

Article

Regulation of ABC Transporters and Ergosterol Biosynthesis by the Transcription Factor FvADS-1 Controls Azole Resistance and Virulence in *Fusarium verticillioides*

Yajing Yin ^{1,*}, Hanxing Zhang ², Zhenying Zhang ² , Mi Zhou ³, Shaojie Li ^{2,4} and Chengcheng Hu ^{2,*} 

¹ Tianjin Key Laboratory of Food Biotechnology, College of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin 300134, China

² State Key Laboratory of Microbial Diversity and Innovative Utilization, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China; zhanghx@im.ac.cn (H.Z.); zhangzy@im.ac.cn (Z.Z.); lisj@im.ac.cn (S.L.)

³ National Institute for Radiological Protection, Chinese Center for Disease Control and Prevention, Beijing 100101, China; zhousmi@nirp.chinacdc.cn

⁴ University of Chinese Academy of Sciences, Beijing 100049, China

* Correspondence: yinyajing@tjcu.edu.cn (Y.Y.); hucc@im.ac.cn (C.H.); Tel.: +86-022-26667547 (Y.Y.); +86-010-64806094 (C.H.)

Abstract

Fusarium verticillioides is a significant agricultural pathogen and an emerging causative agent of invasive fusariosis in clinical settings. *Fusarium* species frequently exhibit resistance to available antifungal agents, yet the molecular mechanisms underlying azole resistance remain poorly characterized. In this study, we identified the Zn(II)₂Cys₆ transcription factor FvADS-1 as a positive regulator of the azole stress response in *F. verticillioides*. The transcription of *FvADS-1* was significantly induced by ketoconazole (KTC), and its deletion increased susceptibility to multiple azole compounds. Mechanistically, FvADS-1 positively regulates the KTC-induced expression of genes encoding ABC transporters and ergosterol biosynthesis enzymes, thereby modulating intracellular KTC accumulation and sterol homeostasis under azole stress. Furthermore, FvADS-1 positively regulates the transcriptional response of peroxisomal genes and contributes to fungal tolerance to oxidative stress. Notably, deletion of *FvADS-1* attenuates the virulence of *F. verticillioides* on maize. The function of ADS-1 is evolutionarily conserved: heterologous expression of *N. crassa ads-1* restored azole resistance in *FvADS-1* deletion mutant, and the deletion of the *F. oxysporum* homolog *FoADS-1* similarly increased azole susceptibility. Collectively, our study demonstrates that the conserved transcription factor ADS-1 plays a central role in regulating azole resistance and virulence in the pathogen *F. verticillioides*, offering new insights into antifungal resistance mechanisms in pathogenic filamentous fungi.

Keywords: FvADS-1; *Fusarium verticillioides*; azoles; drug efflux; ergosterol synthesis



Academic Editor: Teresa Peláez-García de la Rasilla

Received: 17 December 2025

Revised: 13 February 2026

Accepted: 17 February 2026

Published: 22 February 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Fusarium verticillioides is a major fungal pathogen in agriculture, primarily responsible for stalk and ear rot in maize [1]. *Fusarium* stalk rot typically reduces maize yield by approximately 10%, with losses escalating to 30–50% in severely affected regions [2]. Beyond yield reduction, infection leads to the accumulation of fumonisins—mycotoxins produced by *F. verticillioides*—within kernels, posing serious health risks to humans and livestock [3–6]. Furthermore, *F. verticillioides* is an emerging human pathogen, causing invasive fusariosis

with high mortality in immunocompromised patients [7–11]. As one of the most prevalent and virulent *Fusarium* species, its global incidence is increasing. Effective management of *Fusarium* infections remains challenging, largely due to the limited availability of antifungal agents and the rising prevalence of antifungal resistance. Therefore, elucidating the molecular mechanisms of antifungal resistance in *F. verticillioides* is crucial for controlling these infections.

Azoles, such as ketoconazole and voriconazole in clinical settings and triadimefon in agriculture, are the most widely used antifungals. They function by inhibiting ergosterol biosynthesis via targeting lanosterol 14 α -demethylase (ERG11/Cyp51A), an essential cytochrome P450 enzyme required for fungal cell membrane integrity [12–14]. Azoles serve as first-line therapy against *Fusarium* infections. Their agricultural use spans over twenty-five years, representing a significant era in crop protection [15]. Clinically, posaconazole has been successfully employed to treat *Fusarium* infections [16]. However, *Fusarium* species frequently develop resistance to available antifungals [9,17].

