

Article

Histidinol-Phosphate Phosphatase FoHis2 Is Essential for Growth, Stress Responses, and Full Virulence in *Fusarium oxysporum* f. sp. *cubense*

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Abstract

Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is a destructive vascular disease that seriously threatens global banana production. To investigate the contribution of histidine metabolism to Foc growth and pathogenicity, we functionally characterized FoHis2, a putative histidinol-phosphate phosphatase in Foc race 4 (Foc4). Targeted deletion of *FoHis2* severely compromised histidine prototrophy, with the $\Delta FoHis2$ mutant growing slowly on potato dextrose agar and even more slowly on minimal medium (MM, no histidine added). Exogenous histidine fully restored the mutant growth to wild-type (WT) levels, whereas histidinol supplementation rescued the colony size but not the reduced aerial mycelium formation. The $\Delta FoHis2$ mutant exhibited markedly reduced vegetative growth and hyphal branching, and increased sensitivity to elevated H₂O₂ concentrations, compared with the WT strain. Consistent with the oxidative stress phenotype, peroxisome-associated genes were down-regulated in the $\Delta FoHis2$ mutant. FoHis2 was dispensable for conidiation, cell wall integrity, and fusaric acid and beauvericin biosynthesis. Pathogenicity assays showed that the deletion of *FoHis2* severely compromised cellophane penetration and greatly reduced disease incidence and severity on Cavendish banana plantlets, whereas genetic complementation restored the WT phenotypes. These results indicate that FoHis2-mediated histidine biosynthesis is essential for metabolic homeostasis, stress adaptation, and full virulence in Foc4, and highlight histidine metabolism as a potential target for controlling *Fusarium* wilt in banana.

Keywords: banana *Fusarium* wilt; virulence; histidine biosynthesis; stress responses; transcriptome



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1. Introduction

Banana (*Musa* spp.) is the most widely traded fruit globally and a critical staple crop in many tropical and subtropical regions. China, a leading producer and consumer, plays a pivotal role in the global banana industry [1]. However, banana *Fusarium* wilt, caused by the soilborne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), poses a severe and persistent threat to banana production worldwide. Foc is divided into three physiological races based on host specificity. Race 1 (Foc1), which caused the historical “Gros Michel”

epidemics, also infects several AAB cultivars and the ABB cultivar “Pisang Awak”. Race 2 (Foc2) predominantly affects ABB cooking bananas such as “Bluggoe”. Notably, race 4 (Foc4) is highly virulent and capable of infecting nearly all commercially grown banana cultivars [2,3]. The pathogen is highly persistent; its chlamydospores can survive in soil for years and spread through contaminated planting material, soil, and irrigation water [3]. Foc primarily invades the vascular bundles of banana plants, disrupting water and nutrient transport and ultimately causing plant death.

The virulence factors of Foc can be broadly grouped into structural factors, toxins, secreted effectors, and systemic regulatory factors, including transcription factors (TFs), small RNAs, and environmental response regulators. As a hemi-biotrophic fungus, Foc produces cell wall-degrading enzymes that enable penetration through root hairs and subsequent colonization of xylem vessels, leading to vascular occlusion and wilting symptoms [4,5]. The toxins fusaric acid (FA) and beauvericin (BEA) are major virulence determinants that interfere with mitochondrial function, induce host cell apoptosis, and suppress host antioxidant defenses [6–8]. In addition, plant-pathogenic fungi secrete effector proteins that manipulate host immunity. Several Foc effectors, such as the small, secreted proteins FoSsp1 and FSE1, have been functionally characterized and shown to modulate host defense responses and Foc4 virulence [9,10]. Multiple TFs, small RNAs, kinases, and metabolic enzymes have also been implicated in Foc pathogenicity, including FoRlm1 and FoAce2, which are implicated in cell wall regulation and stress responses, the FoQDE2-dependent small RNA miR 87, and proteins such as FoOCH1, FocGCN5, and Focpmi1 [11–18]. Furthermore, environmental response factors associated with metal homeostasis, pH adaptation, oxidative stress responses, and nitrogen metabolism enhance the ability of *F. oxysporum* to adapt to fluctuating conditions and counteract host defenses, for example by neutralizing reactive oxygen species (ROS) [19]. Nitric oxide (NO) has been shown to delay disease progression by modulating banana defense gene expression [20]. Although substantial progress has been achieved in understanding the pathogenicity of Foc, several key issues remain unresolved. The identities and modes of action of many virulence factors, particularly their roles in reshaping host immunity and metabolism, remain unclear.

Amino acids are organic compounds essential for protein synthesis, cell growth and development, and energy metabolism. Increasing evidence indicates that amino acid metabolism is closely linked to fungal growth, sporulation, stress tolerance, and pathogenicity. In the human pathogen *Candida albicans*, genes involved in arginine and aspartate biosynthesis also involved in vegetative growth and virulence [21,22]. In *Botrytis cinerea*, a plant pathogen, the methionine biosynthetic enzyme BcStr2 is essential not only for amino acid synthesis but also for vegetative growth, differentiation, stress adaptation, and pathogenicity [23]. Similar findings have been reported for *Magnaporthe oryzae*, wherein leucine- and arginine-biosynthetic pathway genes, including *LEU1*, *LEU2A*, *LEU4*, *MoLEU3*, *MoARG1*, *MoARG5*, and *MoARG7*, are indispensable for hyphal growth, conidiation, sexual reproduction, and host infection [24–26]. In *Alternaria alternata*, methionine biosynthetic genes *AaMetB*, *AaMetC*, and *AaMetX*, as well as the regulator *AaMetR*, are required for amino acid biosynthesis, ROS tolerance, and fungal virulence [27]. These studies demonstrate that amino acid biosynthetic pathways are closely interconnected with the physiology, development, and virulence of plant-pathogenic fungi, highlighting their potential as molecular targets for antifungal intervention and crop resistance improvement.

Histidine is an essential proteinogenic amino acid and a precursor of several bioactive metabolites involved in cell signaling, pH regulation, metal ion chelation, and redox homeostasis. Consequently, histidine metabolism can profoundly influence the growth, stress adaptation, and pathogenic potential of fungal pathogens. Defects in amino acid metabolism often result in reduced tolerance to oxidative and osmotic stresses and attenu-

ated virulence. In *Gibberella zeae*, *GzHIS7* encodes a bifunctional enzyme in the histidine biosynthetic pathway; loss of *GzHIS7* leads to histidine auxotrophy and severely impaired hyphal growth, sexual reproduction, and pathogenicity, underscoring the importance of histidine biosynthesis to core biological processes [28]. In *Aspergillus fumigatus*, histidine biosynthesis is essential for maintaining metal homeostasis and virulence. Disruption of this pathway reduces resistance to heavy metals and significantly attenuates pathogenicity in multiple infection models [29]. Histidinol-phosphate phosphatase (HolPase) catalyzes the penultimate step in histidine biosynthesis, converting L-histidinol phosphate to L-histidinol. HolPases have been functionally characterized in several bacteria, where they are required for histidine prototrophy, growth, and, in some cases, antibiotic resistance and stress adaptation [30–33].

