Phytopathology Research

REVIEW

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Current progress on pathogenicity-related genes in *Fusarium oxysporum* f. sp. *cubense* tropical race 4

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Abstract

Vascular wilt, a disease caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), is highly destructive to bananas. Identifying genes that contribute to the fungus's virulence is crucial for understanding its pathogenesis. In this review, we provide an overview of recent research on genes involved in various aspects of Foc TR4's pathogenic process. These include signal recognition and transduction, the formation of cellular structures, regulation through microRNA and epigenetic mechanisms, effector secretion, and toxin secretion. We place a particular emphasis on discussing effectors that either facilitate virulence or serve as elicitors of host defense responses. Given the limited understanding of the molecular mechanisms underlying Foc TR4 pathogenesis, summarizing the research on these functional genes is necessary and timely. Our integrative information will facilitate research on identification of more key genes involved in the invasiveness of Foc TR4, contributing to more systemic understanding of pathogenesis of this important pathogen. These findings will, in turn, offer potential targets for the development of effective fungicides or soil disinfectants to combat this devastating disease.

Keywords Vascular wilt, *Fusarium oxysporum*, Foc TR4, Pathogenesis, Effectors

Background

Musa spp. (bananas and plantains) is one of the most important fruit crops in the world, serving not only as a dessert fruit but also staple food to millions of people from subtropical and tropical countries (Butler 2013; Wang et al. 2024). The most important cultivars are triploids with an AAA, AAB, or ABB genome constitution, which evolve from diploid wild pointed-leaf bananas (*Musa acuminata*, AA group) and long-stemmed bananas (*Musa balbisiana*, BB group) through specific intra- and inter- hybridization. However, the annual production and the quality of banana are greatly reduced by the *Fusarium* wilt, one of the devastating diseases caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc). According to the susceptibility of banana cultivars, Foc was divided into four physiological races: race 1 infects bananas of the genome groups AAB and ABB and is responsible for the epidemics on the cultivar Gros Michel in the 1950s. Race 2 is only compatible to cooking bananas cv. Bluggoe (ABB). Race 3 only infects wild bananas (*Heliconia spp*.) but fails to threat cultivated banana varieties. Race 4 infects the Cavendish cultivars (AAA) as well as all the cultivars attacked by races 1 and

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2, and thus is currently considered as the most virulent pathogen of *Fusarium* wilt. Based on regional characteristics of occurrence and range of adaptation to temperature, Foc4 is divided into two types: subtropical race 4 (ST4) affecting Cavendish production in subtropical areas like Canary Islands, South Africa, Australia, and Taiwan of China, and tropical race4 (TR4) was found in the tropical regions of Southeast Asia and Australia (Bai et al. 2013). Of the reported isolates, Foc TR4 is the most destructive Foc variety, as it attacks almost all banana species and has more transmission routes and stronger pathogenicity (Acuña et al. 2022; Mejías et al. 2023).

Foc TR4 produces three types of asexual spores including macroconidia, microconidia, and chlamydospore in its life cycle, enabling it to disperse and survive. The infection cycle is initiated with germination of conidia and formation of hyphae under suitable environment conditions and the favorable host plants. Fungal hyphae then spread and colonize the surface of root. After that, the fungal hyphae surround the epidermis, followed by invading and colonizing the xylem vessels of root. After successfully infecting banana roots, Foc TR4 grows toward the rhizome and pseudostem to proliferate within the vascular tissue, leading to disruption of water translocation and causing the typical wilt symptoms, which include foliage cholrosis, pseudostem longitudinal splitting, necrosis, and ultimate death of the plants (Wang et al. 2024). Finally, fungal hyphae and spores on the debris of banana plant fall into soil through rains and initiate a new infection cycle (Fig. 1). Infection and colonization of banana plants by the fungus always results in wilting and yellowing of the lower part of leaves and discoloration of rhizomes and necrosis of vascular bundles in pseudostem can be observed in the severely infected banana plants (Guo et al. 2014). As a saprophyte, Foc TR4 can survive in soil for long periods in the form of thick-walled chlamydospores (Pietro et al. 2003). Once it perceives the presence of host plants, it begins infecting them from roots (Fig. 1).

Due to the above characteristics of colonization and survival, fungicides are largely ineffective to Foc TR4 (Pietro et al. 2003). Since triploid varieties exhibit high sterility, it is also very difficult to develop wilt resistant banana cultivars by traditional breeding method. To date, few effective solutions have been applied to manage this destructive pathogen. Therefore, it is of great urgency and needs to formulate effective control methods for *Fusarium* wilt of bananas, and this largely requires better understanding of the molecular mechanism underlying Foc TR4 pathogenesis. In the past decades, a few techniques including genomic, transcriptomic, and proteomic methods have been applied to investigate the pathogenesis of Foc TR4. Amongst, as with the sequencing of genomes of the Foc TR4 strain II5, dozens of functional genes have been identified. This review focuses on the recent advances of pathogenic genes of Foc TR4 as well as their roles in pathogenesis and interaction with host bananas.

Genes involved in signaling recognition and transduction

The first step of Foc TR4 in infecting a host is to perceive the presence of host plants, and then to express genes related to the formation of infection structures, in order to successfully infect the plants. Mitogen-activated protein (MAP) kinase cascades have been known to play crucial roles in transducing various extracellular signals and regulating various cellular processes including growth, differentiation, and innate immunity in fungi (Zhao et al.