Fungal azole resistance can be broadly categorized into two interconnected layers: (i) basal (intrinsic) resistance, which reflects the constitutive ability to tolerate the drug prior to exposure, often mediated by pre-existing cellular structures and constitutive expression of defense proteins; and (ii) azole-induced adaptive responses, which involve the rapid transcriptional and metabolic reprogramming triggered by drug stress and are a prerequisite for acquiring full resistance [18–20]. The azole-induced adaptive response processes are mediated by transcription factors such as Zn(II)₂Cys₆ regulators [21,22]. Identifying transcription factors that govern azole-induced responses is therefore valuable for deciphering resistance mechanisms and formulating rational therapeutic strategies. Nevertheless, only a limited number of transcription factors have been functionally characterized in *Fusarium* species [23,24]. Among these, only FgSR, FgAtrR, FgCrz1 and FgCreA in *F. graminearum* have been demonstrated to mediate azole resistance [25–28]. Knowledge in this area remains particularly scarce for the dual-host pathogen *F. verticillioides*.

In this study, transcriptomic analysis of *F. verticillioides* wild-type strain under azole stress revealed that the Zn(II)₂Cys₆ transcription factor FvADS-1 is highly induced and functions as a key regulator of fungal azole susceptibility, oxidative stress response, and virulence. FvADS-1 modulates drug efflux, ergosterol synthesis, and peroxisome function of the adaptive transcriptional response. The role of ADS-1 is evolutionarily conserved: both *Neurospora crassa ads-1* and *F. verticillioides* FvADS-1 complemented the azole-hypersensitive phenotype of the FvADS-1 knockout mutant, while deletion of FoADS-1 in *Fusarium oxysporum* similarly resulted in azole hypersensitivity. Taken together, our findings demonstrate that FvADS-1 plays a central role in the azole stress response by regulating drug efflux and ergosterol biosynthesis in *F. verticillioides*.

2. Materials and Methods

2.1. Strains and Growth Conditions

All strains utilized in this study are detailed in Table S1. PDA medium (2% (*w/v*) glucose, 20% (*w/v*) potato infusion, 1.5% (*w/v*) agar) was used for routine maintenance and antifungal susceptibility assays. YPD medium (2% (*w/v*) glucose, 2% (*w/v*) peptone, 1% (*w/v*) yeast extract) was used for preparing *Fusarium* samples for RNA, sterols, and ketoconazole extraction. Regeneration medium (0.1% (*w/v*) yeast extract, 0.1% (*w/v*) casein enzyme hydrolysate, 0.8 M sucrose, 1.6% agar) was used for protoplast transformation [29]. Antifungal compounds were added as needed. All cultures were incubated at 28 °C.

2.2. Deletion of *FvADS-1* in *F. verticillioides*

F. verticillioides FVEG_06682 (*FvADS-1*) knockout mutant was generated by gene replacement via homologous recombination using the *HygB* resistance gene as a selective marker [30]. Primer pairs Fv06682_Pro_F/Fv06682_Pro_R and Fv06682_Ter_F/Fv06682_Ter_R were used to amplify the upstream and downstream flanking regions of *FvADS-1* gene, respectively (Table S2). The fragment of *HygB* was amplified using primers Fv06682_Hyg_F/Fv06682_Hyg_R (Table S2). The above three DNA fragments were fused by fusion PCR, then inserted into pKSII plasmid digested with *KpnI* and *BamHI* [31]. The obtained plasmid was linearized and transformed into *F. verticillioides* wild-type strain by protoplast transformation, as reported previously [30]. The transformants were screened on PDA medium with Hygromycin B and verified by PCR.

2.3. Complementation of *FvADS-1* Knockout Mutant

The complementary strain of *FvADS-1* knockout mutant was constructed using the Geneticin (*GenR*) resistance gene as a selective marker [30,32]. Firstly, a 4682 bp fragment containing the FVEG_06682 coding sequence (2271bp), 1284 bp upstream region and 1127 bp downstream region was amplified using primers Fvads-1-pKN-ol-F/Fvads-1-pKN-ol-R (Table S2). The fragment was then inserted into pKN plasmid via in-fusion cloning with Clone Express® II One Step Cloning Kit (Vazyme, Nanjing, China), resulting in the complementary plasmid pKN-Fvads1-com. For complementation, pKN-Fvads1-com was then transformed into *FvADS-1* knockout mutant. The transformants were screened on PDA medium with Geneticin and verified by PCR.

