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Article

Keywords: *Aspergillus fumigatus*, af-MFS95, oxidative stress, azole drugs

Posted Date: August 6th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10234383/v1>

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Additional Declarations: No competing interests reported.

Functional study of the MFS transporter gene *af-MFS95* in *Aspergillus fumigatus*

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ABSTRACT

To investigate the role of the *Aspergillus fumigatus* MFS transporter gene *af-MFS95* in fungal growth, stress response, and antifungal susceptibility. A knockout mutant ($\Delta af-MFS95$) and complemented strain ($\Delta af-MFS95-recon$) were constructed via homologous recombination. Azole susceptibility was assessed using M38-A3 broth microdilution and E-test. Strains were cultured under oxidative, osmotic, metal ion, and cell wall stress; colony diameter variations(%) were recorded. Intracellular reactive oxygen species (ROS) were quantified by flow cytometry. Transcriptional profiles of growth- and development-related genes (*tef1*, *mbsA*, *flbA*, *flbD*, *brlA*, *nsdD*) and antioxidant genes (*sodA*, *sodB*, *sodC*, *sodD*) were analyzed by RT-qPCR. *Af-MFS95* deletion did not alter azole susceptibility. However, the mutant exhibited significantly impaired growth under oxidative stress (H_2O_2 , menadione) but enhanced growth under osmotic stress (NaCl, D-sorbitol). No significant differences were observed in response to metal ions, cell wall-disrupting agents, and intracellular ROS levels. RT-qPCR revealed significant downregulation of growth- and development-related genes and antioxidant enzyme genes in $\Delta af-MFS95$. *Af-MFS95* is critical for *A. fumigatus* oxidative stress resistance and osmotic adaptation but dispensable for azole resistance. It likely regulates cellular proliferation and antioxidant defense gene expression, highlighting potential targets for disrupting fungal stress adaptation pathways.

KEYWORDS: *Aspergillus fumigatus*; *af-MFS95*; oxidative stress; azole drugs

1 Introduction

A. fumigatus, a ubiquitous opportunistic pathogenic fungus, causes severe infections, particularly in immunocompromised individuals [7][36][55], such as organ transplant recipients, AIDS patients, and cancer patients undergoing chemotherapy. It often leads to invasive aspergillosis with high mortality, posing a significant clinical challenge [26]. At present, azole antifungals that inhibit ergosterol biosynthesis serve as the primary treatment option; however, their widespread use has contributed to the growing problem of drug resistance [6]. A previous study has demonstrated that drug resistance

in *A. fumigatus* is associated with mutations in the Cyp51A protein, overexpression of target enzymes, dysregulation of ATP-binding cassette (ABC) efflux pumps and major facilitator superfamily (MFS) transporters, as well as the potential contribution of biofilm formation ^[14].

MFS transporters, containing 12-14 transmembrane domains, use proton motive force for drug efflux in nearly all cases ^{[23][38]}. In the *A. fumigatus* genome, 275 MFS transporters were found ^[34], but only a few were identified as drug transporters, also known as multidrug-resistant (MDR) or pleiotropic drug-resistant (PDR) proteins ^{[37][42]}. Many studies suggest that fungal MFS transporters are not limited to drug efflux linked with antifungal resistance, but also involved in essential physiological processes and significantly contribute to fungal development and virulence ^{[21][35]}.

Fungi utilize intricate molecular signaling networks to detect and adapt to varying environmental conditions, such as fluctuations in temperature, pH, nutrient availability, and oxidative or osmotic stress ^[9]. In yeast models, heightened osmotic stress triggers the hyperosmotic glycerol (HOG) pathway, which initiates a multi-faceted stress response that includes transient cell cycle arrest, transcriptional reprogramming, and modifications in protein synthesis to mitigate the risk of growth inhibition or cell death ^[50]. Specifically, salt stress causes ion imbalance and the accumulation of ROS in various fungi, resulting in oxidative damage. In response, fungi regulate ion balance, restore osmotic homeostasis, modulate phytohormone signaling, and remodel the cytoskeleton and cell wall by accumulating Ca²⁺ and activating ROS-mediated signaling cascades ^{[49][52]}. This intricate regulatory network underpins the ability of fungi to synchronize the cell cycle, metabolic adaptation, and structural integrity to endure environmental stressors.

Given the critical importance of environmental stress tolerance for the survival of pathogenic fungi, this study developed a series of knockout strains by systematically targeting MFS transporter genes within the *A. fumigatus* genome. A multidimensional assessment of their growth phenotypes and oxidative stress tolerance was conducted. Screening results revealed that the *af-MFS95* knockout strain exhibited significant

growth restriction and heightened sensitivity to oxidative and osmotic stress. This observation indicates that *af-MFS95* plays an essential and irreplaceable role in maintaining cellular physiological homeostasis and mediating responses to environmental stress. Consequently, this study concentrated on the functional analysis of *af-MFS95*. We successfully generated an *af-MFS95* knockout strain ($\Delta af-MFS95$) and its corresponding complemented strain ($\Delta af-MFS95-recon$) to elucidate the role of this transporter in regulating responses to oxidative damage and osmotic fluctuations. Additionally, we aimed to investigate the potential mechanism by which *af-MFS95* indirectly influences drug tolerance in *A. fumigatus* through the regulation of gene networks associated with growth and development.

2 Materials and Methods

2.1 Strains, plasmids, and culture conditions

The *A. fumigatus* strain AF293, used in the genome project, served as the template for amplifying target DNA fragments. The host strain A1160 ($\Delta KU80$, *pyrG*⁻), a pyrimidine auxotrophic strain obtained from the Fungal Genetics Stock Center (<http://www.fgsc.net/asperg.html>), was employed for fungal transformation [5][11]. The wild strain WT ($\Delta KU80$, *pyrG*⁺) was constructed in our laboratory by randomly introducing the *pyrG* into A1160 and was used as the experimental control. The *pyrG* was obtained from the plasmid pBARGPE1-PyrG-TagRFP and served as a screening marker during the construction of the knockout strain. The hygromycin B-resistant gene (*hph*) and fluorescent marker gene (*egfp*) were obtained from plasmid pCT74, which were used as the screening markers during the construction of the complemented strain. WT was grown on Sabouraud dextrose agar (SDA; HuiBo Bio, Qingdao, China) at 37°C for 3 days. The A1160 strain was cultured under the same temperature for 3 days in Czapek Dox Medium supplemented with uracil (CZA + Uracil; HuiBo Bio). The plasmid pBARGPE1-PyrG-TagRFP was propagated in LB broth (HuiBo Bio) containing 100 mg/L ampicillin at 37°C for 16 h. The plasmid pCT74 was propagated in LB broth containing hygromycin B at 37°C for 16 h [31].

2.2 Construction of the *af-MFS95* knockout mutant strain

The PCR primers used in the experiment are listed in Table 1. Gene knockout was performed using the three-fragment PCR fusion system referred from Zhao et al. [51]. First, genomic DNA from AF293 was extracted with the MolPure® Fungal DNA Kit (18812ES, Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China), and the upstream and downstream flanking regions of *af-MFS95* were amplified with primers P1/P2 and P3/P4, respectively. Then, the *pyrG* fragment was amplified from plasmid pBARGPE1-PyrG-TagRFP, extracted with the MolPure® Plasmid Mini Kit (19001ES) by using primers pyrG-n-F and pyrG-n-R. After verification by 1% agarose gel electrophoresis, all PCR products were purified by using the MolPure® PCR Purification Kit (19106ES) and fused via PCR with primers P5/P6 to generate the knockout cassette (~ 1.7 kb) (Figure 1a).

