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Full Length Article

SIX6 Shows High Divergence in *Fusarium oxysporum* f. sp. *cubense* TR4

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Abstract

Secreted fungal effector proteins and their host targets are good examples to understand the mechanism of host-pathogen co-evolution with genes involved in the interaction undergoing positive selection. *SIX* genes (secreted in xylem) are obtained via horizontal transfer and can be found within the *formae speciales* of *Fusarium oxysporum*. *SIX6* and *SIX9* of *F. oxysporum* f. spp. *cubense* (*Foc*) are predicted to play a role as effectors. However, their involvement in the pathogenicity of *Foc* in banana plants has not been determined yet. In the susceptible banana cultivar, we found that the *SIX6* and *SIX9* genes of *Foc* TR4 were highly expressed in roots, but not in corms or leaves. The host, however, expressed the pathogenesis-related (PR) genes, *PR-1* and *PR-3*, in corms earlier than in the roots. Phylogenetic analysis on *SIX6* and *SIX9* genes of *F. oxysporum* has revealed the separation of *SIX6* and *SIX9* of *Foc* from other *formae speciales*. This leads to detecting genes under positive selection using the ratio nonsynonymous to synonymous substitution rates (Ka/Ks). *SIX6* of *Foc* showed an increase in diversity, but insufficient to drive positive selection. Conversely, *SIX9* of *Foc* showed no divergence in the dN/dS ratio distribution, indicating purifying selection. © 2021 Friends Science Publishers

Keywords: Effector evolution; Ka/Ks ratio; Positive selection; Purifying selection; *SIX* effectors

Introduction

The never-ending battle between pathogens and hosts leads to a co-evolutionary arms race where both evolve to counteract each other (Derbyshire 2020). Hosts develop strategies to recognize pathogens and escape infections whereas pathogens develop ways to avoid host recognition and escape host defenses. The dynamics between secreted fungal effector proteins and their host targets are good examples in understanding the mechanism of host-pathogen co-evolution with genes involved in the interaction undergo positive selection (Presti *et al.* 2015). A successful pathogen must be able to maintain the ability to avoid host recognition but still virulent in the process. This will determine infectivity and host specialization. In order to do this, pathogens will have to pass a series of gene modifications, changes in the expression of existing effector genes, or even generate new effectors (Presti *et al*. 2015).

Generally, effectors are modular proteins. They contain signal peptides that are relatively small in size, rich in Cysteine residues, and do not have similarities with known proteins (Stergiopoulos and Wit 2009; Sonah *et al.* 2016; Dalio *et al*. 2018). In host cells, effectors may suppress host defense systems or deceive host cells to accommodate further infection and colonization (Dodds *et al*. 2009). Fungal pathogens have developed the ability to deliver effectors inside the host cytoplasm as well as in the extracellular space, thus classified as cytoplasmic and apoplastic effectors, respectively (Wang *et al*. 2017).

Banana is the fourth most important export commodity worldwide after rice, wheat, and corn (FAO 2020). However, the sustainability of banana production worldwide is threatened by pests and diseases such as Fusarium wilt caused by *Fusarium oxysporum* f. spp. *cubense* (Dita *et al.* 2018). To counteract this pathogen, molecular studies conducted to identify resistance genes expressed by the host cells and genes involved in virulence or pathogenicity are urgently needed. Until recently, genomic, transcriptomic proteomics analyses have been conducted in *Foc* TR4 (Guo *et al*. 2014; Sun *et al.* 2014) and also on banana cultivars that are susceptible and/or resistant to *Foc* TR4 (Li *et al*. 2012; Bai *et al*. 2013; Sun *et al*. 2019; Zhang *et al*. 2019). These studies are crucial in order to develop effective methods to manage the pathogen while being wary in the emergence of resistance in banana plants. Although host adaptation and specificity within *formae speciales* of diverse pathogenic fungus, including *F. oxysporum*, have been studied extensively (Li *et al*. 2020), the evolutionary origin of the host specificity gene is still undetermined. Ma *et al*. (2010) revealed four lineage-specific

chromosomes in *F. oxysporum,* one of which is the 2-Mb chromosome 14 of *F. oxysporum* f. spp. *lycopersici* (*Fol).* Chromosome 14 consists of genes encoding secreted effectors such as the *SIX* genes, of which many are involved in pathogenicity. It is suggested that the pathogenicity of nonpathogenic *F. oxysporum* strain towards tomato is acquired by the acquisition of *Fol* chromosome 14 by horizontal chromosomal transfer (Mehrabi *et al.* 2011).

