular wilt disease. PLoS One 10, e0117621.

Hou, X., An, B., Wang, Q., Guo, Y., Luo, H., He, C., 2018. SGE1 is involved in conidiation and pathogenicity of *Fusarium oxysporum* f. sp. *cubense*. Can. J. Microbiol. 64, 349–357.

Houterman, P.M., Cornelissen, B.J.C., Rep, M., 2008. Suppression of plant resistance gene-based immunity by a fungal effector. PLoS Pathog. 4, e1000061.

Houterman, P.M., Ma, L., van Ooijen, G., de Vroomen, M.J., Cornelissen, B.J.C., Takken, F.L., Rep, M., 2009. The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein *I*-2 intracellularly. Plant J. 58, 970–978.

Houterman, P.M., Speijer, D., Dekker, H.L., DE Koster, C.G., Cornelissen, B.J.C., Rep, M., 2007. The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. Mol. Plant Pathol. 8, 215–221.

Laurence, M.H., Burgess, L.W., Summerell, B.A., Liew, E.C., 2012. High levels of diversity in *Fusarium oxysporum* from non-cultivated ecosystems in Australia. Fungal Biol. 116, 289–297.

Lievens, B., Houterman, P.M., Rep, M., 2009. Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discrimination from other formae speciales. FEMS Microbiol. Lett. 300, 201–215.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.

Lorang, J.M., Tuori, R.P., Martinez, J.P., Sawyer, T.L., Redman, R.S., Rollins, J.A., Wolpert, T.J., Johnson, K.B., Rodriguez, R.J., Dickman, M.B., Ciuffetti, L.M., 2001. Green fluorescent protein is lighting up fungal biology. Appl. Environ. Microbiol. 67, 1987–1994.

Ma, L., Houterman, P.M., Gawehns, F., Cao, L., Sillo, F., Richter, H., Clavijo-Ortiz, M.J., Schmidt, S.M., Boeren, S., Vervoort, J., Cornelissen, B.J., Rep, M., Takken, F.L., 2015. The AVR2-SIX5 gene pair is required to activate *I*-2-mediated immunity in tomato. New Phytol. 208, 507–518.

Ma, L., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.J., di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P.M., Kang, S., Shim, W.B., Woloshuk, C., Xie, X., Xu, J.R., Antoniw, J., Baker, S.E., Bluhm, B.H., Breakspear, A., Brown, D.W., Butchko, R.A., Chapman, S., Coulson, R., Coutinho, P.M., Danchin, E.G., Diener, A., Gale, L.R., Gardiner, D.M., Goff, S., Hammond-Kosack, K.E., Hilburn, K., Hua-Van, A., Jonkers, W., Kazan, K., Kodira, C.D., Koehrsen, M., Kumar, L., Lee, Y.H., Li, L., Manners, J.M., Miranda-Saavedra, D., Mukherjee, M., Park, G., Park, J., Park, S.Y., Proctor, R.H., Regev, A., Ruiz-Roldan, M.C., Sain, D., Sakthikumar, S., Sykes, S., Schwartz, D.C., Turgeon, B.G., Wapinski, I., Yoder, O., Young, S., Zeng, Q., Zhou, S., Galagan, J., Cuomo, C.A., Kistler, H.C., Rep, M., 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464, 367–373.

Michielse, C.B., Rep, M., 2009. Pathogen profile update: *Fusarium oxysporum*. Mol. Plant Pathol. 10, 311–324.

O'Connell, R.J., Thon, M.R., Hacquard, S., Amyotte, S.G., Kleemann, J., Torres, M.F., Damm, U., Buiate, E.A., Epstein, L., Alkan, N., Altmüller, J., Alvarado-Balderrama, L., Bauser, C.A., Becker, C., Birren, B.W., Chen, Z., Choi, J., Crouch, J.A., Duvick, J.P., Farman, M.A., Gan, P., Heiman, D., Henrissat, B., Howard, R.J., Kabbage, M., Koch, C., Kracher, B., Kubo, Y., Law, A.D., Lebrun, M.H., Lee, Y.H., Miyara, I., Moore, N., Neumann, U., Nordström, K., Panaccione, D.G., Panstruga, R., Place, M., Proctor, R.H., Prusky, D., Rech, G., Reinhardt, R., Rollins, J.A., Rounsley, S., Schardl, C.L., Schwartz, D.C., Shenoy, N., Shirasu, K., Sikhakolli, U.R., Stüber, K., Sukno, S.A., Sweigard, J.A., Takano, Y., Takahara, H., Trail, F., van der Does, H.C., Voll, L.M., Will, I., Young, S., Zeng, Q., Zhang, J., Zhou, S., Dickman, M.B., Schulze-Lefert, P., Ver Loren van Themaat, E., Ma, L., Vaillancourt, L.J., 2012. Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. Nat. Genet. 44, 1060–1065.