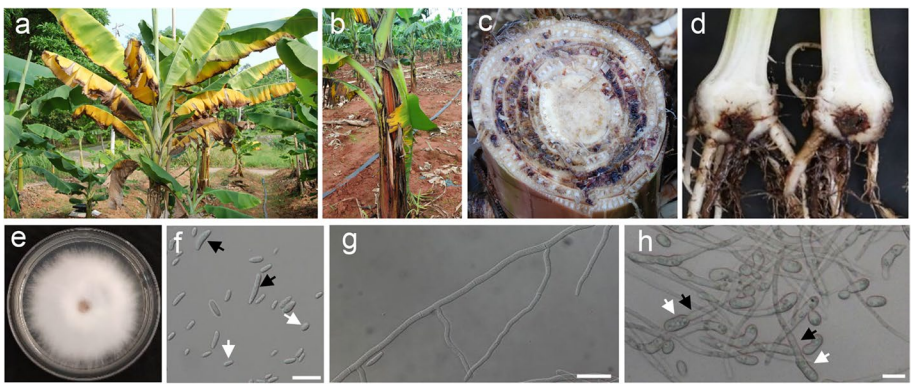

Fig. 1 Symptoms of banana vascular wilt and characteristics of the pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4. **a**, **b** Symptom of the *Fusarium* wilt on the whole banana plant in field. **c**, **d** Symptom of the vascular infected by Foc TR4. **e** Colony growth of the fungus Foc TR4 on the PDA plate cultured for 72 h. **f** Macroconidia and microconidia produced by Foc TR4. The macroconidium and microconidium are indicated with a black arrow and a white arrow, respectively. **g** Vegetative hyphae of Foc TR4. **h** Chlamydospores (indicated by the white arrow) produced by Foc TR4 develop to hyphae (indicated by the black arrow). Scale bars: 10 μm

2005; Johnson et al. 2002; Wu et al. 2024). By homologues sequence analysis with other fungal organisms, the MAP kinase cascade including FocSlt2 (MAPK), FocMkk2 (MAPKK), and FocBck1 (MAPKKK) are identified in Foc. The other two MAPKs are Fmk1 and Hog1 (Wang et al. 2023a) in *Fusarium oxysporum* but have not been reported in Foc. The null mutants Δ*Focslt2*, Δ*Focmkk2*, and Δ*Focbck1* all exhibit flexuous hyphal structures and more branches compared to the wild type (WT). The three mutants show similar morphology of hyphae and colony growth, which are all significantly slower than WT. Additionally, FocSlt2, FocMkk2, and FocBck1 are all important for cell wall integrity and hydrogen peroxide response but are dispensable for osmotic pressure response. Compared with the WT control, the Δ*Focslt2*, Δ*Focmkk2*, and Δ*Focbck1* mutants have less chitin content. Moreover, five out of seven chitin synthases show significantly reduced expression in the three deletion mutants. Additionally, the three kinases are involved in beauvericin biosynthesis by regulating transcription of related genes. The fusaric acid production is significantly reduced in the three mutants by high performance liquid chromatography (HPLC) examination, which is consistent with the observation of lower expression levels of the fusaric acid biosynthesis genes in the mutants compared with WT. Importantly, FocSlt2, FocMkk2, and FocBck1 are all required for full virulence on banana plants. In conclusion, the three mutants by abolishing the MAP kinase-encoding genes show almost identical phenotypes and play vital roles in various biological processes and virulence of Foc TR4 (Ding et al. 2015) (Table 1 and Fig. 2).

Several components of heterotrimeric G-proteins, including Gα, Gβ, and Gγ, have been known to function upstream of the MAP kinase pathway in many fungi (Li et al. 2012). In Foc, the Gα subunit Fga2 and Gβ subunit Fgb1 are also characterized. Phenotypic analyses of the single deletion mutant Δ*fga2* or Δ*fgb1* and the double deletion mutant Δ*fga2*/Δ*fgb1* show that *FGB1* but not *FGA2* is involved in regulation of colony morphogenesis and conidia production. In addition, the Δ*fga2*, Δ*fgb1*, and Δ*fga2*/Δ*fgb1* strains produce more pigments than

Table 1 Genes involved in virulence of Foc TR4

Gene Name	Annotation	Biological Functions	References
SIX1a	Secreted in xylem protein 1a	Virulence	Widinugraheni et al. 2018
SIX8	Secreted in xylem protein 8	Virulence	An et al. 2019
CP1	Cerato-Platanin Family Protein	Virulence	Liu et al. 2019
FSE1	*Fusarium* special effector 1	Virulence elicitor	Yang et al. 2023
SP9	Secreted protein 9	Virulence	Guo et al. 2022
CUPIN1	cupin_1 domain containing protein	Virulence	Yan et al. 2022
SSP1	Small Secreted Protein 1	Conidiation, Virulence elicitor	Wang et al. 2022b
SSP17	Small Secreted Protein 17	Virulence	Wang et al. 2023b
M35_1	Metalloprotease Effector	Virulence	Zhang et al. 2021
OASTL	O-acetylhomoserine(thiol)-lyase	Virulence	Wang et al. 2020
ECM33	GPI-linked cell wall protein	Growth, Virulence	Huang et al. 2022
PGC4	Exo-polygalacturonase 4	Growth, Virulence	Dong et al. 2020
GCN5	General Control Non-derepressible 5	Growth, Conidiation, Virulence	Liu et al. 2022
PMI1	phosphomannose isomerase	Growth, Virulence	Usman et al. 2023
OCH1	α-1,6-mannosyl transferase	Growth, Conidiation, Virulence	Li et al. 2014
GCS	Glucosylceramide synthase	Growth, Conidiation, Virulence	Wang et al. 2022a
SLT2	MAP kinase	Growth, Virulence, BEA synthesis	Ding et al. 2015
MKK2	MAP kinase	Growth, Virulence, BEA synthesis	Ding et al. 2015
BCK1	MAP kinase	Growth, Virulence, BEA synthesis	Ding et al. 2015
FGA2	Gα subunit	Growth, Virulence	Guo et al. 2016
FGB1	Gβ subunit	Growth, Conidiation, Virulence	Guo et al. 2016
SGE1	SIX gene expression 1	Growth, Conidiation, Virulence	Gurdaswani et al. 2020
RLM1	MADS-box TF	Virulence, FSA/BEA synthesis	Ding et al. 2020
ATF1	bZIP transcription factor	Virulence	Qi et al. 2013
PP1	Pheromone precursor 1	Growth, Virulence	Liu et al. 2023
QDE2	AGO protein	Growth, Conidiation, Virulence	Li et al. 2022
DCL1	Dicer protein	Conidiation	Li et al. 2022