The *SIX* effectors initially found in *Fol* that infects tomato were *SIX1* (Rep *et al*. 2004), *SIX2*, *SIX3* and *SIX4* (Houterman *et al*. 2007), *SIX5*, *SIX6* and *SIX7* (Ma *et al*. 2010). In tomato, *SIX1* (also known as *Avr3*) is required for *Fol* virulence (Rep *et al*. 2005) and I-3-mediated resistance (I for immunity) (Rep *et al*. 2004). *SIX1* was found consistently in *Foc* strains, with 3 homologs found in TR4 (*SIX1a, b* and *c*) (Widinugraheni *et al*. 2018). *SIX1* is also known to be involved in *Foc* virulence in Cavendish (Widinugraheni *et al*. 2018). *SIX4* (also known as *Avr1*) plays a role in I-1-mediated resistance but suppresses the I-2 and I-3-mediated resistance (Houterman *et al*. 2008). Similar to *SIX1*, *SIX3* (also known as *Avr2*) is required for *Fol* virulence in susceptible hosts and triggered resistance in tomato plants containing the *I-2* resistance gene (Houterman *et al*. 2009). Furthermore, *SIX8* was reported to be involved in the virulence of *Foc* TR4 into Cavendish (An *et al*. 2019). Up to now, a total of 14 effectors have been identified in bananas (Czislowski *et al*. 2018) and *SIX* gene homologous have been found in *F. oxysporum* infecting other plants, such as tomato, date palm, melon, passionfruit, pea, watermelon, common bean, and cucumber (Thatcher *et al*. 2012; Laurence *et al*. 2015). *SIX6* and *SIX9* of *Foc* have been examined in numerous studies (Czislowski *et al*. 2018; An *et al*. 2019). However, their role in pathogenicity in banana plants has not been determined. In this study, we aimed to provide new evidence to support the hypothesis that *SIX6* and *SIX9* of *Foc* could play a role as effectors.

Materials and Methods

Plant materials and pathogen inoculation

Cavendish "Grand Nain" plantlets were propagated in Murashige and Skoog (MS) media containing 2.5 ppm of benzyl amino purine (BAP). Plantlets with 3–5 leaves were selected for inoculation with *Foc* TR4 isolated from infected banana cv. Bading kayu susu Banana cultivars were grown at room temperature with a 16 h day (approximately 200 μ mol m^{-2} s^{-1} light intensity)/8 h night cycle. *Foc* isolate was grown in Potato Dextrose Agar medium for 7 days at room temperature and prepared as 10^6 spore mL^{-1} suspensions in 0.85% NaCl. Plantlets were acclimatized 2 days prior to infection in MS and inoculated with 1 mL of *Foc* suspension. Samples of roots, corms, and leaves of infected bananas were collected 3, 6, 9 and 14 days post-infection. Each time point is consisted of at least a collection of 2–3 plantlets.

RNA extraction and quantitative real-time PCR

Total RNAs were isolated from the roots, corms, and leaves of the infected banana cv. Cavendish 3, 6, 9 and 14 days post-infection as described by Cordeiro *et al*. (2008). First-strand cDNA synthesis was performed with 1 gram of total RNA employing the iScript cDNA synthesis kit according to the manufacturer's instruction (Biorad, California, USA). The expression of *SIX6, SIX9, PR-1* and *PR-3* genes and *GAPDH* reference gene (Li *et al*. 2015) were quantified using the GoTaq® qPCR master mix (Promega, Wisconsin, USA) in QuantStudio 1 Real-Time PCR System (Applied Biosystem, California, USA) and presented as relative expression (*SIX6* and *SIX9*) and normalized expression (*PR-1* and *PR-3*) (Livak and Schmittgen 2001). Three replicates of each sample were analyzed to ensure reproducibility and reliability.