2.4. Complementation of *FvADS-1* Knockout Mutant with *N. crassa ads-1*

The coding sequence of *N. crassa ads-1* (NCU08899) gene was also used to complement *FvADS-1* knockout mutant. Briefly, primer pairs Fvads-1-pro-ol-F/Fvads-1-pro-ol-R and Fvads-1-ter-ol-F/Fvads-1-ter-ol-R were used to amplify the upstream and downstream regions of *FvADS-1* gene, respectively (Table S2). The fragment of *N. crassa ads-1* gene coding sequence was amplified using primers Ncads-1-com-F/Ncads-1-com-R (Table S2). The three DNA fragments were then inserted into pKN plasmid digested with *EcoRI* and *HindIII* via in-fusion cloning with Clone Express™ MultiS One Step Cloning Kit (Vazyme, Nanjing, China), resulting in complementary plasmid pKN-Ncads-1-com. The constructed plasmid was then transformed into *FvADS-1* knockout mutant by protoplast transformation, as reported previously [30]. The transformants were screened on PDA medium with Geneticin and verified by PCR.

2.5. Deletion of *FoADS-1* in *F. oxysporum*

Knockout mutant FOXG_09079 in *F. oxysporum* (*FoADS-1*) was generated by gene replacement via double homologous recombination using *HygB* (Hygromycin B resistance gene) as a selectable marker [30]. Briefly, primer pairs upFOXG-09079-F/upFOXG-09079-R and downFOXG-09079-F/downFOXG-09079-R were used to amplify the upstream promoter and downstream terminator fragments of *FoADS-1* gene, respectively (Table S2). *HygB* fragment was amplified using primer pair FO09079Hyg_F/FO09079Hyg_R (Table S2). The three DNA fragments were then fused by fusion PCR and the obtained fragment was transformed into *F. oxysporum* wild-type strain by protoplast transformation, as reported previously [30]. The transformants were screened on PDA medium with Hygromycin B and verified by PCR.

2.6. Antifungal Sensitivity Test

Antifungal sensitivity test was conducted as reported previously [33]. Based on published *Fusarium* susceptibility data, sub-inhibitory yet relevant azole concentrations were used throughout this study, including in all RNA-seq and qRT-PCR assays [20,34,35]. After being grown in PDA plates for 7 days, conidia of *Fusarium* strains were harvested with 2 mL of sterile 0.05% Tween 20. The concentration of the conidia suspension was then adjusted and diluted to 10^7 , 10^6 , 10^5 and 10^4 conidia per mL. Two microliters of conidial suspensions were inoculated onto PDA agar plates (diameter, 15 cm) with or without antifungal azoles. All plates were cultured at 28 °C. Ketoconazole (Sigma, St. Louis, MO, USA, KTC) and Voriconazole (Sigma, St. Louis, MO, USA, VRC) were dissolved in dimethylsulfoxide (DMSO) and their final concentrations for *F. verticillioides* were respectively 0.8 and 0.8 µg/mL, while those for *F. oxysporum* were respectively 20 and 2 µg/mL. Triadimefon (aladdin, Shanghai, China, TRA) was dissolved in isopropanol and its working concentrations for *F. verticillioides* was 12 µg/mL, while that for *F. oxysporum* was 30 µg/mL. The experiment was repeated three times independently.

2.7. RNA Extraction and qRT-PCR Analysis

For *F. verticillioides*, 10^7 conidia were inoculated in YPD liquid medium and cultured at 28 °C with shaking (200 rpm) for 20 h. Then, the mycelia were treated with or without 2 µg/mL KTC for 16 h. After harvest, the mycelia were finely grounded into powder in liquid nitrogen. RNA was extracted according to the standard TRIzol method (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into cDNA using FastQuant RT Kit (with gDNase) (TIANGEN, Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a CFX96 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with SYBR green detection (ChamQ Universal SYBR qPCR Master Mix, Vazyme, Nanjing, China). The expression of a specific gene was calculated as the relative expression to its transcript level in wild-type (FvWT) without KTC treatment according to $2^{-\Delta\Delta C_t}$ method [36]. Gene-specific primers are shown in Table S3. The experiment was repeated three times independently.