Ordonez, N., Seidl, M.F., Waalwijk, C., Drenth, A., Kilian, A., Thomma, B.P.H.J., Ploetz, R.C., Kema, G.H.J., 2015. Worse comes to worst: bananas and Panama disease-when plant and pathogen clones meet. PLoS Pathog. 11, e1005197.

Ploetz, R.C., 2005. Panama disease: an old nemesis rears its ugly head. Part 1: the beginnings of the banana export trades. Plant Health Prog. 1–10.

Rep, M., Dekker, H.L., Vossen, J.H., de Boer, A.D., Houterman, P.M., Speijer, D., Back, J.W., de Koster, C.G., Cornelissen, B.J., 2002. Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato. Plant Physiol. 130, 904–917.

Rep, M., Kistler, H.C., 2010. The genomic organization of plant pathogenicity in *Fusarium* species. Curr. Opin. Plant Biol. 13, 420–426.

Rep, M., Meijer, M., Houterman, P.M., van der Does, H.C., Cornelissen, B.J.C., 2005. *Fusarium oxysporum* evades *I*-3-mediated resistance without altering the matching avirulence gene. Mol. Plant Microbe Interact. 18, 15–23.

Rep, M., van der Does, H.C., Meije, M., van Wijk, R., Houterman, P.M., Dekker, H., de Koster, C.G., Cornelissen, B.J., 2004. A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for *I*-3-mediated resistance in tomato. Mol. Microbiol. 53, 1373–1383.

Schmidt, S.M., Houterman, P.M., Schreiver, I., Ma, L., Amyotte, S., Chellappan, B., Boeren, S., Takken, F.L., Rep, M., 2013. MITEs in the promoters of effector genes allow prediction of novel virulence genes in *Fusarium oxysporum*. BMC Genomics 14, 119.

Schumacher, J., 2012. Tools for *Botrytis cinerea*: new expression vectors make the gray mold fungus more accessible to cell biology approaches. Fungal Genet. Biol. 49, 483–497.

Simbaqueba, J., Catanzariti, A.M., González, C., Jones, D.A., 2018. Evidence for horizontal gene transfer and separation of effector recognition from effector function revealed by analysis of effector genes shared between cape gooseberry- and tomato-infecting formae speciales of *Fusarium oxysporum*. Mol. Plant Pathol. 19, 2302–2318.

Stergiopoulos, I., de Kock, M.J., Lindhout, P., de Wit, P.J., 2007. Allelic variation in the effector genes of the tomato pathogen *Cladosporium fulvum* reveals different modes of adaptive evolution. Mol. Plant Microbe Interact. 20, 1271–1283.

Stover, R.H., 1990. *Fusarium* wilt of banana: some history and current status of the disease. In: Ploetz, R.C. (Ed.), Fusarium Wilt of Banana. APS Press, St. Paul, MN, pp. 1–7.

Thatcher, L.F., Gardiner, D.M., Kazan, K., Manners, J.M., 2012. A highly conserved effector in *Fusarium oxysporum* is required for full virulence on *Arabidopsis*. Mol. Plant Microbe Interact. 25, 180–190.

van Dam, P., Fokkens, L., Schmidt, S.M., Linmans, J.H., Kistler, H.C., Ma, L., Rep, M., 2016. Effector profiles distinguish formae speciales of *Fusarium oxysporum*. Environ. Microbiol. 18, 4087–4102.

van der Does, H.C., Rep, M., 2007. Virulence genes and the evolution of host specificity in plant-pathogenic fungi. Mol. Plant Microbe Interact. 20, 1175–1182.

Widinugraheni, S., Niño-Sánchez, J., Does van der, H.C., Dam van, P., García-Bastidas, F.A., Subandiyah, S., Meijer, H.J.G., Kistler, H.C., Kema, G.H.J., Rep, M., 2018. A SIX1 homolog in *Fusarium oxysporum* f.sp. *Cubense* tropical race 4 contributes to virulence towards Cavendish banana. PLoS One 13, e0205896.

Williams, A.H., Sharma, M., Thatcher, L.F., Azam, S., Hane, J.K., Sperschneider, J., Kidd, B.N., Anderson, J.P., Ghosh, R., Garg, G., Lichtenzveig, J., Kistler, H.C., Shea, T., Young, S., Buck, S.A., Kamphuis, L.G., Saxena, R., Pande, S., Ma, L., Varshney, R.K., Singh, K.B., 2016. Comparative genomics and prediction of conditionally dispensable sequences in legume-infecting *Fusarium oxysporum* formae speciales facilitates identification of candidate effectors. BMC Genomics 17, 191.