Bioinfomatics tools for *in silico* study

The signal peptide cleavage site of *SIX6* and *SIX9* homologs was determined using the SignalP (http://www.cbs.dtu.dk/services/SignalP-4.1/). The phylogenetic tree of *SIX6* and *SIX9* in *formae speciales* of *F. oxysporum* was generated using the IQ-TREE (iqtree.cibiv.univie.ac.at). Putative 3D structures of *SIX6* and *SIX9* were generated using trRosetta (https://yanglab.nankai.edu.cn/trRosetta/). SNPs (single-nucleotide polymorphisms) were plotted into the putative 3D structures of *SIX6* and *SIX9* using PyMOL. The Ka/Ks ratio was calculated to identify the site-specific positive selection and purifying selection of *SIX6* and *SIX9* using Selecton (http://selecton.tau.ac.il/index.html). Pairwise Ka (dN; rate of nonsynonymous mutation) and Ks (dS; rate of synonymous mutation) of *SIX6* and *SIX9* genes was analyzed by running pairwise comparisons between CoDing Sequence (CDS) of *SIX6* and *SIX9* from different *formae speciales* using SNAP (Korber 2000) (https://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html).

Results

SIX6 and *SIX9* highly expressed in roots of *Foc*-infected bananas

SIX6 and *SIX9* genes were highly expressed in roots of infected bananas, but not in corms or the leaves (Fig. 1A and B). In Cavendish roots, the expression of the *SIX6* gene was elevated as high as 1.03 at 6 days post-infection (dpi), whereas *SIX9* was 1.07 at 9 dpi. The expression of pathogenesis-related *(PR)* genes *PR-1* and *PR-3* in susceptible cultivar Cavendish was examined during infection. *PR-1* was expressed early in the corms, 3 and 6 dpi, with 8.67 and 8.75-fold expression, respectively (Fig. 1C). In roots, the highest expression was reached at 9 dpi

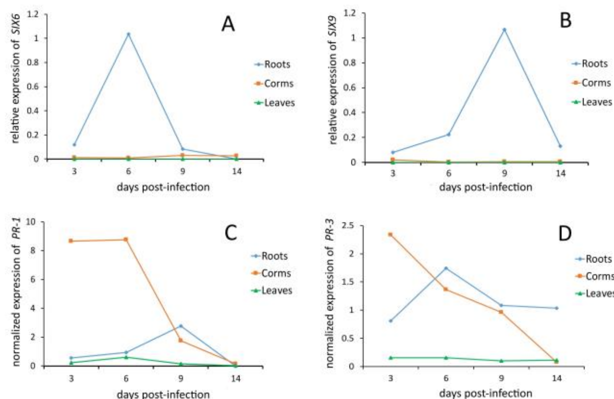

Fig. 1: Gene expression of *SIX6, SIX9, PR-1* and *PR-3* in Cavendish banana after infection with *Foc* TR4. (**A**) Relative expression of *SIX6* and (**B**) *SIX9* gene in roots, corms and leaves of Cavendish 3, 6, 9, and 14 days after infection (dpi) with *Foc* TR4. (**C**) Normalized expression of *PR-1* and (**D**) *PR-3* gene in roots, corms and leaves of Cavendish 3, 6, 9, and 14 dpi with *Foc* TR4. The *GAPDH* was used as a reference gene

with 1.74-fold expression. Similar to *PR-1*, *PR-3* was expressed in the corms 3 dpi with 2.34-fold expression whereas in roots the highest expression was reached at 6 dpi with 1.74-fold expression (Fig. 1D). The expression of both *SIX* and *PR* genes was considerably low in the leaves.