2.8. RNA-Seq and Data Analysis

Transcriptomic profiles of the *F. verticillioides* wild-type and FvADS-1^{KO} strains, either treated with or without KTC, were analyzed by high-throughput RNA sequencing (RNA-seq). Sample preparation and total RNA extraction were performed as described under “RNA extraction and qRT-PCR analysis” above. Transcriptomic profiling was performed with two independent biological replicates per condition, which represents a standard and accepted design for detecting robust transcriptional changes in fungal stress response studies. Total RNA was subjected to the DNBSEQ platform for RNA-seq and data processing. The reference genome of *F. verticillioides* was obtained from FungiDB (https://fungidb.org/fungidb/app/record/dataset/NCBITAXON_334819, accessed on 28 November 2023). Genes with FPKM values <10 across all samples were removed as low-abundance transcripts to reduce noise. Differentially expressed genes (DEGs) were defined as those exhibiting a ≥ 1.5 -fold change (up- or down-regulation) between compared conditions, a commonly used threshold for identifying biologically relevant transcriptional changes in fungal systems [37]. Heatmap construction was performed with TBtools v2.372. GO, KEGG, and functional categories enrichment analysis were performed on DAVID.

2.9. Extraction and Analysis of Sterols and Ketoconazole

Samples for sterols and ketoconazole analysis were prepared as those for RNA extraction. After harvest, the mycelia were heat-dried at 80 °C and grounded into fine

powder. Intracellular sterols and ketoconazole were extracted by chloroform and analyzed by HPLC-MS as previously reported, using ergosterol (J&K Scientific Ltd., Beijing, China) and ketoconazole (Sigma) as standards [38]. This experiment was performed twice independently.

2.10. Virulence Assay of *F. verticillioides* on Maize

Plants of maize cultivar B73 were used for virulence assay. Maize seeds were surface disinfected in 100% bleach (6.15% sodium hypochlorite) for 10 min, washed twice with sterile water and soaked in sterile water for about 4 h [39]. The seeds were then internally sterilized by placing them at 60 °C for 5 min [40]. In order to obtain obvious symptoms, maize seedlings with one set of true leaves and 10–15 cm long tap roots were used for pathogen inoculation. Conidia of *F. verticillioides* were collected from 7-day-old PDA plates by flooding the surface with 2 mL sterile distilled water and adjusted to 10^4 conidia/mL with sterile distilled water. For inoculation, surface-sterilized maize roots were immersed in conidial suspensions of *F. verticillioides* for 30 min (experimental group), while water treatment served as the control. Seedlings were then planted in plastic pots (13 cm diameter) containing twice-autoclaved soil. Each pot contained 8 seedlings, and five replicates were applied for each treatment [41,42]. The pots were watered as needed during the assay, and the assay was performed under environmental conditions that cycled between 30 °C (14 h day) and 20 °C (10 h night).

The number of surviving seedlings, wet and dry weights, diameter of stalks and height of seedlings were documented for each treatment after 10 days of planting. The disease incidence was represented as the mean with standard deviation.

3. Results

3.1. Genome-Wide Transcriptional Response of *Fusarium verticillioides* to Azole Stress

To investigate the transcriptional adaptation of *F. verticillioides* to azole stress, genome-wide RNA-seq analysis was performed. A total of 15,903 transcripts were detected. After filtering out genes with FPKM values below 10 across all samples, 6750 genes were retained for further analysis (Supplemental File S1).

Ketoconazole (KTC) treatment induced the upregulation of 1131 genes and downregulation of 596 genes (Figure 1A, Supplemental Files S2 and S3). Gene Ontology (GO) and KEGG pathway enrichment analyses revealed that upregulated genes were primarily associated with ergosterol biosynthesis, ABC transporters, and peroxisomal function (Figures 1B and S1A). Downregulated genes were enriched in metabolic pathways (e.g., tricarboxylic acid cycle, negative regulation of gluconeogenesis) and protein homeostasis processes (e.g., misfolded/unfolded protein binding, protein dimerization) (Figures 1C and S1B). These findings suggest that *F. verticillioides* adapts to azole-induced sterol biosynthesis inhibition by upregulating sterol biosynthetic and drug efflux genes while suppressing metabolic and translational activity. This response aligns with mechanisms reported in other fungi, including *N. crassa*, *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, and *Candida albicans* [37,43–47], indicating different fungi may cope with azole stress using a similar strategy.