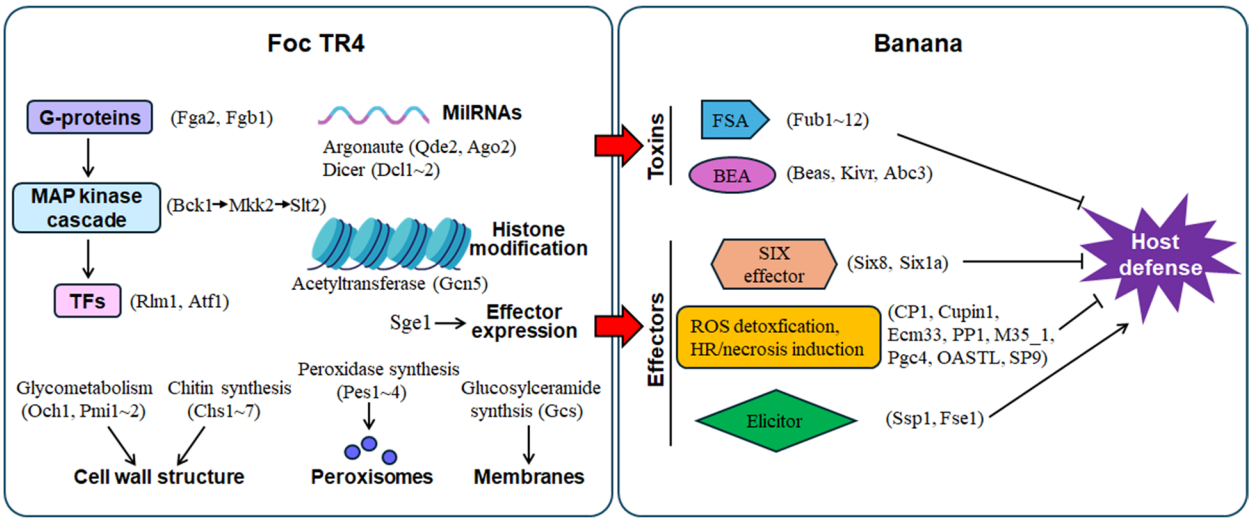

Fig. 2 Schematic representation of proteins involved in different signaling pathways and cellular processes associated with Foc TR4 pathogenicity

the WT on potato dextrose agar (PDA) plates, suggesting that both *FGA2* and *FGB1* negatively regulate pigmentation. Both *FGA2* and *FGB1* have a slight effect on vegetative growth of Foc. Deletion of *FGA2* or *FGB1* enhances heat resistance in Foc. Notably, the intracellular cAMP levels of Δ*fga2*, Δ*fgb1*, and Δ*fga2*/Δ*fgb1* are less than 50% of the WT, suggesting that *FGA2* and *FGB1* are involved in the cAMP pathway and positively regulate intracellular cAMP levels. Pathogen inoculation assays show that G-protein Fga2 and Fgb1 are both required for full virulence of Foc TR4 (Guo et al. 2016) (Table 1 and Fig. 2).

Numerous of transcription factors (TFs) are known as downstream components in the MAP kinase pathway. A MADS-box transcription factor Rlm1 is first reported to play an indispensable role in cell wall integrity as a target of MAPK Mpk1 (Slt2 ortholog) in *S. cerevisiae* (Dodou and Treisman 1997). FocRlm1 is identified to be highly conserved among *Fusarium* species. Deletion of *FocRLM1* leads to reduced aerial hyphal growth and decreased fungal biomass. The *FocRLM1* null mutant exhibits hypersensitivity to H_2O_2, and additionally, by controlling the expression of four peroxidase synthase genes, FocRlm1 is able to regulate anti-oxidation of Foc TR4. Moreover, FocRlm1 is responsible for maintaining cell wall structures of Foc TR4. Notably, FocRlm1 regulates the transcription of fusaric acid biosynthetic genes as well as beauvericin biosynthetic genes. In summary, FocRlm1 is required for full virulence of Foc TR4, and this may result from regulating the expression of genes from RNA-seq of the *FocRLM1* deletion mutant compared to WT (Ding et al. 2020). In filamentous fungi, the MAPK kinases, Foc Slt2 (Ding et al. 2015), *F. graminearum* Mgv1 (Slt2 ortholog), (Hou et al. 2002) and *M. oryzae* Mps1 (Slt2 ortholog) (Xu et al. 1998) play a crucial role in fungal development and virulence. Interestingly, the Δ*Focslt2* and Δ*Focrlm1* mutants display distinct colony phenotype. Unlike Δ*Focslt2*, Δ*Focrlm1* shows only slightly reduced aerial hyphae growth, but exhibits significantly reduced biomass. These results indicate that FocRlm1 may be one of the putative downstream TFs, which regulates the expression of multiple MAPK-associated genes involved in fungal development in Foc. Another transcription factor Atf1 is a downstream target of Osm1 in *M. oryzae* for regulating response to ROS (Li et al. 2012). Osm1 is a mitogen-activated protein kinase homologous to Hog1 (Dixon et al. 1999). Atf1 is also identified in Foc. Disruption of *FocATF1* shows enhanced sensitivity to H_2O_2 and causes oxidative burst of banana plantlets in early stages of infection, indicating that FocAtf1 plays an important role in regulation of oxidative stress response and host innate immunity. Importantly, FocAtf1 is responsible for pathogenicity of Foc TR4 on banana seedlings (Qi et al. 2013) (Table 1 and Fig. 2).