SIX6 and *SIX9* are predicted to be effectors

Both proteins contained a signal peptide that cleaved between amino acids in positions 16 and 17 for *SIX6* and positions 19 and 20 for *SIX9* (http://www.cbs.dtu.dk/services/SignalP-4.1/). Homologs of *SIX6* and *SIX9* were also cleaved at the same site (Fig. 2 and 3, respectively). Eight and six Cysteine (C) residues were identified to be conserved among all *formae speciales* of *F. oxysporum* in *SIX6* and *SIX9*, respectively (Fig. 2 and 3).

SIX6 and *SIX9* of *Foc* are polymorphic compared to other *formae speciales*

Foc SIX6 and *SIX9* shared 51.61 and 44.07% homology to other *formae speciales*, respectively (Fig. 2 and 3). Phylogenetic tree of *SIX6* and *SIX9* genes (Fig. 4 and 5) showed the separation of *Foc SIX6* and *SIX9* from *Fol* and other *formae speciales.* The *Foc SIX6* is in a different clade from all other *formae speciales*, whereas the *Foc SIX9* is in the same group with the *SIX9* of *F. oxysporum* f. spp. *pisi* (accession number MT710731.1), but in the different clade with *Fol* and the other *formae speciales*. This indicates high polymorphisms in the sequences of *Foc SIX6* and *SIX9* genes. The resulted amino acid sequences showed high variations in the signal peptides of *Foc SIX6* and *SIX9*, with 43.75 and 63.16% respectively, polymorphic to other *formae speciales* (Fig. 2 and 3).

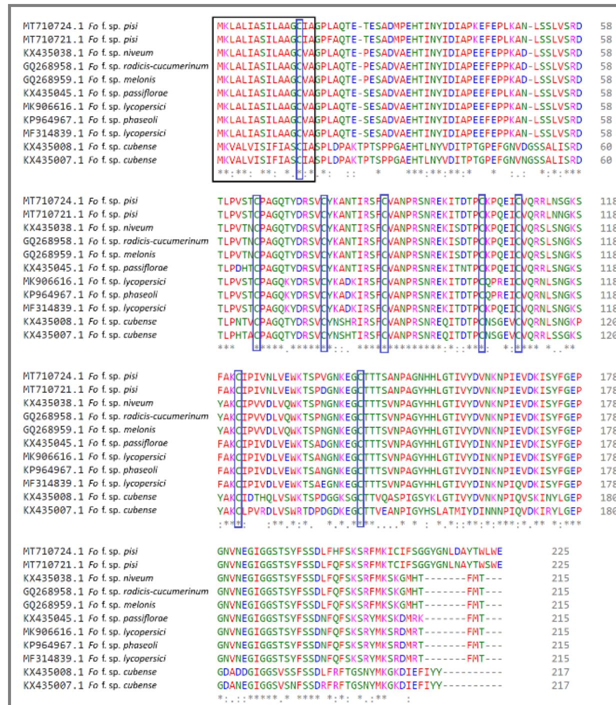

Fig. 2: Alignment of SIX6 in eight formae speciales of *F. oxysporum*, namely f. spp. *pisi,* f. spp. *niveum,* f. spp. *radicis-cucumerinum,* f. spp. *melonis,* f. spp. *passiflorae,* f. spp. *lycopersici,* f. spp. *phaseoli*, and f. spp. *cubense*. Signal peptide is boxed in black, Cysteine (C) residues in blue box. Sequences were aligned using EMBL Multiple Alignment

Fig. 3: Alignment of SIX9 in five formae speciales of *F. oxysporum*, namely f. spp. *pisi,* f. spp. *cubense*, f. spp. *lycopersici*, f. spp. *passiflorae* and f. spp. *niveum.* Signal peptide is boxed in black, Cysteine (C) residues in blue box. Sequences were aligned using EMBL Multiple Alignment

When we compared the sequences of *Foc SIX6* and *SIX9* obtained from the genebank (KX435008.1, and KX435007.1 for *SIX6*, and KX435015.1, KX435016.1 and KX435017.1 for *SIX9*) with other *SIX6* and *SIX9* sequences from different *formae speciales*, we found 14.75 and 13.56% polymorphisms in the Foc *SIX6* and *SIX9* amino acid sequences, respectively. The plotted polymorphisms in the putative 3D structure of Foc *SIX6* and *SIX9* can be seen in Fig. 6A and B. The polymorphic residues in *SIX6* are concentrated in the half downstream of the N-terminus, but the signal peptide residues are conserved. The *SIX9*,