The *af-MFS95* knockout cassette was integrated into A1160 via protoplast-mediated transformation [27]. As *pyrG* was used as the selection marker, transformants were selected on CZA without uracil; correct integration was verified by PCR (primers DIAO-F, DIAO-R and P4) followed by sequencing (Sangon Biotech, Shanghai, China), and the resulting strain was designated $\Delta af-MFS95$.

2.3 Construction and validation of the complemented strain

Based on the method reported by Gong et al. [12], the *af-MFS95* fragment was amplified from AF293 genomic DNA using primers AIM-re-R and AIM-re-F and verified by 1% agarose gel electrophoresis. The plasmid gene fragment containing the *hph* and *egfp* was obtained from pCT74 by enzymatic digestion (restriction endonucleases KpnI and NaeI) and purification (Figure 1b). The *af-MFS95* fragment was genetically recombined in vitro with the double-enzymatic plasmid gene fragment (Hieff Clone® Plus One Step Cloning Kit, 10911ES), then transformed using DH5 α Chemically Competent Cells (11802ES). The transformed cells were then evenly plated onto LB+hygromycin B screening medium. Recombinant plasmid DNA was isolated and validated by PCR using AIM-yz-R and AIM-yz-F primers, then was introduced into

141 protoplasts derived from the knockout strain to complete transformation. The
142 transformants were selected on SDA supplemented with hygromycin B. Genomic DNA
143 was extracted from the transformants and verified by PCR (primers HPH-F/HPH-
144 R) ,then sent to Sangon Biotech (Shanghai) Co., Ltd., for sequencing. The presence of
145 the *hph* gene was considered to indicate a successful gen
146 eration of the Δaf -*MFS95-recon* strain. Primer sequences are listed in Table 1.

147 **Table 1 Primers sequences used in this study.**

Name	Sequence (5'→3')	Function
P1	AGACTTCCGGCATGCACTAAAC	Amplify the upstream
P2	TAGTTCTGTTACCGAGCCGGATCTATTCTTCTATTATTCTATC A	
P3	GCTCTGAACGATATGCTCCAACCTTTGTTTTTTTTTTTATTTC	
P4	ATTGC	Amplify the downstream
P5	TGAGTGGTATCCGTGTCTTGT	Fusion of upstream, downstream, and <i>PyrG</i>
P6	TGACCTTCATTCTCAGCCGTCG	
pyrG-n-F	CCGGCTCGGTAACAGAACTACCGCAGACAATGCTCTCTATC	
pyrG-n-R	GTTGGAGCATATCGTTCAGAGCAATACCGTTACACATTCCA	Amplify the <i>PyrG</i>
DIAO-F	CCAACCATCCCAACCCCTTA	Verify whether the filter marker is inserted into the knockout strain
DIAO-R	TCCGACAGATTCACCCCAGT	
pyrG-F	CTCGGTTGAGTACGCGCGGAAGTA	
pyrG-R	AGATGAGGAAGTTGTGCTTTGTC	Verify the <i>PyrG</i>
AIM-re-R	CTCGAGGGGGGGCCCGAGAGGTGGATAGATAGCCTTTGTAG	Amplify sequence of <i>af</i> -
AIM-re-F	TCTCGCCACGTTGCCCCATGCGTGGACGGAGTC	<i>MFS95</i> (for complementation)
HPH-F1	AGCTCGTCCATGCCGTGAG	Amplify sequence of <i>hph</i> (for verification)
HPH-R1	CAGTGCTTCAGCCGCTACCC	
HPH-F2	ATCGTTATGTTATCGGCACTTTG	
HPH-R2	TGTTGGCGACCTCGTATTGG	Amplify sequence of <i>af-MFS95</i> (for verification)
AIM-yz-F	TCAAACCTTGCTGCTCTTCCAC	
AIM-yz-R	TCATTGTCGCTCTGCTGTCC	

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149 **2.4 Antifungal sensitivity assay**

150 **2.4.1 Microdilution method**

The experimental procedure followed the guidelines outlined in the Clinical and Laboratory Standards Institute (CLSI) protocol M38-A3 for testing the antifungal susceptibility of filamentous fungi ^[45]. *Candida parapsilosis* ATCC 22019 and *Aspergillus flavus* ATCC 204304 served as quality control strains. Spore suspensions of WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ strains were prepared at a concentration of 2×10^4 CFU/mL. The antifungal agents tested included itraconazole (ITR), voriconazole (VOR), and posaconazole (POS), obtained from Selleck Chemicals (USA), each prepared in eight serial dilutions, with maximum concentrations of 8 µg/mL for ITR and VOR and 4 µg/mL for POS. After incubation at 35 °C for 48 h, fungal growth on 96-well plates was observed, and minimum inhibitory concentration (MIC) values were determined. The experiment was performed in triplicate on separate days to reduce experimental error.

2.4.2 E-Test method

Spore suspensions of the mature WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ strains were prepared at a concentration of 1×10^6 CFU/mL. The assay was performed according to the previous procedure ^[20]. Antifungal susceptibility test strips for ITR (Liofilchem, ICZ 92148), VOR (Merck, VCZ 532800), and POS (Liofilchem, PCZ 92152) were used. The inoculated and sealed plates were incubated at 35 °C for 48 h. The experiment was performed in triplicate.

2.5 Phenotypic Assays

2.5.1 Oxidative stress assay

The oxidative stress assay was conducted according to the established protocol ^[30]. Different oxidants (H₂O₂, menadione) were added to the SDA medium at varying concentrations (H₂O₂: 0, 1, 5, 10 mmol/L; menadione: 0, 5, 10, 30 mmol/L). Spore suspensions of the mature WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ strains were prepared at a final concentration of 1×10^6 CFU/mL. The experiment was performed in triplicate. The colony diameter changes (%) were determined using the formula:

Colony diameter change (%) = $[(D_{\text{control}} - D_{\text{treatment}}) / D_{\text{control}}] \times 100$.

2.5.2 Osmotic pressure assay

Osmotic stress assay was performed following the previous protocol^[31]. Briefly, NaCl and D-sorbitol were used at final concentrations of 0.5 mmol/L and 0.8 mmol/L, respectively. Spore suspensions of the mature WT, *Δaf-MFS95*, and *Δaf-MFS95-recon* strains were prepared at a density of 1×10^6 CFU/mL. The experiment was performed in triplicate.

2.5.3 Metal ion interference assay

The metal ion interference test was performed according to the prescribed procedure^[15]. The SDA medium was supplemented with various metal-ion disruptors, including 1.0 mmol/L $\text{Fe}_2(\text{SO}_4)_3$ and 0.5 mmol/L ZnSO_4 . Spore suspensions of the mature WT, *Δaf-MFS95*, and *Δaf-MFS95-recon* strains were prepared at a concentration of 1×10^6 CFU/mL. The experiment was performed in triplicate.

2.5.4 Cell wall disruption assay

The cell wall disruption assay was performed according to the established protocol^[43]. The SDA medium was supplemented with the cell-wall disruptor Congo red (CR). Spore suspensions of the mature WT, *Δaf-MFS95*, and *Δaf-MFS95-recon* strains were prepared at a concentration of 1×10^6 CFU/mL. The experiment was performed in triplicate.