Although HolPases have been extensively characterized in bacteria, their functions in plant-pathogenic fungi remain poorly understood. In *Foc*, the histidine biosynthetic pathway, and in particular the histidinol-phosphate phosphatase *FoHis2*, has not been functionally characterized in relation to fungal biology and pathogenicity. Here, we characterize *FoHis2* in the banana vascular pathogen *Foc4* by combining targeted gene deletion, phenotypic and pathogenicity assays, and transcriptomic analysis to elucidate its roles in fungal growth, stress adaptation, and virulence, as well as its integration into broader regulatory networks.

2. Materials and Methods

2.1. Strains and Culture Conditions

The *Foc4* strain used in this study was isolated from Hainan Province and preserved at Qiongtai Normal University. Cultures were grown on potato dextrose agar (PDA), potato dextrose broth (PDB), minimal medium (MM, no histidine added), or Czapek Dox medium, following protocols described by [17].

2.2. Sequence Analysis

Nucleotide and protein sequences were retrieved from the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/>, accessed on 30 May 2025). Sequence alignments and phylogenetic analyses were performed using MEGA12 with the neighbor-joining algorithm [34].

2.3. Generation of *FoHis2* Deletion Mutants and Complementation

The *FoHis2* gene in the *Foc4* WT strain was disrupted by polyethylene glycol-mediated protoplast transformation using a split-marker polymerase chain reaction (PCR) strategy [35]. In the first PCR round, two overlapping fragments of the hygromycin B phosphotransferase gene *hph* (HP and PH) and the upstream and downstream flanking regions of *FoHis2* were amplified from the corresponding templates. In the second round, fusion PCR was used to generate two chimeric fragments (upstream-HP and PH-downstream), which were co-transformed into *Foc4* protoplasts. Hygromycin-resistant transformants were screened by diagnostic PCR to identify candidates in which *FoHis2* was replaced by the *hph* cassette. To confirm gene deletion via homologous recombination, the transformants were further analyzed by PCR using primer pairs specific for the *hph* cassette, the native *FoHis2* locus, and the upstream and downstream junctions, followed by Southern blotting to verify single, targeted integration at the *FoHis2* locus.

For complementation, a genomic fragment containing the WT *FoHis2* gene together with a 1990-bp native promoter and a 1440-bp terminator was amplified from the WT genomic DNA and cloned into *Hind*III-digested pEX2 NPTII. The construct was verified by sequencing and introduced into *Agrobacterium tumefaciens* strain AGL1 (Weidi

Biotechnology Co., Ltd., Shanghai, China) via electroporation. *A. tumefaciens*-mediated transformation of Foc4 was performed by co-cultivating fresh conidia with recombinant AGL1 cells OD₆₀₀ $\approx$ 0.4 on an induction medium supplemented with 200 μ M acetosyringone (Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 48 h, followed by selection on PDA containing 50 μ g/mL of G418 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 300 μ g/mL of cefotaxime (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). G418-resistant transformants were verified by PCR for the presence of the *FoHis2* complementation construct and the G418 resistance cassette. All primer pairs and probes are listed in Table S1.

2.4. Phenotypic and Stress Response Assays

Vegetative growth was assessed by inoculating 2 μ L of a 2×10^7 conidia/mL suspension onto PDA plates and measuring the colony diameters after 5 days at 28 °C. Microconidial morphology and dimensions were examined and imaged using a Nikon Ni-E fluorescence microscope. Fungal biomass was determined by using a culture of 1 mL of a 10^7 conidia/mL suspension in 100 mL of PDB at 28 °C for 3 days, followed by mycelial drying and weighing. Conidia production was quantified using a hemocytometer after vortexing mycelial plugs in distilled water.

For stress tolerance assays, MM plates were prepared using 4 mM hydrogen peroxide (H₂O₂; Sinopharm Chemical Reagent Co., Ltd.) to test oxidative stress, and 60 μ g/mL of Congo red (CR; Sigma-Aldrich) or 60 μ g/mL of calcofluor white (CFW; Sigma-Aldrich) to test cell wall stress. The Δ *FoHis2* mutant, WT, and complemented strain Δ *FoHis2*-C were inoculated as described above and incubated at 28 °C for 3–5 days. The colony diameter was measured, and growth inhibition was determined by comparing the colony diameter to that on control MM plates. Morphological changes were observed and documented. All experiments were performed in triplicate.

Cellophane penetration assays were performed to assess the penetration ability of Foc strains. Sterile cellophane membranes were placed on PDA plates, and 5 mm mycelial plugs of the WT, Δ *FoHis2*, and complemented strain Δ *FoHis2*-C were inoculated onto the membranes. The plates were incubated at 28 °C for 5 days. The membranes with fungal colonies were then removed, and the agar surface was examined for hyphal growth beneath the membrane. The experiment was repeated three times.

2.5. FA Quantification

FA (Thermo Fisher Scientific Inc.) quantification was performed using a modified version of the protocol described by [17]. In brief, 1 mL of conidial suspension (7×10^8 conidia/mL) from each fungal strain was used to inoculate 120 mL of Czapek Dox medium. The cultures were incubated at 28 °C for 7 days under constant agitation at 170 rpm. Following incubation, mycelia and conidia were removed by filtration. The resulting filtrate was acidified to pH 2.5. Subsequently, FA was extracted with ethyl acetate, and the extract was dried under vacuum. The resulting residue was resuspended in methanol and analyzed via high-performance liquid chromatography (HPLC; Agilent Technologies Inc., Santa Clara, CA, USA). FA was quantified using a standard curve generated with FA standards, with detection at 280 nm. Spiking experiments were performed by adding FA standard to selected extracts prior to HPLC analysis to confirm the identity of the FA peak.

2.6. Virulence Assays

The virulence of the WT, Δ *FoHis2* and complemented strain Δ *FoHis2*-C were assessed on Cavendish banana plantlets using previously described methodologies [17]. In brief, the roots of banana plantlets were immersed in a freshly prepared spore suspension (1×10^6 conidia/mL) for 30 min before being replanted into pots. Internal corm discol-

oration was evaluated 30 days post-inoculation. Disease severity was quantified using metrics for disease incidence and disease index, using sterile water as a negative control.

2.7. Transcriptome and Gene Expression Analysis

WT and Δ FoHis2 mutant strains were grown in the PDB medium at 28 °C with constant shaking at 180 rpm for 18 h. Total RNA was extracted from these cultures and used to generate cDNA libraries, which were then sequenced on the Illumina NovaSeq PE150 platform (Illumina, San Diego, CA, USA). The resulting high-quality clean reads were mapped to the reference genome of Foc 4 strain II5 (NRRL 54006, NCBI accession no. GCA_000260195.2) using the HISAT2 software (2.0.5). Transcript assembly was subsequently conducted with StringTie. Differential expression genes (DEGs) were determined using DESeq2, with statistical significance established by an adjusted p -value of ≤ 0.05 and an absolute log2 fold change of ≥ 1 . Functional enrichment analyses, including Gene Ontology (GO) annotation and KEGG pathway enrichment, were performed using the clusterProfiler package in R, applying a significance threshold of $p < 0.05$.

To confirm the transcriptomic findings, quantitative real-time PCR (qRT-PCR) was employed. Relative expression levels of selected genes were calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to an internal reference gene. All qRT-PCR experiments were run in triplicate, encompassing three independent biological replicates. Primer sequences designed for qRT-PCR are listed in Table S1.