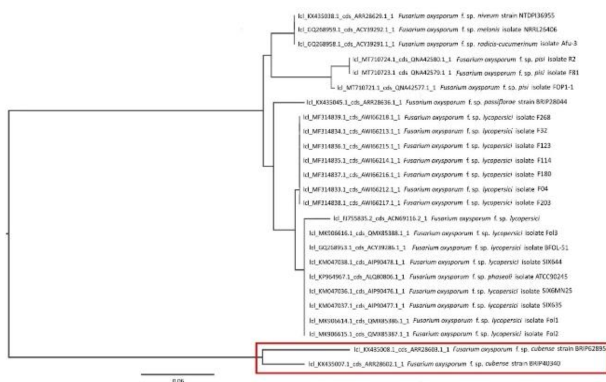

Fig. 4: Phylogenetic tree of SIX6 in formae speciales of *F. oxysporum*. The tree was generated using IQ-TREE (iqtree.cibiv.univie.ac.at)

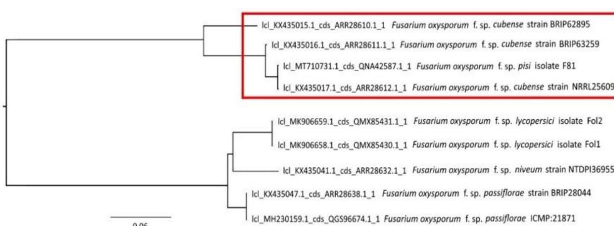

Fig. 5: Phylogenetic tree of SIX9 in formae speciales of *F. oxysporum*. The tree was generated using IQ-TREE (iqtree.cibiv.univie.ac.at)

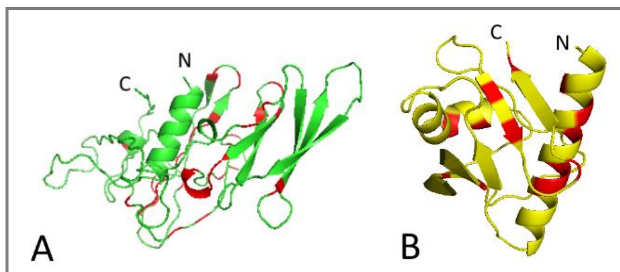

Fig. 6: Putative 3D structure of SIX6 (**A**) and SIX9 (**B**) of *Foc*. SIX6 and SIX9 of *Foc* are small proteins with the size of 217 and 118 amino acids, respectively. The structures were generated using trRosetta software (https://yanglab.nankai.edu.cn/trRosetta/). Polymorphisms of SIX6 and SIX9 in *Foc* were plotted in red colour using PyMOL. The C- and N-terminus were indicated

however, has 6 polymorphic residues in the signal peptide whilst the rest are scattered.

SIX6 of *Foc* is highly diverse compared to other *formae speciales* based on the rate of synonymous mutation

Polymorphisms in *SIX6* and *SIX9* of all *formae speciales* were observed at the amino acid level using Selecton analysis (Fig. 7). We found that *SIX6* is more diverse compared to *SIX9* with 60 residues with a sign of positive

Fig. 7: Site-specific positive selection and purifying selection of *SIX6* (**A**) and *SIX9* (**B**) proteins. Positive selection (orange, level 1) indicates high level for polymorphisms whereas purifying selection (purple, level 7) indicates low level for polymorphisms

selection (yellow to orange scale), comprising 26.67% of the length of the protein (Fig. 7A). Conversely, *SIX9* did not show any sign of positive selection (Fig. 7B). We further studied the distribution of Ka and Ks values by conducting a pairwise comparison between different *SIX6* and *SIX9* sequences using SNAP. The distribution of Ka and Ks values between *SIX6* sequences from different *formae speciales* indicates that *SIX6* underwent a purifying selection where the rate of Ks is higher than the rate of Ka between all the sequences of *SIX6* in the alignment (Fig. 8A). Interestingly, the majority of the high Ka and Ks values observed in the distribution were contributed by the two *Foc SIX6* sequences (KX435008.1 and KX435007.1). The exclusion of the two *Foc SIX6* sequences resulted in the change in the distribution of the Ka and Ks values (Fig. 8B). This would indicate that in the case of the *SIX6* gene, there is a high degree of diversity between *Foc* and other *formae speciales*.