2.6 2,7-dichlorofluorescein diacetate (DCFH-DA) staining

Determination of intracellular ROS levels was performed via 2,7-dichlorofluorescein diacetate (DCFH-DA) staining^{[4][10]}. DCFH-DA (Yeesen Biotechnology, Shanghai, China) was added to SDA broth at a final concentration of 0.1% (v/v). Flow cytometry data were generated using a BD FACSCanto II flow cytometer and analyzed with FlowJo v10 software. The excitation wavelength was 488 nm, and the emission wavelength was 525 nm. The experiment was performed in triplicate.

2.7 Reverse-transcriptase quantitative fluorescence PCR (RT-qPCR)

WT and Δaf -MFS95 strains were grown on solid SDA medium for 2 days. Total RNA was then extracted using TRIzol reagent (TRIeasy™ LS Total RNA Extraction Reagent, Yeasen) and subsequently reverse-transcribed into cDNA using the Hifair® II 1st Strand cDNA Synthesis Kit (Yeasten). RT-qPCR was performed with primers (Table 2) targeting *A. fumigatus* growth- and development-related genes (*tefl*, *mbsA*, *flbA*, *flbD*, *brlA*, *nsdD*) and anti-oxidant enzyme genes (*sodA*, *sodB*, *sodC*, *sodD*) to measure expression levels. Relative gene expression levels ($2^{-\Delta\Delta CT}$) were calculated using *tubA* as a control [29][47]. To reduce experimental error, the experiment was performed in triplicate, each including three mutual control groups. Data were analyzed using multiple t-tests to calculate *P* values, evaluating differences in relative gene expression between WT and Δaf -MFS95.

216 **Table 2 Real-time PCR primers table.**

Name	Sequence (5'→3')
<i>tubA-q-f</i>	CGGTGATCAGTTCAGTGGTATGTTCC
<i>tubA-q-r</i>	CTCCTCGCCGTACTCCTCCTCAC
<i>RT-tef1-F</i>	CCATGTGTGTCGAGTCCTTC
<i>RT-tef1-R</i>	GAACGTACAGCAACAGTCTGG
<i>RT-sodA-F</i>	AACTTCGAGACCGATGCTG
<i>RT-sodA-R</i>	TTCTTGGACTCCTCGTTGC
<i>RT-sodB-F</i>	CTTACCACAAGAACCACCAC
<i>RT-sodB-R</i>	GGCGATCTGAGACGCAATG
<i>RT-sodC-F</i>	GTCTCAAAGCAGCCATCG
<i>RT-sodC-R</i>	CGGTCATAACCAACCCCAT
<i>RT-sodD-F</i>	CGGTTTTGTATGGCTGGCTAA
<i>RT-sodD-R</i>	AAGAGGGGCATCGGGACTGT
<i>RT-mbsA-F</i>	GGGCTGGCACCAGGAAAATA
<i>RT-mbsA-R</i>	GCCCATCACCTTAGCCTTCA
<i>brlAfl</i>	TCATCTTCAGCATCGTCGGG
<i>brlAr1</i>	CAGCCATTCCGCTGCATTAC
<i>nsdDfl</i>	CCCATCCTCCACATGCTCTC
<i>nsdDr1</i>	ATAGATGCGAGGCTTGACCG
<i>flbAfl</i>	TTGTCGTCTTCCAGCCATCC
<i>flbAr1</i>	ACGGAATAGCAGTCGCAGAG
<i>flbDfl</i>	AGAACCTGAAGCCCTCGTTG
<i>flbDr1</i>	GGTTCATGCTGCCATTCCAC
<i>RT-af-MFS95-F</i>	ATGGGCGGTCTGTTGTTT
<i>RT-af-MFS95-R</i>	TACTTGCCGTTGCTGTACTC

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218 **2.8 Data processing software**

Graphs were generated using GraphPad Prism 9, and statistical analyses were conducted with SPSS 19.0. In each assay, at least three biological replicates were done to measure each parameter in each condition, avoiding biased results. Error bars included in all the graphs represent standard deviation. ANOVA or t-test was used depending on if we did multiple comparisons or compared punctual data, respectively.

3 Results and analyses

3.1 Bioinformatics analysis of af-MFS95

The *af-MFS95* gene is situated on chromosome 7, spanning 1,724 base pairs, and encodes a putative MFS monosaccharide transporter. The gene comprises only two exons (Figure 2).

3.2 Identification of the transduction candidate

PCR was performed to validate the transduction candidate. Genomic DNA was extracted, and successful amplification of the *pyrG* gene was obtained. However, no band corresponding to the target gene was detected, confirming the absence of *af-MFS95* in the transduction candidate (Figure 3).

3.3 Drug-Susceptibility Analysis

After 48 h of cultivation, the colony patterns of WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ in the 96-well plate were observed. The results showed that there were no significant changes in the sensitivity of $\Delta af-MFS95$ and WT to VOR, ITR, and POS (Table 3). Compared with WT, $\Delta af-MFS95$ showed relative sensitivity to POS. The MIC value of WT was 0.5 $\mu\text{g/mL}$, while that of $\Delta af-MFS95$ was 0.25 $\mu\text{g/mL}$. However, the MIC values for ITR and VOR were the same for both strains. Following 48 h of incubation, the MIC values were determined by measuring the points where the drug-susceptibility strips met the inhibition zones (Figure 4). The MIC of POS was 0.094 $\mu\text{g/mL}$ for the WT strain and 0.047 $\mu\text{g/mL}$ for $\Delta af-MFS95$. For ITR, the MIC was 0.5 $\mu\text{g/mL}$ in WT and 0.25 $\mu\text{g/mL}$ in $\Delta af-MFS95$. For VOR, the MIC values were 0.047

μg/mL for WT and 0.064 μg/mL for *Δaf-MFS95*. The results revealed no significant differences in MICs for ITR, VOR, and POS between WT and *Δaf-MFS95*. This showed that the knockout of the *af-MFS95* gene did not change the strain's sensitivity to triazole drugs.

Table 3 Statistical data of drug sensitivity test.

Strain	MIC (μg/mL)					
	Via broth microdilution			Via E-test		
	POS	ITR	VOR	POS	ITR	VOR
WT	0.5	2	0.25	0.094	0.5	0.047
<i>Δaf-MFS95</i>	0.25	2	0.25	0.047	0.25	0.064
<i>Δaf-MFS95-recon</i>	0.25	0.125	0.03	0.5	0.5	0.125

MIC: Minimum inhibitory concentration

3.4 Phenotypic assays

3.4.1 Determination of oxidative stress

After 48 hours of incubation, the colony diameter changes (%) were measured (Figure 5b, d). The analysis showed that exposure to 5 mmol/L H₂O₂ and menadione at concentrations of 5, 10, and 30 mmol/L caused significant changes in the colony diameters of the WT and *Δaf-MFS95* strains (Figure 5a, c; $P < 0.05$). The difference between the two strains was more significant at menadione levels of 10 and 30 mmol/L (Figure 5c; $P < 0.0001$). These observations suggest that deleting *af-MFS95* reduces the ability of *A. fumigatus* to resist oxidative agents such as H₂O₂ and menadione. Following the reintroduction of the *af-MFS95* gene into the mutant strain, the strain's capacity to grow under 5 mmol/L H₂O₂ stress was partially restored, further confirming that the deletion of *af-MFS95* impairs cellular resistance to oxidative stress. However, under concentration gradients of 5, 10, and 30 mmol/L menadione, the trends in colony diameter changes for both *Δaf-MFS95-recon* and *Δaf-MFS95* were highly consistent,

and both exhibited significant differences from the WT strain. Quantitative analysis of *af-MFS95* transcription levels indicated that expression of this gene in the complemented strain was significantly lower than in the WT (Figure 5e). This under-expression may result from the random integration of the exogenous gene during the complemented process; non-specific insertion into the original genomic site, along with factors such as positional effects, variations in copy number, or partial integration, likely prevented *af-MFS95* from achieving wild-type expression levels. Consequently, these factors led to differences in the degree of phenotypic recovery under varying oxidative stress conditions.