To identify key FoHis2-responsive proteins with central roles in protein–protein interaction (PPI) networks, we first selected the 250 most up-regulated and 250 most down-regulated DEGs between the WT and Δ FoHis2 strains according to log2 fold-change values. Because Foc is not included in the STRING database, the corresponding orthologous proteins of *F. oxysporum* f. sp. *lycopersici* (Fol) were retrieved and used to construct a PPI network in STRING. The resulting PPI network was imported into Cytoscape (version 3.10.4) for visualization and network analysis. Hub proteins were identified using the CytoHubba plugin with the Maximal Clique Centrality (MCC) algorithm, and the top-ranking nodes were defined as FoHis2-associated hub proteins (and their corresponding genes in Foc).

3. Results

3.1. Phylogenetic Analysis and Conserved Domain Identification of FoHis2

A phylogenetic tree was constructed in MEGA11 to investigate the evolutionary relationships of the FoHis2 protein and its homologs in other fungi. The analysis revealed that the amino acid sequence of FoHis2 is identical (100% similarity) to that of Foc4 (XP_031066978.1). Additionally, FoHis2 shows close similarity to the homologous proteins of other *Fusarium* species, such as *F. oxysporum* f. sp. *lycopersici* (XP_018246367.1) and *F. verticillioides* (XP_018752505.1). In contrast, FoHis2 displays more distant evolutionary relationships with the proteins of non-*Fusarium* species, such as *Saccharomyces cerevisiae* (NP_116681.1), sharing only 28.48% sequence similarity (Figure 1a). These findings suggest that FoHis2 exhibits minimal sequence variation among *Fusarium* species.

The analysis of the conserved domain demonstrated that the homologous FoHis2 proteins belong to the PHP_HisPPase_Hisj_like (PHP) superfamily, indicating a high degree of structural and functional conservation among the fungal His2 proteins (Figure 1b). This conservation underscores the potential critical role of His2 in fungal biology and virulence.

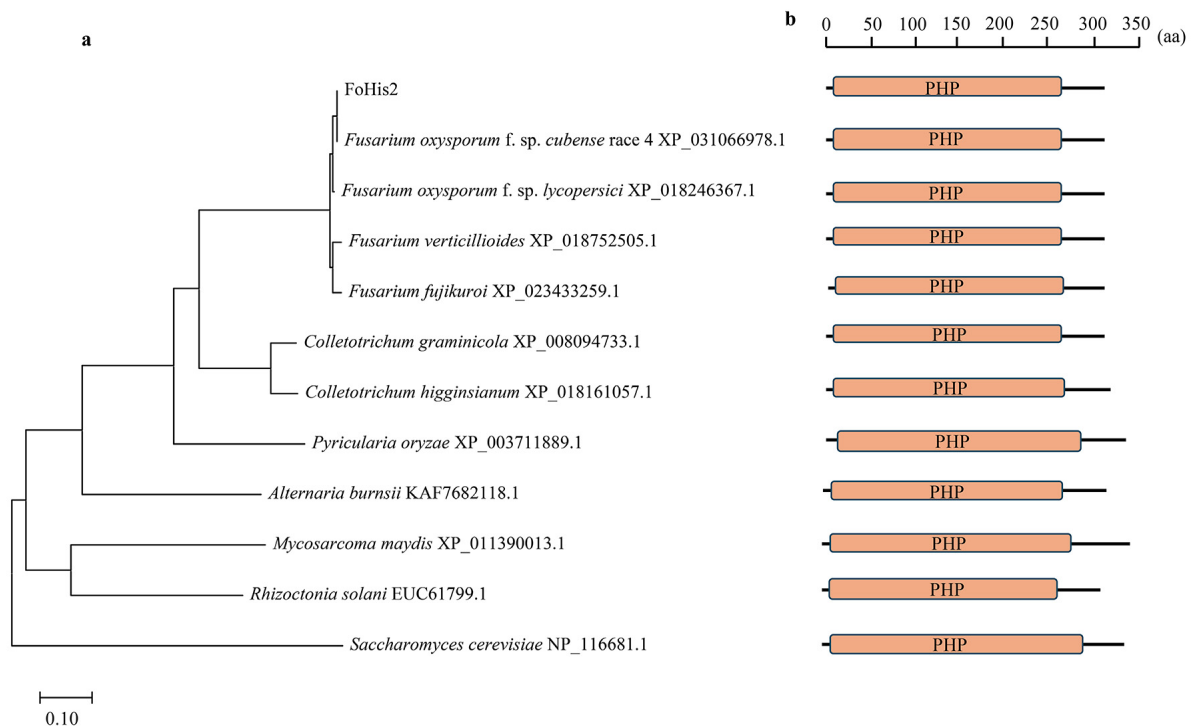


Figure 1. Phylogenetic relationships and conserved domain architectures of FoHis2 homologs in fungi. (a) A phylogenetic tree depicting the evolutionary relationships of FoHis2-like proteins across diverse fungal species; (b) Schematic representation of conserved domains in FoHis2 homologs, emphasizing the PHP_HisPPase_Hisj_like (PHP) superfamily domain (indicated in light pink).

3.2. Generation and Verification of FoHis2 Deletion Mutants and Complementation

FoHis2 deletion mutants were obtained in the Foc4 background WT by replacing the *FoHis2* coding region with the hygromycin B phosphotransferase *hph* cassette via homologous recombination. PCR screening confirmed the presence of the *hph* cassette and the loss of the native *FoHis2* allele in three independent transformants, whereas the WT strain retained the intact *FoHis2* locus. Junction-specific PCR further verified correct recombination at both flanking regions (Figure 2a–e; Table S1). Southern blot analysis revealed hybridization patterns in the transformants consistent with the predicted replacement of *FoHis2* by a single *hph* cassette, while the WT strain showed the expected band for the intact locus (Figure 2a,h,i). These results demonstrate that the *FoHis2* locus was precisely disrupted by homologous recombination. Because the three $\Delta FoHis2$ mutants— $\Delta FoHis2$ -1, $\Delta FoHis2$ -2, and $\Delta FoHis2$ -3—exhibited indistinguishable phenotypes, $\Delta FoHis2$ -1 was selected for subsequent analyses.

For genetic complementation, the WT *FoHis2* gene under its native regulatory elements was introduced into the $\Delta FoHis2$ mutant. PCR analysis confirmed the presence of the native *FoHis2* locus in both the WT strain and in the complemented strain $\Delta FoHis2$ -C, while the deletion mutant remained negative for the native allele (Figure 2a,f,g). Amplification of the G418 resistance cassette was detected only in $\Delta FoHis2$ -C but not in the WT or $\Delta FoHis2$ strains, confirming successful integration of the complementation construct. Thus, both a stable *FoHis2* deletion mutant and a corresponding complemented strain were generated for downstream phenotypic and pathogenicity assays.