Genes required for development of infection-related subcellular structures

During the pathogenic process, synthesis of cell wall is necessary for the fungus invading into host cells. The outermost cell wall, consisted by a crosslink of polysaccharides, is crucial for fungal viability and adaptation to surrounding environment. Phosphomannose isomerase is a key enzyme involved in the biosynthesis of GDP-mannose, an important precursor of the fungal cell wall. Two genes encoding phosphomannose isomerase in Foc, the *FocPMI1* and *FocPMI2*, are identified from homologue analysis with *S. cerevisiae*. *FocPMI1*, but not

FocPMI2, is highly expressed in the conidial germination and the mycelium stage, indicating that it may play an important role in development of Foc. Notably, the *FocPMI1* rather than the *FocPMI2* deletion mutant is unable to grow in the absence of mannose, further confirming its role in biosynthesis of GDP-mannose. The optimal concentration of exogenous mannose to support growth of Δ*Focpmi1* is 5 mM. Interestingly, fructose or other carbon sources are ineffective to ensure vegetative growth of Δ*Focpmi1*. In addition, FocPmi1 is involved in cell wall integrity and stress tolerance. Transcriptome analyses show that FocPmi1 regulates expression of genes involved in metabolism of carbohydrates, amino acids, and lipids. Importantly, the pathogen inoculation assay shows that Focpmi1 is responsible for infection and virulence on banana seedlings (Usman et al. 2023). The α-1,6-mannosyltransferase OCH1 is identified and characterized from a T-DNA insertion mutant of Foc. Loss of *FocOCH1* leads to impaired fungal growth and reduced number of cell wall proteins. *FocOCH1* is involved in cell wall integrity of the fungus. In addition, the *FocOCH1* null mutant reduces the ability in hyphal attachment and colonization, and finally results in decreased virulence on banana plantlets (Li et al. 2014) (Table 1 and Fig. 2).

Chitin, a microfibrillar β-1,4-linked homopolymer of N-acetylglucosamine (GlcNAc), is one of the main components of fungal cell wall (Lenardon et al. 2010; Kong et al. 2012). Chitin constitutes the main structure of the fungal cell wall and provides the rigidity (Bowman et al., 2006; Latgé 2007). Chitin synthases are key enzymes catalyzing the polymerization of GlcNAc and are reported to be involved in fungal growth and virulence (Odenbach et al. 2009; Kong et al. 2012). Similar to the model pathogenic fungus *Magnaporthe oryzae*, there are seven chitin synthases in Foc, the FOIG_07229 (Chitin synthase 1, Chs1), FOIG_10825 (Chs2), FOIG_09216 (Chs3), FOIG_00580 (Chs4), FOIG_06735 (Chs5), FOIG_06738 (Chs6), and FOIG_06723 (Chs7). The expression levels of them have been considered as a standard parameter to evaluate various effects on cell wall biosynthesis (Ding et al. 2020) (Table 2 and Fig. 2).

Membrane lipids, which are arranged continuously or as a double layer, construct the main skeleton of membranes within the cell, and are crucial for transportation of nutrients and maintenance of osmotic pressure. Glucosylceramides are a class of membrane lipids that serve as vital structural and signaling molecules in eukaryotes. A glucosylceramide synthase named FocGCS has been identified in Foc TR4. FocGCS is highly expressed in germinating conidia and the early infection stage of Foc TR4. The *FocGCS* null mutant lacks glucosylceramide by sphingolipid profiling analysis. Disruption of *FocGCS* results in severely abnormal development and substantial loss of virulence. Transcriptome analysis shows FocGCS is closely related to transmembrane transport (Wang et al. 2022a).