3.4.2 Determination of osmotic pressure

Colony diameter variation (%) was measured after 48 h of culture (Figure 6b). When exposed to NaCl and D-sorbitol, a significant difference in colony diameter variation was observed between the WT and $\Delta af-MFS95$ strains (Figure 6a; $P < 0.05$). Under these osmotic conditions, *af-MFS95* transcript levels in WT and $\Delta af-MFS95-recon$ were analyzed by RT-qPCR. The results showed that *af-MFS95* expression in the $\Delta af-MFS95-recon$ strain was lower than in the WT strain (Figure 6c). These findings suggest that deleting *af-MFS95* increases the strain's ability to tolerate osmotic stress.

3.4.3 Metal ion tolerance test

After 48 h of incubation, the change in colony diameter (%) was recorded (Figure 7b). After the addition of $Fe_2(SO_4)_3$ and $ZnSO_4$, no significant differences were observed in colony diameter among $\Delta af-MFS95$, $\Delta af-MFS95-recon$, and WT strains (Figure 7; $P > 0.05$). These findings suggest that deletion of the *af-MFS95* gene does not alter the strain's tolerance to metal ions.

3.4.4 Cell wall disruption test

Colony diameter changes (%) were recorded after 48 h of incubation (Figure 8b). After treatment with the cell wall disruptor Congo red, no significant difference was observed between $\Delta af-MFS95$ and WT strains (Figure 8a; $P > 0.05$). Under Congo red exposure,

af-MFS95 expression in WT and $\Delta af-MFS95-recon$ was analyzed by RT-qPCR, which revealed significantly lower transcript levels in $\Delta af-MFS95-recon$ compared with WT (Figure 8c; $P < 0.0001$). These findings indicate that deletion of *af-MFS95* does not alter susceptibility to cell-wall stress.

3.5 Measurement of intracellular ROS levels

Flow cytometry analysis showed no significant difference in intracellular ROS levels between WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ (Figure 9; $P > 0.05$). Oxidative stress phenotypic assays clearly demonstrated that the deletion of *af-MFS95* significantly heightened the strain's sensitivity to H₂O₂ and menadione. This phenotypic dissociation — characterized by reduced oxidative stress tolerance without an increase in steady-state ROS levels — indicates the existence of complex compensatory antioxidant defense mechanisms within the cell. Specifically, potential alterations at the transcriptional level or the redistribution of enzyme activity may sustain steady-state ROS levels, yet these mechanisms prove inadequate in managing acute exogenous oxidative stress.

3.6 RT-qPCR analysis

RT-qPCR analysis showed that, compared to WT, the expression of growth-related genes *brlA* [48], *nsdD* [24][25], *mbsA* [53], *tefl* [19][32], *flbA*, *flbD* [13], and antioxidant enzyme genes *sodA*, *sodB*, *sodC*, *sodD* [22] in $\Delta af-MFS95$ were significantly down-regulated (Figure 10). *BrlA* showed the largest expression change among the growth-related genes, decreasing by 5.3-fold. *SodC* showed the most significant change for the antioxidant enzyme genes, reducing its expression by 14-fold.

4 Discussion

In this study, the $\Delta af-MFS95$ and $\Delta af-MFS95-recon$ strains were generated to investigate the function of *af-MFS95* in *A. fumigatus*, with emphasis on its roles in growth, environmental stress adaptation, and antifungal susceptibility. The results

indicated that deletion of *af-MFS95* did not affect sensitivity to triazole drugs, but impaired growth under oxidative stress (H₂O₂ and menadione) and increased tolerance to osmotic stress (NaCl, D-sorbitol). Furthermore, transcript levels of antioxidant enzyme genes (*sodA*, *sodB*, *sodC*, *sodD*) and growth-related genes (*tefl*, *mbsA*, *flbA*, *flbD*, *brlA*, *nsdD*) were significantly downregulated. These findings suggest that *af-MFS95* contributes to fungal environmental resilience by modulating antioxidant defense and developmental pathways.

A comprehensive analysis of the experimental results indicated that, while there was no statistically significant difference in intracellular ROS levels between the Δ *af-MFS95* and WT, the transcriptional levels of its antioxidant enzyme genes (*sodA*, *sodB*, *sodC*, *sodD*) were significantly diminished. Furthermore, Δ *af-MFS95* demonstrated increased sensitivity to oxidants. Superoxide dismutase (SOD), a crucial component of the fungal antioxidant system, mitigates ROS-induced toxicity by catalyzing the breakdown of ROS [8][44]. The antioxidant enzyme genes (*sodA*, *sodB*, *sodC*, *sodD*) of *A. fumigatus* encode four putative SOD subunits: SodA (Cu/Zn-sod), SodB (mitochondrial Mn-sod), SodC (cytoplasmic Mn-sod), and SodD (a putative sod with a C-terminal Mn-sod domain) [3][16][17][22][40]. Previous studies have demonstrated that *sodD* is essential for *A. fumigatus* survival, and the deletion of *sodA* or *sodB* increases oxidative stress sensitivity [22]. A similar trend was observed in this study, where deletion of the *af-MFS95* gene led to a significant reduction in *sodA/B/C/D* expression; ultimately, this increased sensitivity to oxidative agents. ROS plays a dual role in fungal biology: at low concentrations, it acts as a key signalling molecule involved in physiological regulation; at high concentrations, its accumulation leads to DNA damage, lipid peroxidation and protein oxidation [33][41]. To maintain this balance, *A. fumigatus* has developed a multilayered antioxidant defense system comprising superoxide dismutases (SODs), catalases (CATs), peroxidases (e.g., Prx1), thioredoxin reductases (TrxR), and transcriptional regulators such as Yap1 and Skn7 [1][22][39]. However, the complex compensatory mechanisms that exist between antioxidant enzyme systems mean that the regulation of ROS homeostasis is not a simple linear

relationship. For example, the loss of OxrA heightens sensitivity to H₂O₂ and elevates intracellular ROS levels; however, this effect can be reversed by overexpression of CAT family members (such as *CatA* or *CatI*)^[50], suggesting the existence of a finely tuned feedback regulatory network. In this study, the transcriptional levels of *sodA-D* were significantly downregulated, and oxidative sensitivity increased; however, there were no notable fluctuations in intracellular ROS levels. This occurrence of "phenotypic-biochemical dissociation" implies that the oxidative stress defect resulting from the *af-MFS95* deletion is not solely attributable to ROS accumulation. Instead, the gene may regulate the expression of antioxidant enzyme genes through a non-ROS-dependent pathway or mediate the oxidative stress response by influencing non-oxidative damage repair mechanisms, such as Fe-S cluster maintenance and glutathione pool homeostasis. Consequently, the role of *af-MFS95* in the oxidative stress defense of *A. fumigatus* may extend beyond the conventional focus on ROS scavenging, involving more intricate cellular adaptive regulatory networks.