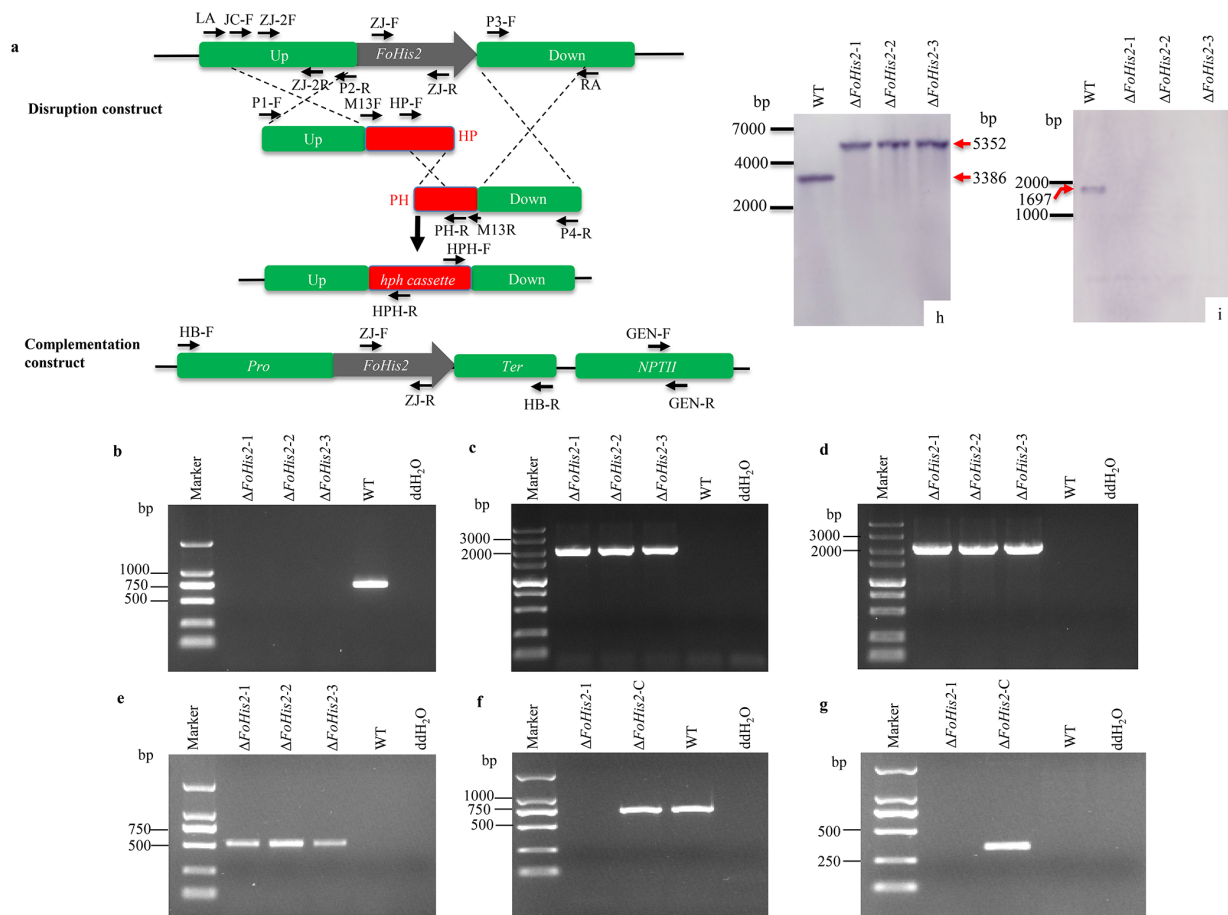


Figure 2. Generation and validation of *FoHis2* deletion and complementation mutants in *Foc*. (a) Gene replacement and complementation strategy, indicating primer locations for deletion, PCR, and Southern blot analysis; (b–e) PCR confirmation of three $\Delta FoHis2$ mutants: (b) Amplification of a 740-bp *FoHis2* fragment with primers ZJ-F/ZJ-R; (c) Junction PCR with primers JC-F/HPH-R (expected ~2100 bp); (d) Junction PCR with primers HPH-F/RA (expected ~2200 bp); (e) Amplification of a 518-bp *hph* fragment with primers HP-F/PH-R; (f,g) PCR confirmation of the complemented strain $\Delta FoHis2$ -C: (f) Restoration of the *FoHis2* fragment (740 bp) detected with primers ZJ-F/ZJ-R; (g) Amplification of a 350-bp *NPTII* (G418 resistance) fragment with primers GEN-F/GEN-R; (h,i) Southern blot verification of the $\Delta FoHis2$ mutants: (h) A DIG-labeled probe targeting the 5' upstream homologous arm of *FoHis2* (ZJ-2F/ZJ-2R) was hybridized to XbaI/KpnI-digested genomic DNA; (i) A probe amplified with ZJ-F/ZJ-R was hybridized to BamHI/EcoRI-digested genomic DNA. Red arrows indicate the fragment sizes detected in the indicated strains. WT: wild-type.

3.3. *FoHis2* Is Required for Histidine Prototrophy and Vegetative Growth

To assess the role of *FoHis2* in fungal growth, the WT, $\Delta FoHis2$ mutant, and complemented strain $\Delta FoHis2$ -C were cultured on PDA plates. After 3 days at 28 °C, the $\Delta FoHis2$ mutant exhibited markedly slower colony expansion and formed more compact colonies than the WT and $\Delta FoHis2$ -C strains did (Figure 3a,b). Microscopic observation revealed markedly reduced hyphal branching in the $\Delta FoHis2$ mutant compared with that in the WT and $\Delta FoHis2$ -C strains (Figure 3c), indicating that *FoHis2* contributes to normal hyphal morphogenesis. Similarly, when cultured in PDB, the $\Delta FoHis2$ mutant produced significantly less mycelial biomass than the WT and $\Delta FoHis2$ -C strains did (Figure 3d). In contrast, no significant difference in conidiation was observed among the three strains (Figure 3e), suggesting that *FoHis2* is dispensable for asexual sporulation under these conditions.