Table 2 Genes related to several key biosynthetic pathways

Gene Name	Gene ID	Biosynthetic pathway	References
CHS1	FOIG_07229	Chitin biosynthesis	Ding et al. 2020
CHS2	FOIG_10825	Chitin biosynthesis	Ding et al. 2020
CHS3	FOIG_09216	Chitin biosynthesis	Ding et al. 2020
CHS4	FOIG_00580	Chitin biosynthesis	Ding et al. 2020
CHS5	FOIG_06735	Chitin biosynthesis	Ding et al. 2020
CHS6	FOIG_06738	Chitin biosynthesis	Ding et al. 2020
CHS7	FOIG_06723	Chitin biosynthesis	Ding et al. 2020
PES1	FOIG_08821	Peroxidase synthase	Ding et al. 2020
PES2	FOIG_07465	Peroxidase synthase	Ding et al. 2020
PES3	FOIG_04532	Peroxidase synthase	Ding et al. 2020
PES4	FOIG_09161	Peroxidase synthase	Ding et al. 2020
FUB1	FOIG_16450	Fusaric acid biosynthesis	Liu et al. 2020
FUB2	FOIG_16451	Fusaric acid biosynthesis	Liu et al. 2020
FUB3	FOIG_16452	Fusaric acid biosynthesis	Liu et al. 2020
FUB4	FOIG_16453	Fusaric acid biosynthesis	Liu et al. 2020
FUB5	FOIG_16454	Fusaric acid biosynthesis	Liu et al. 2020
FUB6	FOIG_16456	Fusaric acid biosynthesis	Liu et al. 2020
FUB7	FOIG_16458	Fusaric acid biosynthesis	Liu et al. 2020
FUB8	FOIG_16459	Fusaric acid biosynthesis	Liu et al. 2020
FUB9	FOIG_16460	Fusaric acid biosynthesis	Liu et al. 2020
FUB10	FOIG_16461	Fusaric acid biosynthesis	Liu et al. 2020
FUB11	FOIG_16462	Fusaric acid biosynthesis	Liu et al. 2020
FUB12	FOIG_16463	Fusaric acid biosynthesis	Liu et al. 2020
BEAS	FOIG_15793	Beauvericin biosynthesis	Ding et al. 2015
KIVR	FOIG_15792	Beauvericin biosynthesis	Ding et al. 2015
ABC3	FOIG_15791	Beauvericin biosynthesis	Ding et al. 2015

Another important subcellular structure required for infection in Foc TR4 is peroxisome. Peroxisomes are single-layer and membrane-bound organelles that are ubiquitously distributed in eukaryotic cells, playing vital roles in β-oxidation of fatty acid to provide cellular energy (Smith and Aitchison 2013). As the marker enzyme of peroxisomes, the peroxidase is a type of oxidoreductases which uses hydrogen peroxide (H_2O_2) as an electron acceptor to catalyze multiple reactions including fatty acid β-oxidation and the glyoxylate cycle, thereby playing an important role in energy metabolism (Chen et al. 2017). Moreover, during interactions of pathogens and host plants, peroxidases catalyze the decomposition of host-derived ROS, which is developed to defend pathogen attacks by plants. In Foc TR4, four genes including FOIG_08821 (*PES1*), FOIG_07465 (*PES2*), FOIG_04532 (*PES3*), and FOIG_09161 (*PES4*) that are responsible for peroxidase synthesis have been identified. The expression levels of these four peroxidase synthases define the

sensitivity of oxidative stress responses (Ding et al. 2020) (Table 2 and Fig. 2).

Key regulators of infection-related genes

Epigenetic regulation plays important roles in the regulation of biological processes, such as growth, development, tissue differentiation, diseases, and interactions between organisms and the environment (Bird 2007; Handel et al. 2010; Verdin et al. 2015). Histone acetylation, catalyzed by histone acetyltransferases (HATs), is one of the main types of epigenetic modification in eukaryotes. FocGcn5 is characterized from homologue sequence blast of Gcn5 in *S. cerevisiae* and contains a histone acetyltransferase domain Acetyltransf_1, indicating it as a histone acetyltransferase. The null mutant of *FocGCN5* shows pleiotropic defects including restricted vegetative growth, dramatically reduced conidiation, and significantly decreased pathogenicity. RNA-seq analysis indicates that the expression levels of around 1500 genes are down-regulated in the *FocGCN5* null mutant. Among them, 27 of 29 genes involved in fungal cell wall synthesis, 14 of 16 effector-coding genes, 22 of 23 plant cell wall degrading enzymes-coding genes, and 114 of 137 major facilitator superfamily (MFS) protein-coding genes are confirmed to express at a lower level in Δ*Focgcn5* by qRT-PCR analysis. Further analyses demonstrate that *FocGCN5* is responsible for environmental stress tolerance and polarity of deposition during cell wall biosynthesis. RNA-seq analysis suggests that FocGcn5 acts as an important regulator of virulence, although it also plays important roles in growth and development (Liu et al. 2022) (Table 1 and Fig. 2).

MicroRNAs (miRNAs), a type of small non-coding single-stranded RNAs primarily found in plants and animals, are known to play crucial roles in diverse biological processes by regulating gene expression. Fungi produce microRNA-like RNAs (milRNAs) that are structurally similar to miRNAs (Sunkar 2010; Ebert et al. 2012). Several proteins including Dicers, QDE2 (Quelling Deficient 2), the exonuclease QIP (QDE2 interacting protein), and MRPL3 (RNAse III domain-containing protein), are involved in milRNAs production (Lee et al. 2010; Chang et al. 2012; Xue et al. 2012). Two Argonaute-coding gene *FocQDE2* and *FocAGO2*, and two Dicer-coding genes *FocDCL1* and *FocDCL2* are identified in Foc. Phylogenetic analysis shows that FocQde2 and FocAgo2 in Foc are conserved with other *Fusarium* fungi. Together with *FocDCL1* and *FocDCL2*, the expression of *FocQDE2* but not *FocAGO2* is significantly up-regulated at 24 h after inoculation on host banana plants, suggesting that sRNAs synthesis mediated by the three proteins including FocQde2, FocDcl1, and FocDcl2 may play an active role during Foc pathogenesis. Phenotypic analyses of deletion mutants showed that *FocQDE2*, but not *FocDCL1* or *FocDCL2*, is important for colonial morphology. *FocQDE2* and *FocDCL1* are related to conidia production. Furthermore, *FoQDE2* is required for tolerance to oxidative stress. Importantly, the virulence assay indicates that *FocQDE2* is required for invasive growth, while *FocDCL1* or *FocDCL2* is dispensable for full virulence. Based on sRNA sequencing together with molecular methods, a micro-like RNA milR-87 is identified as the target of FocQde2. MilR-87 regulates oxidative tolerance and is involved in pathogenicity of Foc. Further analysis suggests that a glycosyl hydrolase gene (FOIG_15013) is one of the targets of milR-87 and the target site locates at the GH-79C domain in the open reading frame (ORF) region. Moreover, the mutant Δ*FOIG_15013* is more virulent than WT, demonstrating it as a negative regulator of Foc pathogenicity. MilR-87 may promote Foc pathogenicity by suppressing FOIG_15013 expression. Meanwhile, further investigation indicates that milR-87 suppresses defense responses likely by silencing the expression of FOIG_15013 (Li et al. 2022) (Table 1 and Fig. 2).