Given the central role of transcription factors (TFs) in regulating gene expression, we identified TFs among the KTC-responsive genes. GO enrichment analysis of upregulated genes revealed ten significantly enriched TFs (Figure 1B, Table 1). All identified TFs possess homologs in *Fusarium* species, and several are highly conserved across filamentous fungi, including *N. crassa* and *A. fumigatus* (Table 1). Notably, the protein encoded by FVEG_06682 and its homologs showed a universally conserved response to azoles across all mentioned fungal species above. This protein showed high conservation with ADS-1

(NCU08899) from *N. crassa* OR74A (36% sequence identity [235/648] and 52% sequence similarity [341/648]) and was designated FvADS-1. ADS-1 is a transcription factor essential for fungal responses to azole stress by regulating drug efflux and ergosterol metabolism in *N. crassa* and *Aspergillus flavus* [37]. Consequently, this study focuses on elucidating the function and underlying molecular mechanisms of FVEG_06682 in mediating azole stress responses.

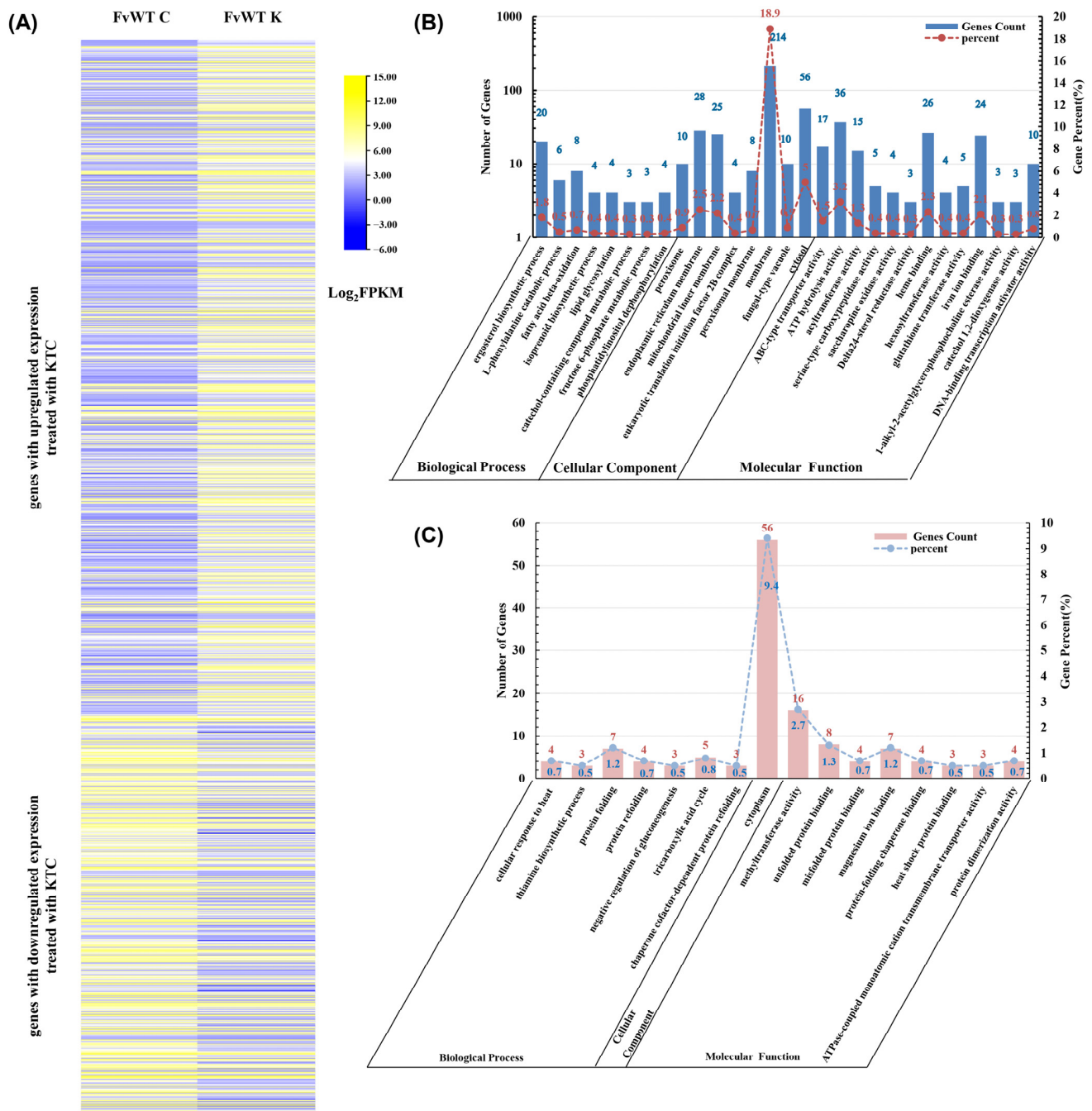


Figure 1. Characterization of azole-responsive genes in FvWT and Gene Ontology (GO) enrichment analysis. (A) Heatmap of differential gene expression. Transcriptional profiles of FvWT strain under control (FvWT C) and ketoconazole-treated (FvWT K) conditions are shown. Log-transformed and scaled average FPKM values were used for heatmap construction, visualized with TBtools. (B,C) GO enrichment analysis of KTC-upregulated genes (B) and KTC-downregulated genes (C). Processes with a significance of $p < 0.05$ are shown. Gene counts are represented by bar charts, and the percentage of genes associated with each term is represented by a line chart.

Table 1. Differentially expressed transcription factors in FvWT strain under KTC stress, identified by RNA-seq analysis.