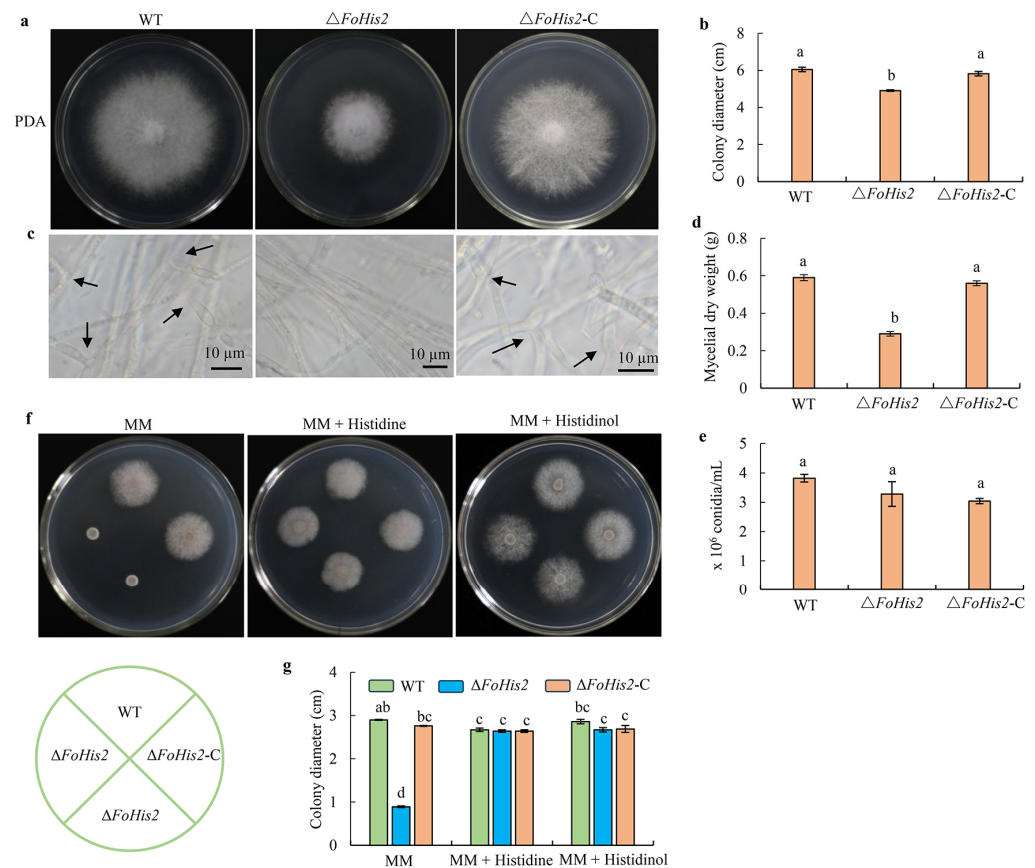


Figure 3. Impact of *FoHis2* deletion on growth and conidiation in *Foc*. (a) Colony diameter and microconidial dimensions of the indicated strains cultured on PDA media at 28 °C for 3 days; (b) Quantitative analysis showed smaller colony diameters in the $\Delta FoHis2$ mutant compared with WT and $\Delta FoHis2-C$; (c) Microscopic observation showing reduced hyphal branching in the $\Delta FoHis2$ mutant compared with WT and $\Delta FoHis2-C$. Black short arrows denote hyphal branching; (d) Quantification of mycelial dry weight in MM cultures showing significantly reduced biomass in the $\Delta FoHis2$ mutant relative to WT and $\Delta FoHis2-C$; (e) Quantification of conidial concentration in liquid cultures; (f) Colony morphology on MM (no histidine added), MM supplemented with histidine, and MM supplemented with histidinol after 3 days of incubation at 28 °C; (g) Statistical analysis of colony diameter under the indicated conditions. Different letters above columns denote statistically significant differences ($p \leq 0.05$), and error bars show the standard error based on three replicates. WT: wild-type.

In striking contrast to its growth on PDA, the $\Delta FoHis2$ mutant exhibited a pronounced growth defect on MM (no histidine added), producing much smaller colonies than those produced by the WT and $\Delta FoHis2-C$ strains (Figure 3f,g). Supplementation of MM with exogenous histidine fully restored the growth defects of the $\Delta FoHis2$ mutant to WT levels, while supplementation with histidinol rescued colony size but not the development of aerial mycelium (Figure 3f,g). These results indicate that $\Delta FoHis2$ is a histidine auxotrophy and support the crucial role of *FoHis2* in histidine biosynthesis.

3.4. *FoHis2* Is Essential for Antioxidant Defense but Not for Maintaining Cell Wall Integrity

Fungi are constantly exposed to diverse environmental stresses during growth and infection, including oxidative stress, and perturbations in cell wall structure. To investigate the role of *FoHis2* in stress adaptation, the sensitivities of the WT and $\Delta FoHis2$ mutant strains to H_2O_2 and cell wall inhibitors CR and CFW were systematically evaluated. The $\Delta FoHis2$ mutant failed to grow on MM plates supplemented with 4 mM H_2O_2 , whereas the WT and complemented strain $\Delta FoHis2-C$ exhibited normal growth. Interestingly,

supplementation of MM with histidine restored the growth of the $\Delta FoHis2$ mutant to the WT level under the same oxidative stress conditions (Figure 4a). Consistent with this phenotype, transcriptomic analysis revealed that 19 genes associated with the peroxisome pathway were significantly downregulated in the $\Delta FoHis2$ mutant (Figure 4b). In contrast, no significant difference in growth was observed between the WT and mutant strains on MM plates containing CR or CFW (Figure S1), indicating that FoHis2 is essential for antioxidant defense but dispensable for maintaining cell wall integrity.

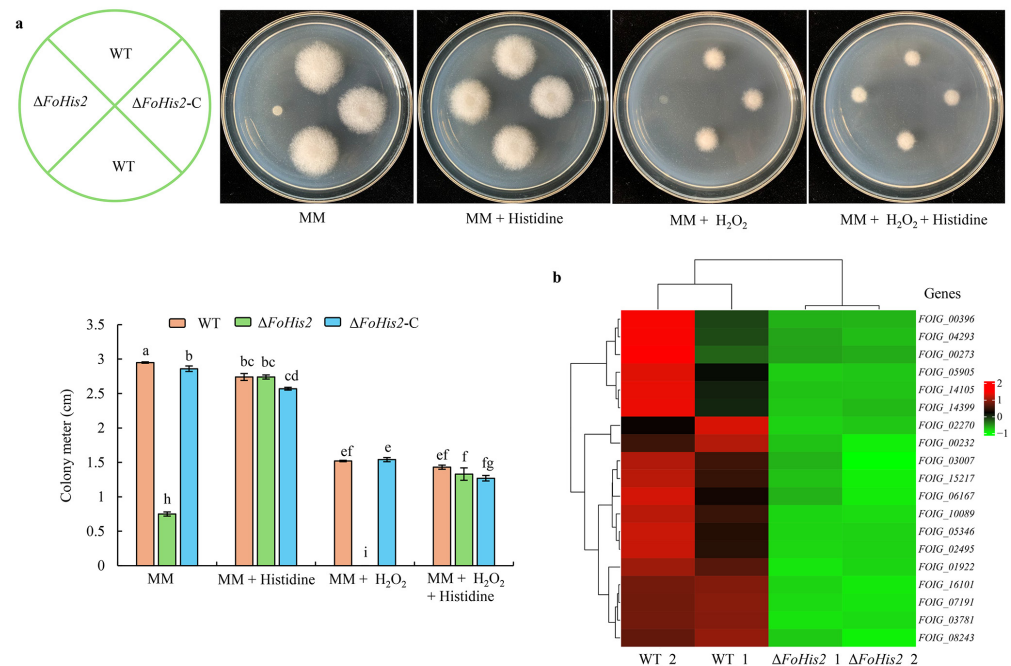


Figure 4. Analysis of oxidative stress responses in WT, $\Delta FoHis2$ mutant and complemented strain $\Delta FoHis2$ -C. **(a)** Growth phenotypes of the wild-type (WT), the $\Delta FoHis2$ mutant, and the complemented strain $\Delta FoHis2$ -C on MM (no histidine added), MM supplemented with histidine, MM supplemented with 5 mM H₂O₂, and MM containing both H₂O₂ and histidine. Plates were photographed after incubation at 28 °C for 3 days. The schematic on the left indicates the placement of each strain on the plates. Bars labeled with different letters indicate statistically significant differences ($p \leq 0.05$). Error bars represent the standard error from three independent replicates; **(b)** Heatmap showing the expression patterns of differentially expressed genes (DEGs) associated with the peroxisome pathway in WT and $\Delta FoHis2$ strains. WT_1, WT_2 and $\Delta FoHis2$ _1, $\Delta FoHis2$ _2 represent two biological replicates each of the WT and $\Delta FoHis2$ mutant, respectively.