Foc remains as a saprophyte or survives as chlamydospores in the soil in the absence of host bananas, while exists as a pathogen in the presence of banana roots. During this transition, numerous genes related to pathogenicity are required to be reprogrammed, which is governed by several master transcriptional regulators responsible for controlling the gene expression profile. Sge1 (SIX gene expression 1), which was first found in *Fusarium oxysporum* f. sp. *lycopersici* (Fol), is one of these regulators capable of regulating expression of effector-encoding genes. The expression of Sge1 is induced in Foc during infection on banana. Phenotypic analyses of the *FocSGE1* deletion mutant reveal that FocSge1 affects vegetative growth, conidiation, hydrophobicity, and mycelial pigmentation, probably because FocSge1 is related to the cAMP-PKA signaling since phosphorylation of Sge1 by PKA is required for its function in Fol (Michielse et al. 2009). After 6 weeks of inoculation of banana plantlets, the symptoms including yellowing of the older leaves and cracking of the pseudostem are not observed in the *FocSGE1* null mutant-inoculated plants. In comparison, such symptoms are clearly found in banana plantlets inoculated with WT at the third week. This result demonstrates that FocSge1 is required for pathogenicity on banana plants. Moreover, FocSge1 is also related to biosynthesis of fusaric acid (Gurdaswani et al. 2020) (Table 1 and Fig. 2).

Genes encoding secreted proteins and effectors

Foc TR4 invades host plants by secreting a large number of effector proteins to assist in acquiring nutrients, suppressing plant immunity, and thereby promoting its own

reproduction and infection. Deciphering the functions of these effector proteins is an important mechanism research of Foc TR4 pathogenesis and has also been a hot topic and frontier research field recently. With protein extraction and secretome analysis, 129 and 105 secretory proteins are found from Foc TR4 cultured alone and cultured with banana roots, respectively. The protein numbers are 120 and 109 from Foc TR1 under the same conditions. Secretory proteins related to hydrolase activity, oxidoreductase activity, and transferase activity are commonly found between Foc TR1 and Foc TR4. Notably, in culturing with banana roots, Foc R1 and Foc TR4 secrete many novel secretory proteins, of which approximately 90% (Foc R1, 57/66; Foc TR4, 50/55) are unconventional secretory proteins which lack signal peptides. In addition, a high proportion of secretory proteins specifically found in Foc TR4 are associated with a number of metabolic pathways such as cysteine and methionine metabolism, carbon metabolism, and phenylalanine metabolism (Wang et al. 2020). Effectors are classified into three categories based on their main roles during the infection.

Effectors for promoting infection or immunity suppression

A cysteine biosynthesis enzyme O-acetylhomoserine (thiol)-lyase (OASTL) is one of the most abundant and inducible Foc TR4-specific secretory protein in presence of plant roots. OASTL is important for pathogenicity but dispensable for vegetative growth of Foc TR4. This is likely because OASTL is responsible for biosynthesis of cysteine, which is the precursor of of the well-known sulfur-containing defense compounds (Wang et al. 2020). The cerato-platanin (CP) protein family was first described and found in *Ceratocystis fimbriata*, an ascomycete pathogen of the European plane tree (Pazzagli et al. 1999). Since then, CP proteins have been reported in many different filamentous fungi and have also been identified as secreted proteins both in *Ascomycota* and *Basidiomycota*. CP proteins are known to be secreted into the culture filtrate, but they are also found in the cell wall of ascospores, conidia, and hyphae (Boddi et al. 2004). Transcriptomic analysis of resistant and susceptible banana roots that are inoculated with Foc TR4 had led to the discovery of plenty of candidate effectors, including a CP protein FocCP1. FocCP1 is highly expressed during the spore germination and infection process. It is localized to plant apoplast and can induce necrosis in banana leaves. Notably, loss of *FocCP1* markedly reduces the pathogenicity of Foc TR4 on bananas and restricts invasive growth in banana roots (Liu et al. 2019).

Another novel effector protein FocSP9 has a functional signal peptide but does not contain known motifs or domains. FocSP9 is dispensable for asexual development but required for full virulence by affecting invasive growth in the rhizomes of bananas. RNA-seq analysis show FocSP9 affected the expression of many virulence-associated genes (Guo et al. 2022). FoSsp17 is one of the candidate effectors identified from a streamlined screening system (candidate effector prediction, RNAseq-based expression level analysis, and cell death manipulative activity assessment based on transient expression in *N. benthamiana*) of Foc. Disruption of *FoSSP17* leads to reduced virulence but has little effect on vegetative growth and conidiation. Moreover, FoSsp17 suppresses pattern-triggered immunity in plants by inhibiting reactive oxygen species (ROS) accumulation, reducing callose deposition, and suppressing the expression of *NbLOX* (associated with the jasmonic acid-pathway) and *NbERF1* (related to the ethylene-pathway) (Wang et al. 2023b). FocM35_1, a secreted metalloprotease, was significantly induced during the early stage of Foc TR4 infection. FocM35_1 reduced banana chitinase activity by interacting with the banana chitinase MaChiA. Expression of FocM35_1 could not only facilitate Foc TR4 infection in bananas, but also suppress the INF1-induced hypersensitive response in *N. benthamiana*. These results indicate that metalloprotease effector FocM35 contributes to pathogen virulence by inhibiting the host immunity (Zhang et al. 2021).