Locus ^a	Fun-Anno ^b	FPKM ^c FvWT	FPKM ^c FvWTK	Fold ^d (FvWTK/FvWT)	<i>N. crassa</i> OR74A Homolog	Azole Responsive [20]	<i>A. fumigatus</i> Af293 Homolog	Azole Responsive [43,48]	<i>A. fumigatus</i> A1163 Homolog	Azole Responsive [49]	<i>F. graminearum</i> PH-1 Homolog	Azole Responsive [28,50]
FVEG_17328	hypothetical protein	5.59	16.92	3.03	NCU03931	NO	AFUA_3G00930	Up	AFUB_092100	UP	FGSG_09464	NO
FVEG_00309	hypothetical protein	12.51	18.96	1.52	NCU03686	NO	AFUA_6G14150	NO	AFUB_044930	NO	FGSG_11736	NO
FVEG_03527	hypothetical protein	3.62	13.63	3.77	NCU10597	Up	AFUA_5G09890	NO	AFUB_000040	NO	FGSG_04873	Up
FVEG_15459	hypothetical protein	7.92	50.33	6.36	NCU08594	Up	AFUA_5G09890	NO	AFUB_000040	NO	FGSG_05332	NO
FVEG_04360	hypothetical protein	12.09	21.08	1.74	N.A.		N.A.		N.A.		FGSG_07834	NO
FVEG_10357	hypothetical protein	2.23	6.52	2.92	NCU09549	NO	AFUA_8G07000	NO	AFUB_080790	NO	FGSG_04170	NO
FVEG_10599	hypothetical protein	1.83	49.20	26.96	NCU12126	Up	AFUA_3G03315	NO	AFUB_044930	NO	FGSG_12584	NO
FVEG_00760	hypothetical protein	7.68	14.22	1.85	NCU03931	NO	AFUA_3G00930	Up	AFUB_044930	NO	FGSG_00313	NO
FVEG_10002	hypothetical protein	3.58	17.00	4.75	NCU12126	Up	AFUA_6G14150	NO	AFUB_000040	NO	FGSG_12584	NO
FVEG_06682	hypothetical protein	132.98	276.74	2.08	NCU08899	Up	AFUA_1G03800	odrA/mdu2, Up	AFUB_004210	Up	FGSG_09014	Up

NOTE: FvWT: *F. verticillioides* wild type; “K” denotes treatment with ketoconazole. ^a: gene ID of transcription factors involved in azoles adaptation. ^b: Function annotations were obtained from the Fungi Functional Genomics Database (<http://fungidb.org>). ^c: FPKM: Fragments Per Kilobase of transcript per Million fragments mapped; values represent the mean of two biological replicates. ^d: Fold change in expression. N.A., Not Available, meaning no homologous protein detected in the target strain.