3.5. FoHis2 Is Not Essential for Mycotoxins Biosynthesis

Recent studies have demonstrated that FA and BEA, two well-characterized mycotoxins, are critical for the virulence of *Fusarium* species, including Foc. FA functions by suppressing host defense mechanisms and exacerbating disease symptoms in banana plants [8], while BEA exhibits significant cytotoxic and apoptotic effects, contributing to plant cell death and tissue necrosis [36].

To examine the potential regulatory role of FoHis2 in FA biosynthesis, we compared FA production by the WT strain and the $\Delta FoHis2$ mutant using HPLC analysis of the cultures grown in Czapek Dox medium. FA production was normalized to mycelial biomass (mg FA per g mycelial dry weight) to account for potential differences in growth. The $\Delta FoHis2$ mutant exhibited no significant change in FA production compared with that shown by the WT strain (Figure S2a,b). Furthermore, qRT-PCR analysis of key FA biosynthetic genes (*FUB1*–*FUB5*, *FUB10*) revealed no significant difference in expression between the $\Delta FoHis2$ mutant and the WT strain (Figure S2c).

Additionally, we analyzed the expression of three BEA biosynthetic gene homologs, previously identified in *F. oxysporum* f. sp. *lycopersici* [37], using qRT-PCR. The expression levels of all three genes also showed no significant differences between the $\Delta FoHis2$ mutant and the WT strain (Figure S2d). These results suggest that FoHis2 does not play a critical regulatory role in FA or BEA biosynthesis.

3.6. FoHis2 Is Essential for Full Virulence

To further investigate the role of FoHis2 in fungal infection ability, a cellophane penetration assay was performed. The WT and complemented strain $\Delta FoHis2$ -C were able to penetrate the cellophane membrane covering the culture medium and subsequently grow on the underlying agar. In contrast, the $\Delta FoHis2$ mutant was restricted to growing on the surface of the membrane and failed to penetrate the medium (Figure 5a). These results indicate that deletion of *FoHis2* severely impairs the ability of Foc4 to breach physical barriers, suggesting that FoHis2 is crucial for host invasion.

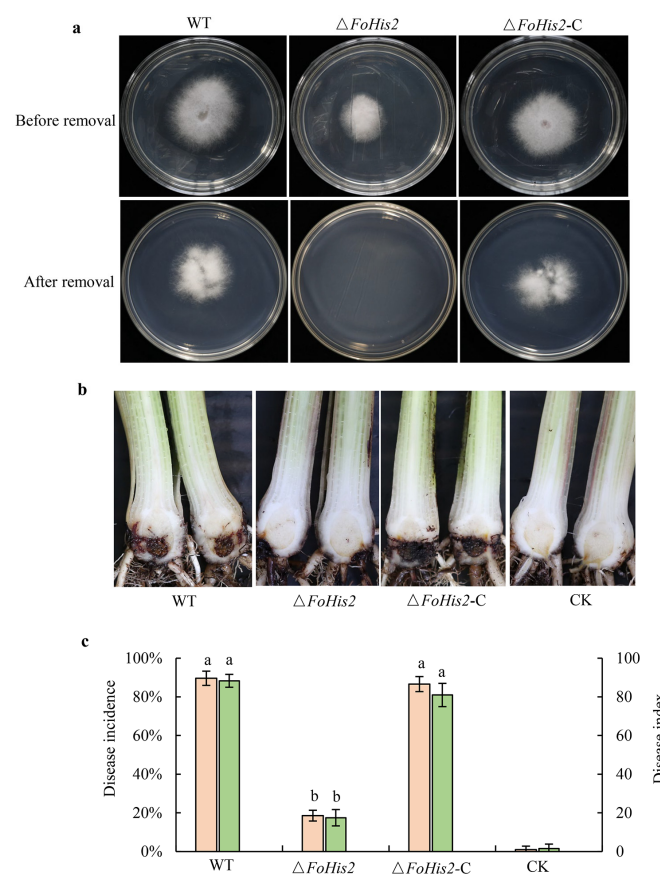


Figure 5. Reduced penetration ability and virulence of $\Delta FoHis2$ mutant. (a) Cellophane penetration assay of the wild-type (WT), $\Delta FoHis2$, and complemented strain $\Delta FoHis2$ -C; (b) Disease symptoms at 30 days post-inoculation (dpi). Banana plantlets at the fully expanded fourth-leaf stage were inoculated with WT, $\Delta FoHis2$, and complemented strain $\Delta FoHis2$ -C. Photos illustrate representative disease appearances; (c) Disease incidence and index measured at 30 dpi. Different letters above columns suggest statistically significant differences ($p \leq 0.05$). Error bars indicate the standard error from three replicates.

To assess whether FoHis2 influences virulence in plants, tissue-cultured Cavendish banana plantlets were inoculated with the WT and complemented strain $\Delta FoHis2$ -C or the $\Delta FoHis2$ mutant strain by root dipping for 30 min. After 30 days, the degree of browning at the base of the pseudostem was evaluated (Figure 5b). Plantlets inoculated with $\Delta FoHis2$ mutant exhibited significantly lower disease incidence and disease index compared with

those inoculated with the WT and complemented strains (Figure 5c). These findings demonstrate that FoHis2 is essential for full virulence of Foc and suggest that its loss compromises the pathogen's ability to infect and colonize the host.

3.7. Transcriptome Analysis

To explore how FoHis2 regulates fungal virulence through gene expression networks linked to virulence, we performed a transcriptome analysis comparing the WT and $\Delta FoHis2$ mutant. Among the 10,071 genes identified, 1077 were uniquely expressed in the WT strain, while 637 were specifically expressed in the $\Delta FoHis2$ mutant. Additionally, 8357 genes were found to be expressed in both strains (Figure 6a). A total of 3481 DEGs (1854 down-regulated genes and 1627 up-regulated) were detected between the WT and $\Delta FoHis2$ mutant (Figure 6b,c). The 20 most significantly up-regulated and 20 most significantly down-regulated genes are listed in Table S2. GO analysis revealed that DEGs were significantly enriched in several biological processes, including small-molecule, organic acid, oxoacid, and carboxylic acid metabolic processes. In terms of molecular function, the enriched categories included adenylyl nucleotide binding, adenylyl ribonucleotide binding, ATP binding, and transmembrane transporter activity (Figure 7a).