A Cupin type-1 domain-containing protein (named as FoCupin1) was identified as another candidate effector. FoCupin1 has a signal peptide but does not have transmembrane domain or GPI-anchor site. Expression of FoCupin1 is significantly induced at the early stage of Foc TR4 infection on bananas. Importantly, FoCupin1 is required for full virulence of Foc TR4 and shows little effect on fungal growth, conidiation, fusaric acid production, and cell wall integrity. In addition, deletion of *FoCupin1* results in activation of multiple defense responses and ROS accumulation in banana root, which restricts fungal penetration and proliferation, and finally decreases virulence of Foc TR4 on bananas (Yan et al. 2022). An exo-polygalacturonase (exo-PG), Pgc4, is purified from a culture of Foc4. The expressed recombinant Pgc4 is capable of causing tissue maceration and necrosis, indicating that Pgc4 may act as a virulence factor in Foc4. Compared with the WT, the *PGC4* deletion mutant shows slightly slower hyphal growth and significantly reduced virulence on the banana plantlets (Dong et al. 2020).

Glycosylphosphatidylinositol (GPI)-anchored proteins are important ester modified proteins and are commonly found in eukaryotes. Multiple functions have been reported for the GPI-anchored proteins in filamentous fungi, such as serving as enzymes, cell surface antigens, cell wall proteins, and extracellular matrix proteins.

ECM33 is identified as a GPI-anchored protein in Foc TR4. FocECM33 is highly expressed at the early stage of infection on banana roots. Disruption of *FocECM33* leads to the defected vegetative growth, the wrinkling hyphae and conidia, and the weakened colonization ability. The *FocECM33* deletion mutant shows almost two-folds of endogenous ROS levels as the WT. Three ROS synthesis-related genes all show higher transcription levels in the *FocECM33* deletion mutant compared to the WT and the complementary strain. The high endogenous ROS production may cause cell damage of the mycelia in the *FocECM33* deletion mutant. Moreover, loss of *FocECM33* not only results in increased cell wall sensitivities and chitin synthesis, but also triggers stronger PTI in banana plantlets. Transcriptome analysis reveals that *FocECM33* is closely associated with the membrane biological process and chitin accumulation of Foc TR4 (Huang et al. 2022).

F. oxysporum harbors a putative protein with the hallmarks of fungal-pheromone precursors and secretes an-pheromone to act as a growth regulator, chemoattractant, and quorum-sensing signaling molecule. Foc4-PP1, ortholog of the-pheromone precursor gene in TR4 has been identified. Knocking out of Foc4-PP1 leads to slightly lower colony growth and significantly reduced virulence on banana roots. The *FocPP1* deletion mutant is more sensitive to Congo red and H_2O_2, suggesting that Foc4-PP1 plays an essential role in cell wall integrity and oxidative response. When transiently expressed in *N. benthamiana*, Foc4-PP1 is found to localize in both the nucleus and cytoplasm of plants. In addition, Foc4-PP1 and its signal peptide-deficient variant (Foc4-PP1$^{\Delta sp}$) can inhibit BAX-induced cell death, suggesting its role in modulating plant immunity (Liu et al. 2023) (Fig. 2).

SIX effectors

Unlike foliage pathogens, Foc TR4 causes typical symptoms in xylem. It is a good solution to seek secreted pathogenic proteins in xylem of host plants after invasion of this pathogen. "*Secreted In Xylem*" (*SIX*) genes were first identified in *F. oxysporum* f. sp. *lycopersici* (Fol) with proteomic analysis in xylem sap (Rep et al. 2002). A total of 15 *SIX* genes have been identified in all *F. oxysporum* formae speciales (Rep et al. 2004; Houterman et al. 2007; Lievens et al. 2009; Ma et al. 2010; Rep and Kistler 2010; Thatcher et al. 2012; Schmidt et al. 2013; Ma et al. 2015; Williams et al. 2016; Czislowski et al. 2018; Simbaqueba et al. 2018). The protein sequences of SIXs display little homology with each other or known proteins, and the biological functions of SIXs remain largely unknown (An et al. 2019). In Foc, seven *SIX* genes, the *SIX1*, *SIX2*, *SIX6*, *SIX7*, *SIX8*, *SIX9*, and *SIX13*, have been identified based on homologous sequence alignment with Fol. Among these genes, *SIX2* and *SIX8* have only been detected in Foc race 4. *SIX8* has been proved to be a functional gene which distinguishes race 4 from race 1 and 2 isolates. Moreover, SNP variations of *SIX8* can further differentiate tropical and subtropical race 4 isolates (Fraser-Smith et al., 2014). In Foc TR4, functions of several *SIX* genes have been clarified. *SIX8* can be secreted to the extracellular space and is required for virulence. In comparison, *SIX2* is dispensable for pathogenicity since its deletion mutant causes similar disease symptom as WT when inoculating the banana plantlets (An et al. 2019). In Focub II5 genome, three homologues of Fol-*SIX1* are annotated as *SIX1a*, *SIX1b*, and *SIX1c*. These genes show over 80% nucleotide identity to Fol *SIX1*, and the corresponding proteins exhibit around 70% amino acid identity to Fol Six1. Knocking out of *SIX1a* in FocTR4 severely reduced its virulence to Cavendish banana. The role of *SIX1b* and *SIX1c* in FocTR4 virulence remains to be investigated (Widinugraheni et al. 2018) (Fig. 2).