We also performed a similar pairwise analysis of Ka and Ks with *SIX9* sequences and we observed a similar distribution of Ka and Ks, which also suggests that purifying selection was acted upon the *SIX9* gene. However, exclusion of the three *Foc SIX9* sequences (KX435015.1, KX435016.1 and KX435017.1) did not produce a significant difference in the distribution of *Ka* and *Ks*, which suggests that the diversity of the *SIX9* gene between different *formae speciales* is relatively low (Fig. 9).

Discussion

Two effector candidates from *F. oxysporum* were investigated in this study, *SIX6* and *SIX9*. Both genes showed expressions that are specific only in the roots and

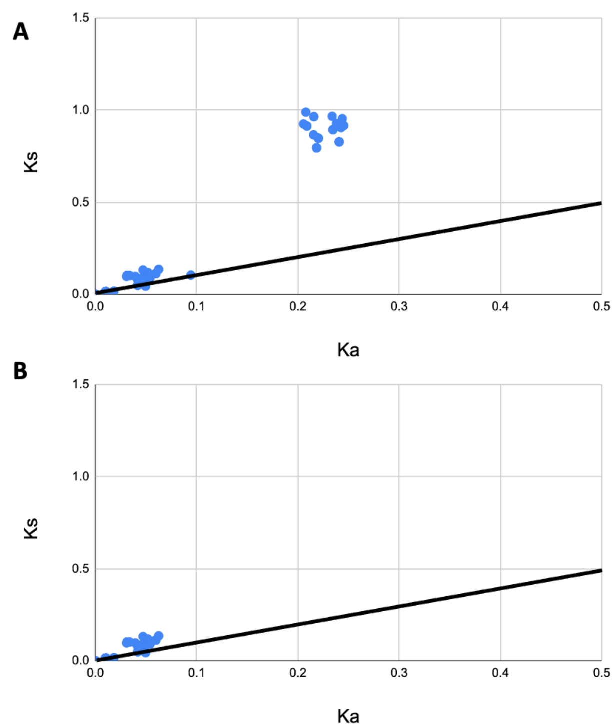

Fig. 8: Distribution of synonymous (Ks/dS) and nonsynonymous (Ka/dN) substitution rate across different SIX6 sequences. Displayed are the distribution of Ka and Ks with two sequences of *Foc* SIX6, KX435008.1 and KX435007.1, included (**A**) or excluded (**B**) from pairwise analysis of Ka and Ks. Black line represents Ks = Ka