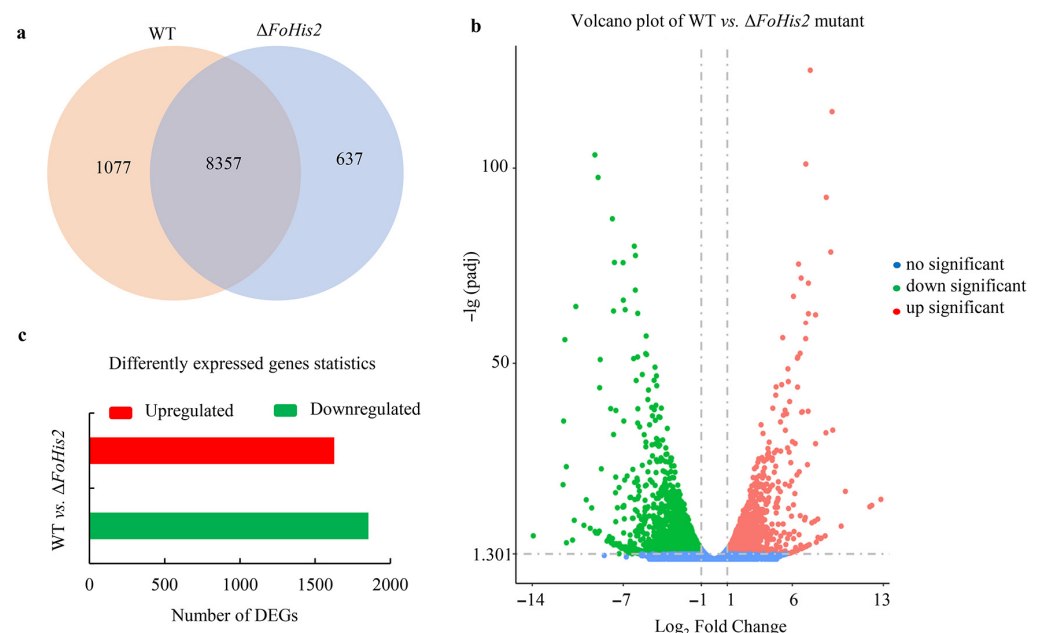


Figure 6. Transcriptomic analysis of wild-type (WT) and $\Delta FoHis2$ strains. (a) Venn diagram illustrating overlapping and non-overlapping genes between the two strains. (b) Volcano plot displaying the distribution of differential expression genes (DEGs). (c) Summary of up-regulated and down-regulated DEGs in WT versus $\Delta FoHis2$.

Furthermore, KEGG analysis showed that 461 DEGs were enriched in pathways. Among the 20 main significantly enriched pathways, most were associated with amino acid metabolism and related biosynthetic pathways, including alanine, aspartate and glutamate metabolism, arginine, and proline metabolism, cysteine and methionine metabolism, and valine, leucine, and isoleucine biosynthesis. In addition, the pathways “Biosynthesis of amino acids” and “Biosynthesis of secondary metabolites” exhibited strong enrichment, indicating that the deletion of *FoHis2* led to notable alterations in multiple metabolic processes. Moreover, pathways related to energy metabolism and redox regulation, such as peroxisome and glutathione metabolism, were also significantly enriched. Taken together, these results suggest that *FoHis2* deficiency may broadly impact the metabolic regulation of the fungus (Figure 7b). The related data are deposited in the National Genomics Data

Center (<https://ngdc.cncb.ac.cn/gsa/>, accessed on 16 December 2025) with accession number PRJCA053742.

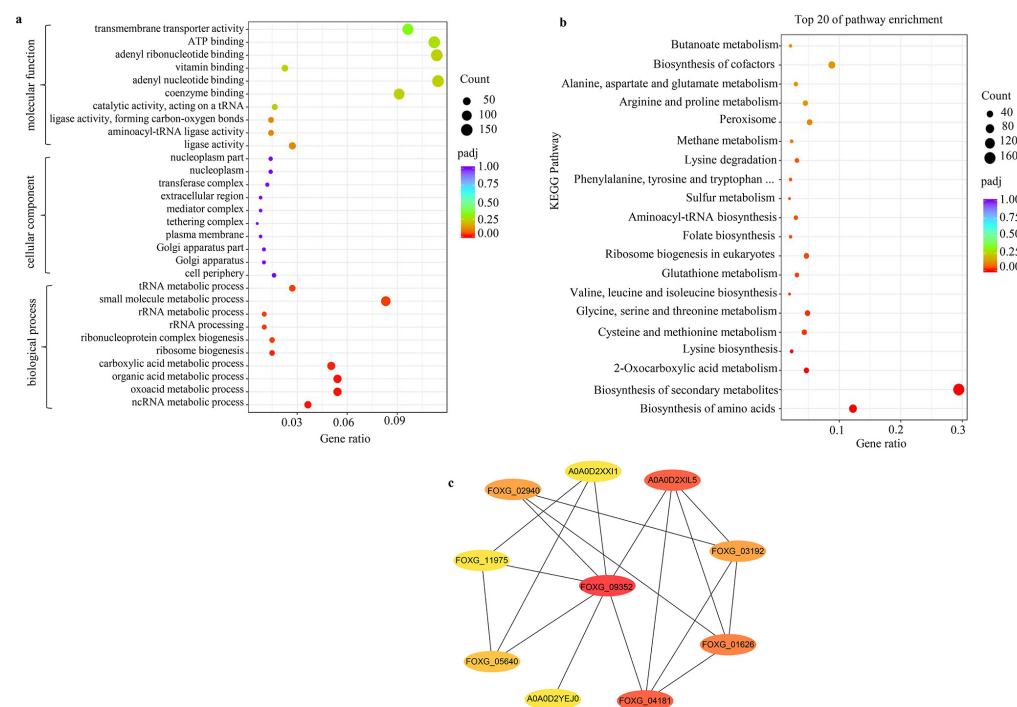


Figure 7. Enrichment analyses of differential expression genes (DEGs) and prediction of FoHis2-regulated hub genes. (a) GO enrichment, where the Rich factor represents the ratio of DEGs to the total number of genes assigned to each GO term; (b) KEGG pathway enrichment, where the Rich factor indicates the ratio of DEGs to the total genes within each pathway; (c) Subnetwork highlighting the top 10 hub genes centered on FOXG_09352. The network was inferred from *F. oxysporum* f. sp. *lycopersici* (Fol) orthologs due to the absence of Foc data in STRING. All ten Fol genes correspond to homologous Foc genes identified in our dataset. Node color intensity reflects degree centrality.

The PPI network of Foc was inferred based on its orthologs in *F. oxysporum* f. sp. *lycopersici* (Fol) using the STRING database (Figure S3), because Foc itself is not included in STRING. The top 10 hub proteins in the Fol-based PPI network (encoded by FOXG_09352, A0A0D2XIL5, FOXG_04181, FOXG_01626, FOXG_02940, FOXG_03192, FOXG_05640, A0A0D2XXI1, FOXG_11975, and A0A0D2YEJ0) correspond to the Foc homologs FOIG_05133, FOIG_14490, FOIG_06720, FOIG_02886, FOIG_01910, FOIG_02194, FOIG_13732, FOIG_12285, FOIG_12792 and FOIG_09629, respectively (Figure 7c; Table S3). These orthologous relationships were used to infer potential hub proteins in Foc.

Functional annotation revealed that eight of these ten FoHis2-responsive hub proteins are enzymes involved in nitrogen and amino acid metabolism, including anthranilate synthase component II (FOIG_05133), two nitrate reductases (FOIG_14490 and FOIG_06720), nitrite reductase (FOIG_02194), NADP-specific glutamate dehydrogenase (FOIG_02886), an aromatic amino acid aminotransferase (FOIG_13732) and two branched-chain amino acid aminotransferases (FOIG_12285 and FOIG_12792). FOIG_01910 encodes a hypothetical protein similar to nitrilase, whereas FOIG_09629 is annotated as a hypothetical protein (Table S3). These functional categories are highly consistent with the GO and KEGG enrichment results obtained from the global DEG set (Figure 7), particularly pathways related to amino acid metabolism and biosynthesis of secondary metabolites. To validate the reliability of the RNA-seq data, the expression levels of ten FoHis2-responsive hub genes were further examined by qRT-PCR. The results revealed expression trends consistent with those observed in the transcriptome analysis (Figure S4), supporting the robustness of the

RNA-seq dataset. Taken together, the transcriptomic and network analyses indicate that *FoHis2* deficiency causes extensive reprogramming of nitrogen and amino acid metabolism, secondary metabolite biosynthesis, and redox-related pathways. Such changes are in line with the impaired vegetative growth, reduced virulence, and increased sensitivity to H_2O_2 observed in the $\Delta FoHis2$ mutant, suggesting that *FoHis2* regulates fungal pathogenicity at least in part by controlling the core metabolic and stress-adaptation networks in *Foc*.