Plant defense elicitors

Foc4 secrets a type of proteins called elicitors which activate plant immunity. A small secreted protein FocSsp1 is proven to be a putative elicitor that negatively regulates pathogenicity of Foc4. Knocking out of *FocSSP1* leads to increased virulence and conidiation. In addition, expressions of four pathogenesis-related genes (*PR1*, *PR3*, *PR5*, and *PR10*) of bananas that are infected with the *FocSSP1* deletion mutant are down-regulated in comparison with that of WT. Moreover, *PR1b* and *LOX* which are known to be related to the salicylic acid signaling and the jasmonic acid signaling respectively, are both significantly induced at 48 h after inoculation with the *FocSSP1* null mutant on *N. benthamiana*. These results show that FocSsp1 is likely to be an elicitor which triggers plant defense response (Wang et al. 2022b). Another plant defense elicitor is the *Fusarium* special effector 1 (Fse1). It is significantly induced after colonizing the banana root in Foc TR4. *FSE1* is not required for vegetative growth and conidiation. Disruption of *FSE1* increases the disease index, while overexpression of *FSE1* decreases virulence on banana plantlets. Additionally, Fse1 interacts with a MaEFM-like MYB transcription factor in plant nucleus, and can induce cell death in tobacco leaves (Yang et al. 2023).

Toxins

Fusaric acid (FSA) is a phytotoxin produced by several *Fusarium* species and known to show strong phytotoxicity on plants and to facilitate infection (Fakhouri et al. 2003; Liu et al. 2020). In Foc TR4, FSA is also proven to be associated with plant disease development (Liu et al. 2020). Similar to other *Fusarium* species including *F.*

verticillioides and *F. fujikuroi*, a total of 12 genes designated as *FUB*1–12 have been identified in the fusaric acid biosynthetic (FUB) cluster in Foc TR4 (Brown et al. 2015; Liu et al. 2020). Of the twelve genes, *FUB1*, *FUB2*, *FUB3*, *FUB4*, *FUB5*, and *FUB10* have been successfully knocked out and are all found to be critical for FSA production in *Foc* TR4. Moreover, the pathogen inoculation assay shows that pretreatment with FSA promotes *Foc* TR4 invasion. In addition, FSA inhibits mitochondria function by regulating the expression of related genes and facilitates disease development by inhibiting the accumulation of host-derived ROS. In conclusion, FSA functions as a positive virulence factor of Foc TR4 (Liu et al. 2020) (Table 2 and Fig. 2).

Beauvericin (BEA) is a cyclohexadepsipeptide mycotoxin produced by many fungi such as *Beaveria bassiana* and *Fusarium* spp. (Wang et al. 2012). BEA exhibits various bioactivities including insecticidal, anti-tumor, and anti-microbial activity. BEA has been reported to be produced by Foc. Three BEA biosynthetic genes, *FOIG_15793* (BEAS ortholog), *FOIG_15792* (KIVR ortholog), and *FOIG_15791* (ABC3 ortholog), have been identified according to orthologues analysis of the BEA biosynthetic genes in *F. oxysporum* f. sp *lycopersici* race 2 (Lopez-Berges et al. 2013; Ding et al. 2015) (Table 2 and Fig. 2).

Conclusions and perspectives

The *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 is the destructive disease in bananas. Identification of functional genes associated with virulence of Foc TR4 is crucial to understand the pathogenesis, and is necessary for further developing effective strategies to protect bananas from the vascular wilt. In this review, we summarize the current knowledge of functional proteins involved in four stages during the pathogenic process including signal recognition and transduction, cellular synthesis, gene expression regulation, effectors, and toxin secretion. Because of the limited information of pathogenesis and functional research of genes, the presented summary will help researchers focus on banana *Fusarium* wilt and will advance more systemic research on elucidating pathogenesis of Foc TR4. Overall, based on the increasing knowledge on this fungus and advancing method such as omics and gene editing, a variety of Foc TR4 pathogenicity-related genes will be identified and explored. Research on key enzyme-mediated pathways or posttranslational modifications will be the focus in future. Utilization of these genes as targets to develop specific soil disinfectant or fungicide will be facilitated. In conclusion, more pathogenic genes will be identified to reveal pathogenesis of Foc TR4 and to be utilized to control this devastating disease.

Abbreviations

BEA	Beauvericin
bZIP	Basic leucine zipper
CP	Cerato-platanin
exo-PG	Exo-polygalacturonase
Foc	*Fusarium oxysporum* F. sp. *cubense*
Fol	*Fusarium oxysporum* F. sp. *lycopersici*
FSA	Fusaric acid
FUB	Fusaric acid biosynthetic
GlcNAc	N-acetylglucosamine
GPI	Glycosylphosphatidylinositol
HPLC	High performance liquid chromatography
MAP	Mitogen-activated protein
MFS	Major facilitator superfamily
milRNAs	MicroRNA-like RNAs
miRNAs	MicroRNAs
OASTL	O-acetylhomoserine (thiol)-lyase
QIP	QDE2 interacting protein
SIX	Secreted In Xylem
TR4	Tropical race 4

Acknowledgements
Not applicable.

Authors' contributions
DC, XC, and JP designed the manuscript; DC and MJ drafted the manuscript; XC, JP, and JX revised the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by Central Public-interest Scientific Institution Basal Research Fund (1630042024018) and the earmarked fund for CARS-31.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 12 May 2024 Accepted: 8 July 2024
Published online: 15 October 2024

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