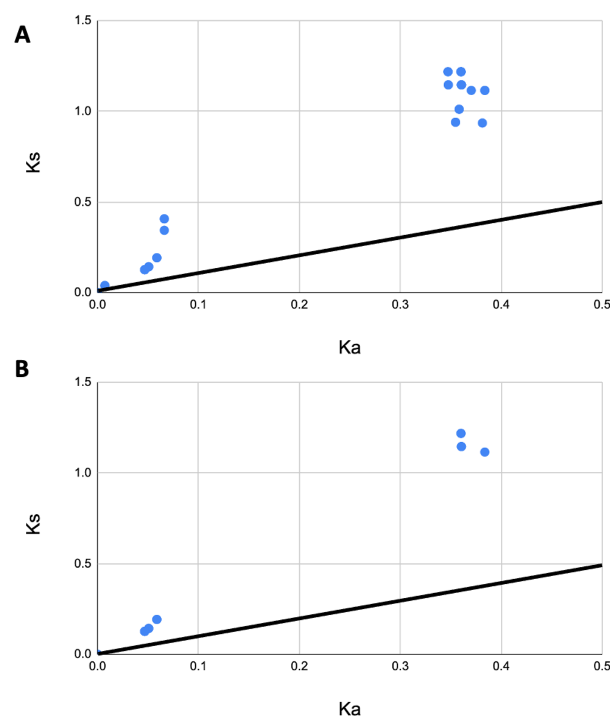

Fig. 9: Distribution of synonymous (Ks/dS) and nonsynonymous (Ka/dN) substitution rate across different SIX9 sequences. Displayed are the distribution of Ka and Ks with three sequences of *Foc* SIX9, KX435015.1, KX435016.1 and KX435017.1, included (**A**) or excluded (**B**) from pairwise analysis of Ka and Ks. Black line represents Ks = Ka

happened at the earliest stage of infection peaking at 6 dpi and 9 dpi for *SIX6* and *SIX9*, respectively. The difference in the expression peak between *SIX6* and *SIX9* would indicate the different stages in which each gene plays its part during the infection of the host. Both *SIX6* and *SIX9* exhibited a degree of conservation across different *formae speciales* of *F. oxysporum* infecting a wide range of hosts. We also observed several features that support the hypothesis that *SIX6* and *SIX9* are effectors by the existence of signal peptide at the N terminal of the protein sequence of both proteins, along with the high number of conserved cysteine residues.

Based on the calculated pairwise Ka and Ks values we observed that both *SIX6* and *SIX9* genes were under purifying selection. However, based on the distribution of Ka and Ks values, the diversity between *Foc* and other *formae speciales* is higher in *SIX6* compared to *SIX9*. The clustering of the Ka and Ks values in the plot mimics the clades in the phylogenetic tree with the two clusterings of the Ka and Ks. The distribution of the Ka and Ks values suggests that both *SIX6* and *SIX9* underwent purifying selection across the different *formae speciales*.

The suggestion that *SIX6* underwent purifying selection might at first seem to contradict the result from the Selecton analysis where *SIX6* was reported to undergo positive selection. This discrepancy can be explained by the difference in the way Ka and Ks were measured between the two methods. In pairwise comparison using SNAP, the Ka and Ks were measured across all the codon sites within the gene, while in the case of Selecton, Ka and Ks were measured in a codon-by-codon manner. This would allow Selecton to identify sites that are undergoing either positive balancing or purifying selection.

While in general *SIX6* gene across different *formae speciales* is under purifying selection, we observed that the *SIX6* gene showed a great degree of diversity between *Foc* and other *formae speciales* as shown both by the phylogenetic tree and the distribution of Ka and Ks value. These results in combination with the result from Selecton analysis lead us to believe that in the context of *Foc* clade, we are observing a degree of relaxation in the purifying selection acting on the gene. Whether the hypothesized relaxation of purifying selection in *Foc* clade corresponds to the specificity of *Foc SIX6* to a certain host is a question that remains to be answered. In the case of *SIX9*, both the analysis of pairwise Ka and Ks distribution and Selecton analysis agreed on the possibility of purifying selection acting on the *SIX9* gene. The peak of *SIX9* gene expression also happened at a later day compared to *SIX6* (Fig. 1B), suggesting that *SIX9* might be mediating the infection at the later stage of the infection compared to *SIX6*.

Based on the difference in the pattern of nucleotide and amino acid diversity between *SIX6* and *SIX9* when we compared *Foc* and other *formae speciales*, it is suggested that the *SIX6* gene in *Foc* have gained a degree of adaptation that is specific to the main host of *Foc*. The low degree of both nucleotide and amino acid diversity in *SIX9* would suggests that the role that it plays during the infection is non-*formae speciales* specific and conserved across different *formae speciales*. Further study by disrupting the expression of either the gene, and in the case of *FocSIX6*, the expression under different *formae speciales*, would be needed to further dissect the roles of *SIX6* and *SIX9* as an effector of *F. oxysporum*.