4. Discussion

This study identified the histidinol phosphatase *FoHis2* and, based on phenotypic analyses, demonstrated that it plays a crucial role in vegetative growth, stress responses, and virulence in *Foc*. Furthermore, our transcriptomic and network analyses indicated that *FoHis2* may promote *Foc* pathogenicity in banana plantlets by modulating the core primary metabolic pathways.

Phylogenetic analysis revealed that *FoHis2* is highly conserved among *Fusarium* species, and that the *FoHis2* protein is identical to the annotated *Foc4* homolog (XP_031066978.1) at the amino acid level. *FoHis2* shows high sequence similarity with the homologs obtained from *F. oxysporum* f. sp. *lycopersici* and *F. verticillioides*; however, it markedly diverges from the corresponding protein in *Saccharomyces cerevisiae*. These features are consistent with *FoHis2* functioning as a conserved metabolic component in *Fusarium*. Conserved domain analysis further showed that *FoHis2* and its homologs belong to the PHP_HisPPase_HisJ_like (PHP) superfamily, supporting their conserved role in histidine biosynthesis. Similar conservation patterns have been reported for other key metabolic enzymes in plant-pathogenic fungi. Such enzymes are considered promising targets for the development of broad-spectrum antifungal strategies [27,29].

Deletion of *FoHis2* severely impaired the vegetative growth of *Foc* and altered the hyphal morphology, while supplementation with exogenous histidine or histidinol restored these phenotypes. These findings confirm that *FoHis2* catalyzes the penultimate step in histidine biosynthesis in *Foc*, consistent with the functions of its homologs in *Bacillus subtilis* and *Pseudomonas aeruginosa* [30,32]. In addition, the $\Delta FoHis2$ mutant is hypersensitive to hydrogen peroxide, with growth completely inhibited under hydrogen peroxide stress. However, this defect was rescued by exogenous histidine, which indicates that histidine availability is essential for resistance to oxidative damage. Consistent with this perspective, our transcriptome analysis showed that 19 peroxisome-associated genes were significantly downregulated in the $\Delta FoHis2$ mutant, providing molecular evidence for its increased sensitivity to oxidative stress. Histidine and its derivatives, particularly histidine-containing dipeptides such as carnosine, have been shown to exhibit antioxidant activity in multiple biological systems [38,39]. These physicochemical properties likely contribute to the roles of *FoHis2* in stress tolerance and metal homeostasis. Iron homeostasis is a well-established determinant of fungal virulence, as pathogens must precisely regulate the acquisition, storage, and detoxification of metals to counteract host nutritional immunity and oxidative stress [40,41]. Histidine biosynthesis has been proposed as an antifungal target in fungi, including *Aspergillus fumigatus* [29]. Our data show that *FoHis2* is essential for histidine production and contributes to oxidative-stress resistance in *Foc*. This study did not assess copper, zinc, siderophore pathways, or iron handling; future work should evaluate these metal-homeostasis processes to clarify *FoHis2*-associated phenotypes and their translational potential for plant disease control.

Deletion of *FoHis2* caused multiple defects in virulence, including reduced penetration capacity and attenuated pathogenicity in banana plantlets. Given the pronounced growth defect, the attenuated virulence of $\Delta FoHis2$ may be largely attributable to reduced fitness. Notably, the deletion did not affect the production of FA or BEA, indicating that

the reduced virulence is not due to impaired toxin biosynthesis. Instead, our transcriptomic and network analyses suggest that FoHis2 may influence pathogenicity, at least in part, through broader metabolic reprogramming. In the FoHis2-responsive PPI network, eight of the top ten hub proteins were enzymes involved in nitrogen and amino acid metabolism, including nitrate reductase, nitrite reductase, glutamate dehydrogenase, and several transaminases. These enzymes occupy key nodes at the interface of nitrogen assimilation and amino acid biosynthesis. Their prominence within the FoHis2-dependent network points to tight integration between histidine metabolism and wider nitrogen metabolic pathways. Comparable network-based approaches using STRING-derived PPI maps and CytoHubba centrality analyses have successfully identified virulence-associated hub factors in plant pathogenic fungi, including *F. oxysporum* [42,43]. Such coordinated metabolic regulation likely enhances the efficiency of nutrient utilization by the pathogen during host colonization. Importantly, the major defects observed in Δ FoHis2 were restored by genetic complementation and exogenous histidine, supporting that these phenotypes are attributable to FoHis2-dependent histidine biosynthesis.

The extensive transcriptomic alterations observed in the Δ FoHis2 mutant, encompassing 3481 DEGs, highlight the profound global impact of histidine metabolism on fungal physiology. These genes were highly enriched in multiple amino acid metabolic pathways, particularly those involving alanine, aspartate, glutamate, arginine, proline, cysteine, methionine, and branched-chain amino acids, as well as secondary metabolic pathways. Such coordinated perturbations mirror the metabolic reprogramming documented during host infection by plant-pathogenic fungi. Transcriptomic studies on such perturbations have reported that stage-specific remodeling of primary and secondary metabolism is closely tied to virulence and plant adaptation [44,45].

Links between amino acid metabolism and fungal pathogenicity have been firmly established across diverse plant pathogens. For example, disruption of leucine biosynthesis genes in the rice blast fungus *M. oryzae* substantially impairs virulence in rice and barley [25,26]. Likewise, genes associated with arginine biosynthesis are essential for the pathogenicity of various fungal species [22,24]. Our results extend these observations by showing that FoHis2-mediated histidine metabolism acts as a central metabolic hub, orchestrating multiple pathways critical for virulence.

5. Conclusions

In summary, this study identifies FoHis2 as an important virulence-associated factor in Foc, positioned at the interface of primary metabolism, stress responses, and pathogenicity. Loss of FoHis2 causes multiple phenotypic defects, underscoring the tight interconnection among metabolic pathways and their importance for disease development. Future work should clarify how FoHis2-mediated histidine metabolism regulates the identified hub proteins and downstream virulence factors. Such knowledge will provide a solid scientific basis for developing sustainable strategies to manage banana Fusarium wilt.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jof12020121/s1>, Figure S1: Comparison of cell wall stress responses in wild type (WT), Δ FoHis2 mutant and complemented strain Δ FoHis2-C; Figure S2: Influence of FoHis2 deletion on mycotoxin biosynthesis in Foc; Figure S3: Full protein–protein interaction (PPI) network of hub protein candidates in orthology-based Foc using *F. oxysporum* f. sp. *lycopersici* (Fol); Figure S4: Validation of transcriptomic data by qRT-PCR; Table S1: Primers used in this study; Table S2: Putative functions of the top 20 upregulated and 20 downregulated differential expression genes (DEGs) in Δ FoHis2 mutant; Table S3: Orthologous relationships between *Fusarium oxysporum* f. sp. *lycopersici* (Fol) and *F. oxysporum* f. sp. *cubense* (Foc) hub proteins with their putative functional annotations.

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