The *Δaf-MFS95-recon* failed to fully restore WT phenotype in experiments assessing oxidative stress tolerance, osmotic stress response, and cell wall disruption. Moreover, the transcriptional level of the *af-MFS95* gene remained significantly lower in the complemented strain compared to the WT, indicating inadequate expression of this gene in the complemented strain. This deficiency may stem from positional effects due to the random integration of the gene during the complemented process, partial integration, or insufficient copy numbers. Given that a single gene often cannot simultaneously regulate all relevant pathways, the degree of recovery observed in *Δaf-MFS95-recon* varies across different stress conditions; for instance, it demonstrated only partial recovery at a concentration of 5 mmol/L H₂O₂ and in the presence of CR. The oxidative stress induced by H₂O₂ and menadione is fundamentally distinct. H₂O₂ primarily generates peroxide stress, which can be mitigated through pathways such as catalase and peroxidase^[18]. In contrast, menadione predominantly produces superoxide anions (O₂⁻) through the generation of ROS and may also cause ROS-independent damage, such as protein alkylation^[54]. Consequently, when the transcriptional level of

the *af-MFS95* gene is low, H₂O₂ levels are more likely to partially recover, while menadione levels do not. This observation suggests that the *af-MFS95* gene may play a role in regulating catalase or the peroxide clearance pathway. CR-induced cell wall stress demonstrates multifactorial effects; its growth-inhibitory action is linked not only to β -1,3-glucan binding but also to alterations in cell membrane permeability, transporter-mediated drug influx and efflux, and the regulation of CR entry into cells by cell surface polysaccharides, such as glycosaminoglycans (GAGs) [28]. Consequently, when the transcriptional level of the *af-MFS95* gene is low in $\Delta af-MFS95-recon$, partial recovery under CR stress may still occur due to improvements in cell wall integrity or associated regulatory networks.

The expression of *mbsA* was significantly down-regulated in $\Delta af-MFS95$. *MbsA* encodes a transcription factor of the APSES family that regulates biological processes such as growth, morphogenesis, development, and secondary metabolism in *A. fumigatus* [53]. The deletion of *mbsA* results in weakened radial mycelial growth and reduced conidial yield, indicating its crucial role in mycelial expansion and proliferation [2]. *MbsA* integrates growth, development, and stress responses by regulating *abaA*, *rodA*, CHS family genes, and the SakA pathway, establishing itself as a key node for fungal environmental adaptation [2]. Moreover, its regulatory role may also act in concert with the selective translation mechanism of *tefl* to help maintain cellular stability under stress conditions. Furthermore, the expression levels of *flbA* and *flbD* were significantly reduced. These genes are critical components of the developmental activation pathway in *Aspergillus*, where they initiate conidiation by activating the central regulatory gene *brlA* within the Central Developmental Pathway (CDP) [13]. *BrlA*, which encodes a C2H2-type zinc finger protein, is a core regulatory gene for asexual development [48]. *FlbA*, as a regulator of the G protein signaling pathway (RGS protein), negatively regulates G α subunits (such as *fadA*), therefore influencing the activity of the cAMP-PKA pathway and playing a central role in fungal development and stress responses [48]. *FlbD*, a bZIP-type transcription factor, directly regulates genes related to conidiation and functions under H₂O₂ stress [13][48].

Simultaneously, a significant reduction in *nsdD* expression was observed. *NsdD* encodes a GATA-type transcription factor that downregulates *brlA* as a repressor, thus regulating the development and biosynthesis of secondary metabolites [24][25]. Therefore, based on the experimental findings, it is predicted that *af-MFS95* interacts with specific transcription factors, functioning as a negative regulator of salt stress tolerance and a positive regulator of oxidative stress tolerance in coordination with antioxidant enzyme genes (*sodA*, *sodB*, *sodC*, *sodD*) in *A. fumigatus*, resulting in increased tolerance of the $\Delta af-MFS95$ strain to osmotic stress (NaCl, D-sorbitol), while significantly impairing its growth under oxidative stress conditions (H₂O₂, menadione). Although the initial hypothesis proposed that *af-MFS95* might indirectly affect drug resistance, our results showed no significant change in azole susceptibility. This discrepancy may be due to the absence of specific stress conditions (e.g., combined oxidative and drug stress) under which *af-MFS95* could modulate drug resistance-related genes, which requires further investigation.

Conclusion

In conclusion, based on these findings, *af-MFS95* appears to play a crucial role in the environmental adaptation and growth regulation of *A. fumigatus*. Future studies could use techniques such as EMSA or ChIP-seq to determine whether *af-MFS95* directly influences the transcription of genes associated with oxidative and osmotic stress responses. Further mechanistic insights may be obtained through transcriptomic, proteomic, and metabolomic analyses conducted under stress conditions. Moreover, the function of *af-MFS95* within host-like environments could be validated using in vitro co-culture systems and animal infection models.

Af-MFS95 is critical in maintaining fungal tolerance to oxidative stress and osmotic fluctuations. However, it does not appear to influence sensitivity to azole antifungal agents. Instead, it may regulate the expression of growth-associated proteins and antioxidant enzymes.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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

Funding: This work was supported by the Jingzhou Science and Technology Plan Project [grant numbers 2024HD34]; the Open Research Fund Program of the State Key Laboratory of Virology of China [grant numbers 2023KF006]; the Yangtze University Science and Technology Aid to Tibet Medical Talent Training Program Project [grant numbers 2023YZ06]; the Key Research and Development program of Hubei Province [grant numbers 2024BCB043]; the Natural Science Foundation of Hubei Province [grant numbers 2024AFC034] and the Yangtze River Delta University Innovation Training Programme [grant numbers Yz2023325].

Author contributions: All authors contributed to the research in this report. Individual contributions are as follows: YS designed the study; JQX and ZPF developed and optimised the model; MS and TYM performed the experiments and wrote the manuscript; YPC and YZZ interpreted data and carried out statistical analyses. YS, HZ, and JFT made the final critical revision for important intellectual content. All authors approved the final version of the paper.

Acknowledgments: We thank everyone who contributed to the success of this research, including colleagues, institutions, and funding bodies.