F. oxysporum has attracted plant pathologists across the globe due to its devastating impact on the economy of many countries and also because of its evolutionary quests affecting different hosts, hence the name *F. oxysporum* species complex (FOSC) (Di *et al*. 2016). The soil-borne fungus in FOSC includes both nonpathogenic and pathogenic strains (Gordon 2017). In banana and many other important crops, the pathogenic strains invade roots and cause wilting via colonization of xylem tissues (Dita *et al*. 2018). More than 120 *formae speciales* have been identified in pathogenic *Fo* strains (Edel-Hermann and Lecomte 2019). The *formae speciales* refers to narrow host specificity, where each *forma specialis* infects specific plant species (Gordon 2017). However, this host range was subsequently found to be wider in many *formae speciales* not only in plants (Edel-Hermann and Lecomte 2019) but also in humans (Zhang *et al*. 2020). The *Fo* pathogenic strains usually are hemi-biotrophs, performing a biotrophic lifestyle at early stages of infection and at later stages release toxins in order to kill the host cells and obtain nutrients on the dead tissue (Michielse and Rep 2009; Horbach *et al*. 2011).

The co-evolutionary arms race between pathogens and hosts can be observed in a interplay between genes involved in the interaction, namely resistance *(R)* genes in host plants and avirulence *(Avr)* genes in pathogens (Jones and Dangl 2006). Avr genes are known as effectors that have the ability to manipulate the host immune system to avoid detection and optimizing the virulence function (Presti *et al*. 2015). Host plants evolved by recognizing these specific proteins *via* R genes (Derbyshire 2020). *SIX* genes have been reported involved in virulence and host manipulations in susceptible cultivars (Rep *et al*. 2005; Houterman *et al*. 2009; Widinugraheni *et al*. 2018; An *et al*. 2019). However, in resistant cultivars, these genes mediated and triggered resistance (Rep *et al*. 2004; Houterman *et al*. 2008, 2009). *SIX6* was reported to contribute to the virulence of *Fol* and suppresses I-2-mediated cell death (Gawehns *et al*. 2014). However, its role in *Foc*-banana pathosystem has not been determined yet. To overcome the fungal attack, the host cells were expressing the pathogenesis-related *(PR)* genes which are crucial components of the plant innate immune system especially systemic acquired resistance, thus extensively utilized as markers for defense signaling pathways (Ali *et al*. 2018). The over expression of the *PR-1* gene was reported to enhance resistance in plants during bacterial and fungal attacks (Chandrashekar *et al*. 2018; Lu and Edwards 2018; Tosarini *et al*. 2018; Akbudak *et al*. 2020). *PR-3* gene encodes a chitinase that disintegrates chitin in fungal cell walls and inhibits the fungal growth (Takahashi *et al*. 2016; Chandrashekar *et al*. 2018). In susceptible banana cultivar, these *PR* genes were highly expressed in the corms and subsequently in the roots. In this study, we have shown that both *PR-1* and *PR-3* are displaying expression patterns that are antagonistic to *SIX6* and *SIX9* genes despite the spatial difference in which they are expressed. This difference can be attributed to the nature of the effector itself, which can trigger virulence response in tissues other than of the initial site of infection. The underlying molecular mechanism in which PR-1 and PR-3 proteins from the host interact with SIX6 and *SIX9* proteins from the pathogen and trigger virulence is still an open question that remains to be answered.

Conclusion

We found that in the susceptible banana cultivar, *SIX6* and *SIX9* of *Foc* TR4 are highly expressed in roots, but not in corms or the leaves. The host, however, expressed the pathogenesis-related *(PR)* genes, *PR-1* and *PR-3*, in corms earlier than in the roots. We also discovered that *SIX6* and *SIX9* of *Foc* are polymorphic compared to other *formae speciales*. Based on the rate of synonymous mutation, *SIX6* of *Foc* showed an increase in diversity, but insufficient to drive positive selection. Conversely, *SIX9* of *Foc* showed no divergence in the distribution of the dN/dS ratio, indicating purifying selection.

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Author Contributions

RRE and NF planned the experiments, reviewed and edited the manuscript, INPA and MBB analyzed the data, NF and MBB write the manuscript and made illustrations.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

We hereby declare that all data reported in this paper are available and will be produced on demand.

Ethics Approval

Not applicable.

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