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Figures legends:

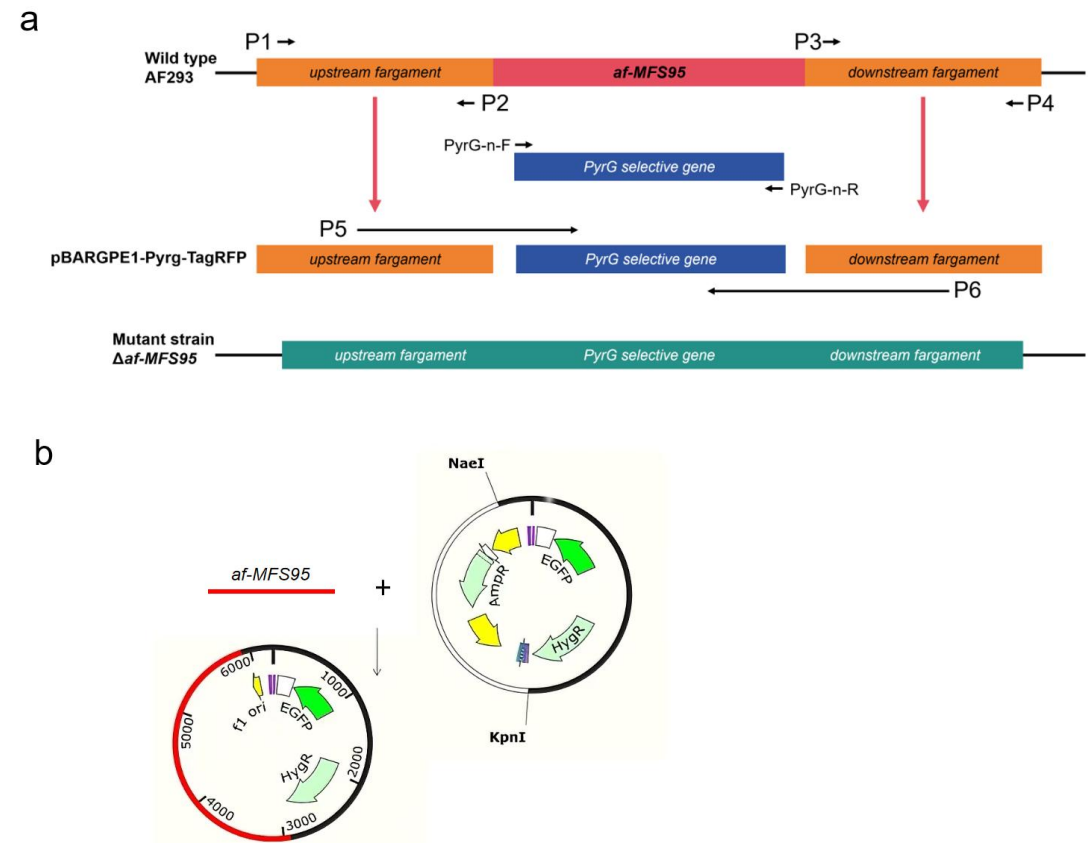


Figure 1. The location of the primer used to confirm the transformants.

a: Construction of the *af-MFS95* knockout mutant strain. b: Construction of the *af-MFS95*

complemented strain.

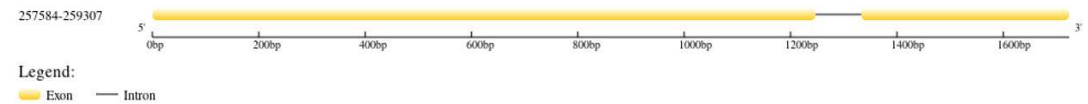


Figure 2. Schematic structure of *A. fumigatus*'s *af-MFS95* gene

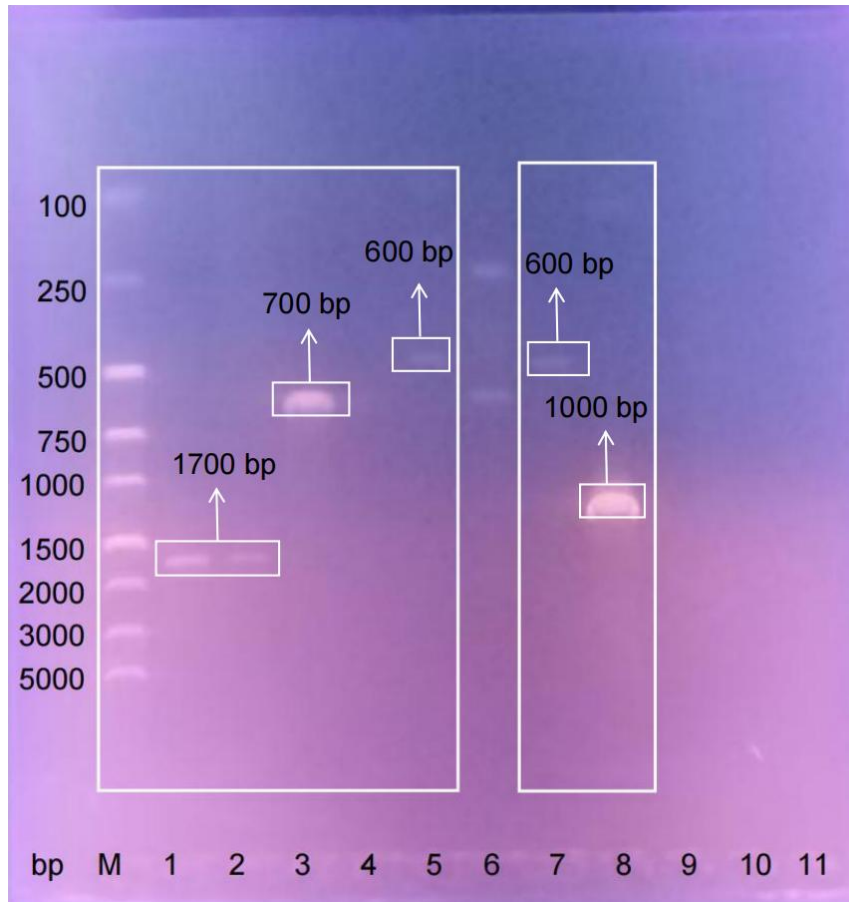


Figure 3. Gel electrophoresis for validation of *A. fumigatus*'s transduction candidate.

Note: White boxes highlight lanes relevant to the main analysis. Lanes outside the white boxes contain data not pertinent to the current study. Lane M represents the DNA marker. Lanes 1 and 2 show amplification of the gene knockout verification band (~1700 bp) using primers P1+DIAO-R and P4+DIAO-F on DNA from $\Delta af-MFS95$. Lane 3 shows amplification of the *pyrG* fragment (~700 bp) with primers pyrG-F and pyrG-R. Lane 4 shows amplification with AIM-yz-R and AIM-yz-F, shows no detectable band (0 bp) from $\Delta af-MFS95$, confirming deletion of the *af-MFS95* gene. Lanes 5 and 7 indicate amplification of the *hph* complemented verification fragment (~600 bp) using primers HPH-F1/HPH-R1 and HPH-F2/HPH-R2 on DNA from $\Delta af-MFS95-recon$. Lane 8, amplified with AIM-yz-R and AIM-yz-F from $\Delta af-MFS95-recon$, produces a band (~1000 bp), confirming restoration of the *af-MFS95* gene. Representative gel images are shown.

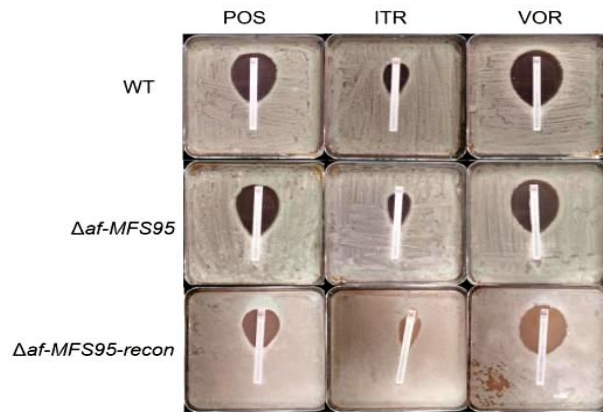


Figure 4. The results of the E-test.

Note: POS: Posaconazole; ITR: Itraconazole; VOR Voriconazole.

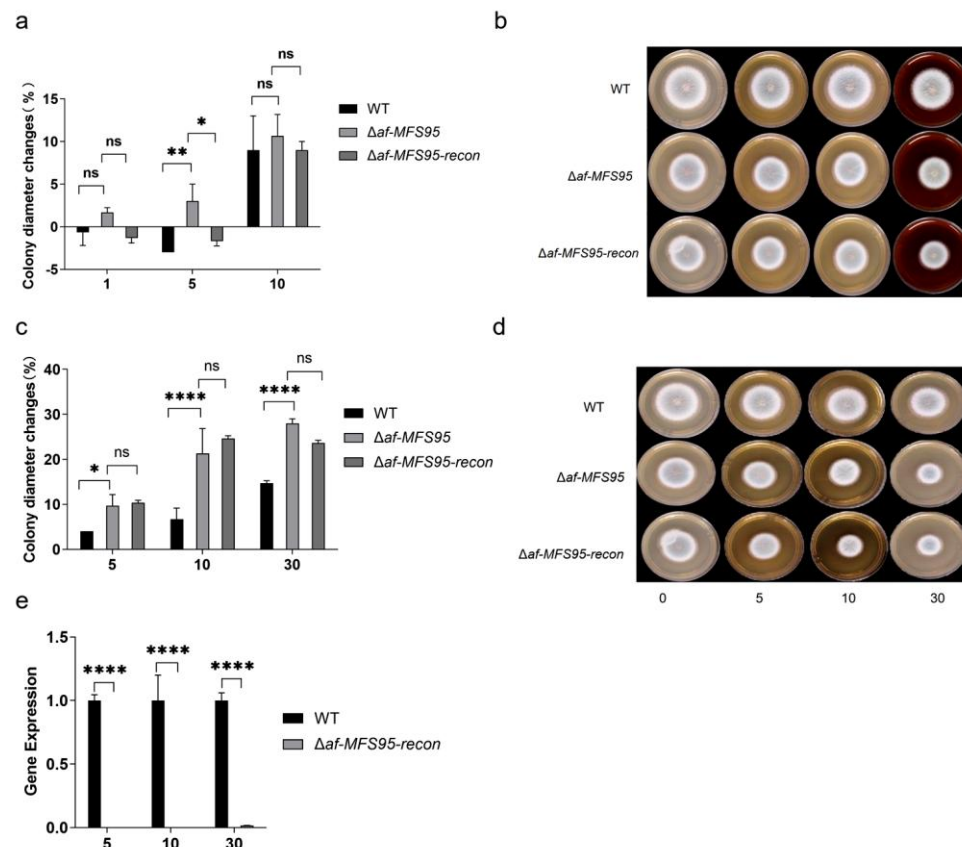


Figure 5. Results of the oxidative stress test.

Note: a: Histogram of the percentage differences in colony diameters for WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ in the presence of different concentrations of H_2O_2 . Statistical analysis was performed using two-way ANOVA with Tukey's test for multiple comparisons. b: Comparison of colony diameters between WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ in different H_2O_2 concentrations. c: Histogram of the percentage differences in colony diameters for WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$.

recon in the presence of different concentrations of menadione. Statistical analysis was performed using two-way ANOVA with Tukey's test for multiple comparisons. d: Comparison of colony diameters between WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ in the presence of different concentrations of menadione. e: Histogram showing *af-MFS95* expression levels in WT and $\Delta af-MFS95-recon$ under different concentrations of menadione. Statistical analysis was performed using a one-tailed, paired t test compared to the control condition. **** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$; ns, $P > 0.05$.

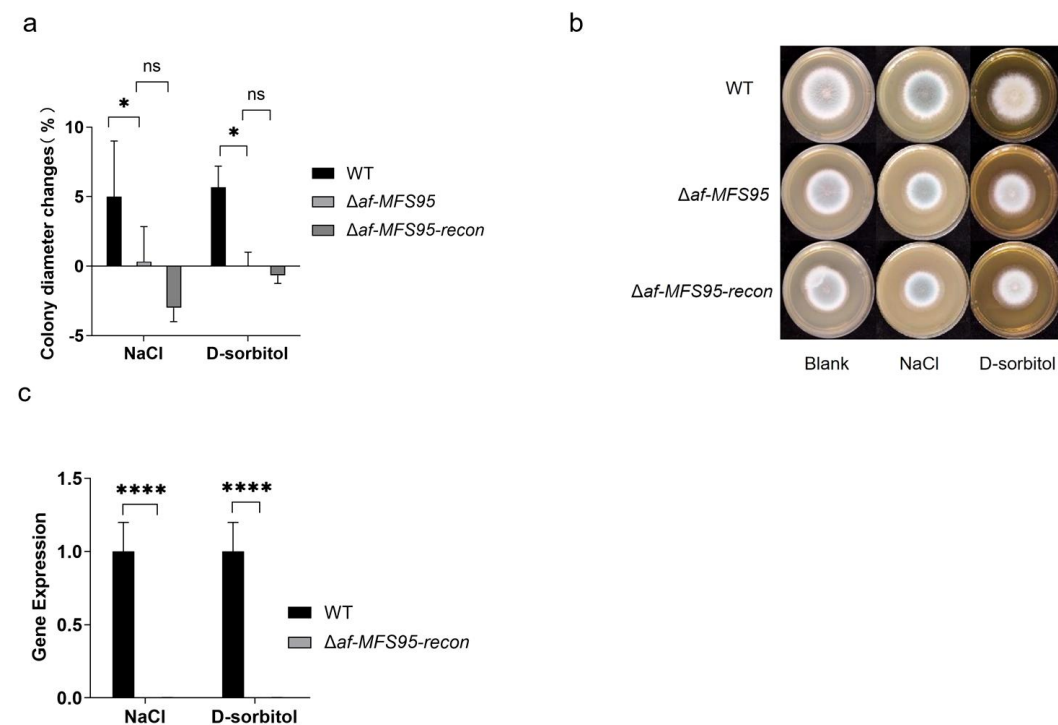


Figure 6. Results of the osmotic pressure test.

Note: a: Histogram of the percentage difference in the colony diameters of WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ in the presence of NaCl and D-sorbitol. Statistical analysis was performed using two-way ANOVA with Tukey's test for multiple comparisons. b: Comparison of colony diameters for WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ in the presence of NaCl and D-sorbitol. c: Histogram showing *af-MFS95* expression levels in WT and $\Delta af-MFS95-recon$ under NaCl and D-sorbitol treatment. Statistical analysis was performed using a one-tailed, paired t test compared to the control condition. **** $P < 0.0001$; * $P < 0.05$; ns, $P > 0.05$.

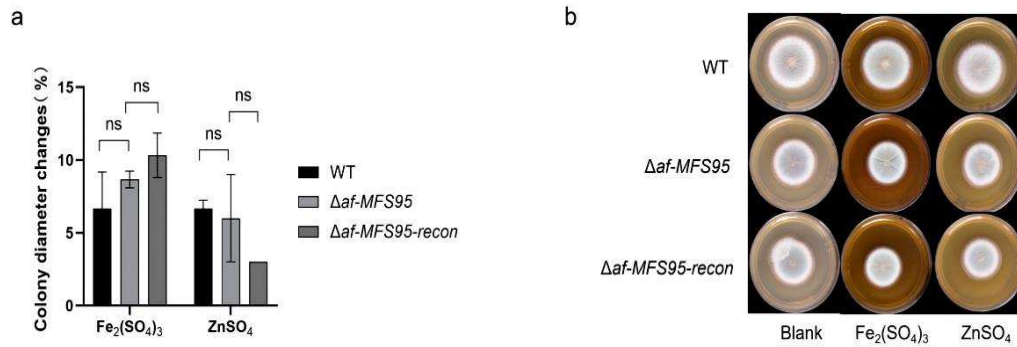


Figure 7. Result of metal ion interference test.

Note: a: Histogram of the percentage difference in the colony diameters of WT, $\Delta af\text{-MFS95}$, and $\Delta af\text{-MFS95-recon}$ in the presence of $\text{Fe}_2(\text{SO}_4)_3$ and ZnSO_4 . Statistical analysis was performed using two-way ANOVA with Tukey's test for multiple comparisons. b: Comparison of colony diameters for WT, $\Delta af\text{-MFS95}$ and $\Delta af\text{-MFS95-recon}$ in the presence of $\text{Fe}_2(\text{SO}_4)_3$ and ZnSO_4 . ns, $P > 0.05$.

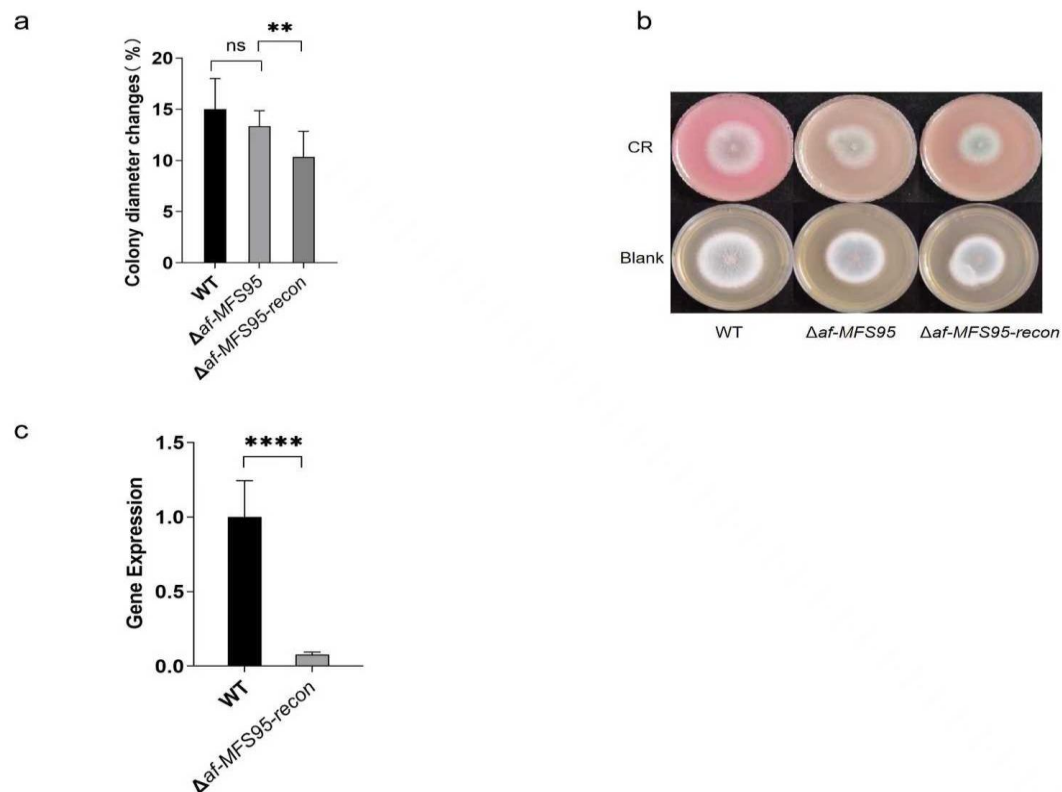


Figure 8. Result of Cell wall disruptor test.

Note: a: Histogram showing the percentage difference in the colony diameters of WT, $\Delta af\text{-MFS95}$, and $\Delta af\text{-MFS95-recon}$ following exposure to Congo red. Statistical analysis was performed using two-way ANOVA with Tukey's test for multiple comparisons. b: Comparison of colony diameters

for WT, $\Delta af-MFS95$, and $\Delta af-MFS95-recon$ in the presence of Congo red. c: Histogram of *af-MFS95* gene expression levels in WT and $\Delta af-MFS95-recon$ in the presence of Congo red. Statistical analysis was performed using a one-tailed, paired t test compared to the control condition. **** $P < 0.0001$; **, $P < 0.01$; ns, $P > 0.05$.

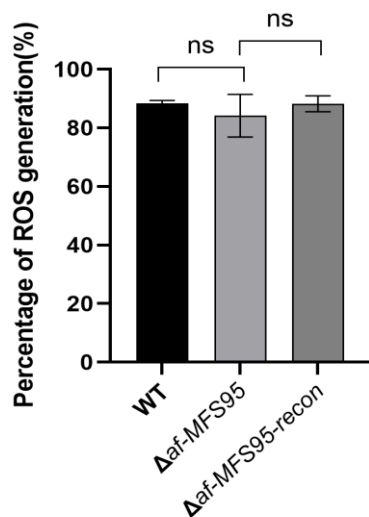


Figure 9. Results of intracellular ROS levels.

Note: Statistical analysis was performed using two-way ANOVA with Tukey's test for multiple comparisons. ns, $P > 0.05$.

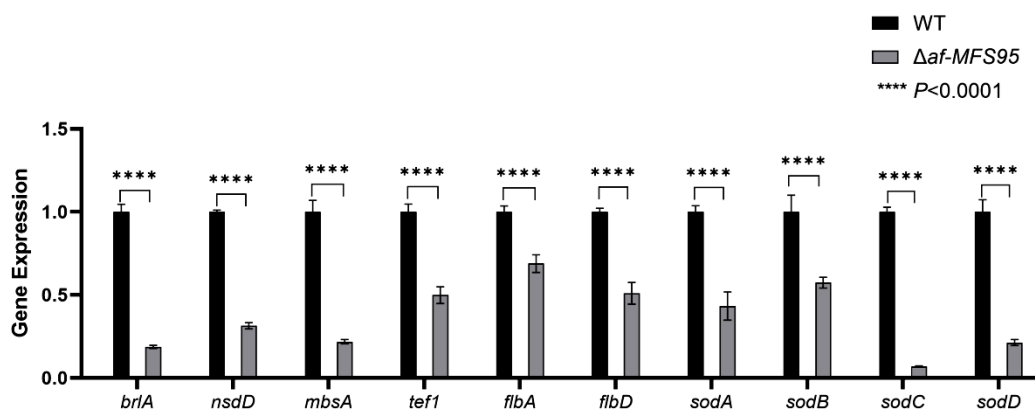


Figure 10. Histogram of gene expression levels related to growth and development in WT and $\Delta af-MFS95$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryInformation.pdf](#)