3.2. *FvADS-1* (FVEG_06682) Is Required for KTC Resistance in *F. verticillioides*

SMART protein analysis (<https://smart.embl.de/>, accessed on 28 November 2023) indicated that FVEG_06682 encodes a 707-amino-acid protein containing a conserved GAL4-like Zn(II)₂Cys₆ binuclear cluster DNA-binding domain and a fungal-specific transcription factor domain (Figure S2A). The transcription factor is widely conserved across multiple filamentous fungal pathogens, including *Aspergillus flavus*, *A. oryzae*, *A. fumigatus*, *F. graminearum*, *Verticillium dahliae*, *Pyricularia oryzae* (Figure S2B). Transcriptomic data showed a 2.08-fold induction of *FvADS-1* upon KTC treatment (Table 1), which was further verified by qRT-PCR (Figure 2A). To assess its role in azole resistance, a knockout mutant (*FvADS-1*^{KO}) was generated via protoplast transformation. qRT-PCR confirmed the complete absence of *FvADS-1* transcription in the mutant (Figure 2A). While colony morphology on PDA without azoles was similar between wild-type (*FvWT*) and *FvADS-1*^{KO}, the mutant exhibited significantly reduced growth on media containing 0.8 µg/mL KTC or 0.8 µg/mL voriconazole (VRC), two clinical antifungal azoles (Figure 2B). A similar hypersensitive phenotype was observed with the agricultural azole triadimefon (TRA; 12 µg/mL) (Figure 2C). These results indicate that deletion of *FvADS-1* confers hypersensitive phenotype to azoles.

To further verify the role of *FvADS-1* under antifungal azole stress, we constructed a *FvADS-1* complement strain (*FvADS-1*^{COM}). As shown in Figure 2B,C, the *FvADS-1*^{COM} strain exhibited the similar growth phenotype as *FvWT* on plates with each of these azoles, indicating that *FvADS-1* is essential for resistance to the antifungal azoles KTC, VRC and TRA in *F. verticillioides*.

We also examined the strains' sensitivity to other stresses. However, deletion of *FvADS-1* did not significantly alter the sensitivity to osmotic stress (2 M NaCl or 2 M KCl) and extreme temperature (40 °C (lethal) or 4 °C) (Figure S3A,B). In addition, conidiation on PDA slants between *FvWT* and *FvADS-1*^{KO} were not significantly distinguishable ($p > 0.05$, both) (Figure S3C). All above data indicate that *FvADS-1* specifically modulates the responses to azoles.

3.3. *N. crassa ads-1* Functionally Complements Azole Susceptibility of *FvADS-1*^{KO} Strain

Given the sequence homology between *FvADS-1* and *N. crassa* ADS-1 (Figure S2B), we tested functional conservation by heterologously expressing *N. crassa ads-1* in the *FvADS-1*^{KO} strain. The resulting strain (*FvADS-1*^{KO}; *Ncads-1*^{COM}) exhibited azole resistance comparable to *FvWT* (Figure 2B,C), demonstrating that the regulatory function of ADS-1 in azole stress response is evolutionarily conserved.

3.4. *FvADS-1* Modulates the Genome-Wide Transcriptional Responses to KTC

To elucidate the molecular mechanisms by which *FvADS-1* mediates the azole stress response, we compared the transcriptomes of *FvWT* and *FvADS-1*^{KO} strains with and without KTC treatment. In the absence of KTC, the transcriptional profiles of the two strains were highly correlated (correlation coefficient >0.989 ; Figure S4), indicating that the influence of *FvADS-1* in genome-wide transcription were very limited in the absence of KTC stress. Therefore, we focused on differentially expressed genes (DEGs) induced by KTC.

Among the 1131 KTC-upregulated genes, 96 showed attenuated induction due to *FvADS-1* deletion (Supplemental File S4, Figure 3A). Conversely, among the 596 KTC-downregulated genes, 191 exhibited reduced repression due to *FvADS-1* deletion (Supplemental File S5, Figure 3A). These 287 genes were defined as *FvADS-1*-dependent responders to azole stress.

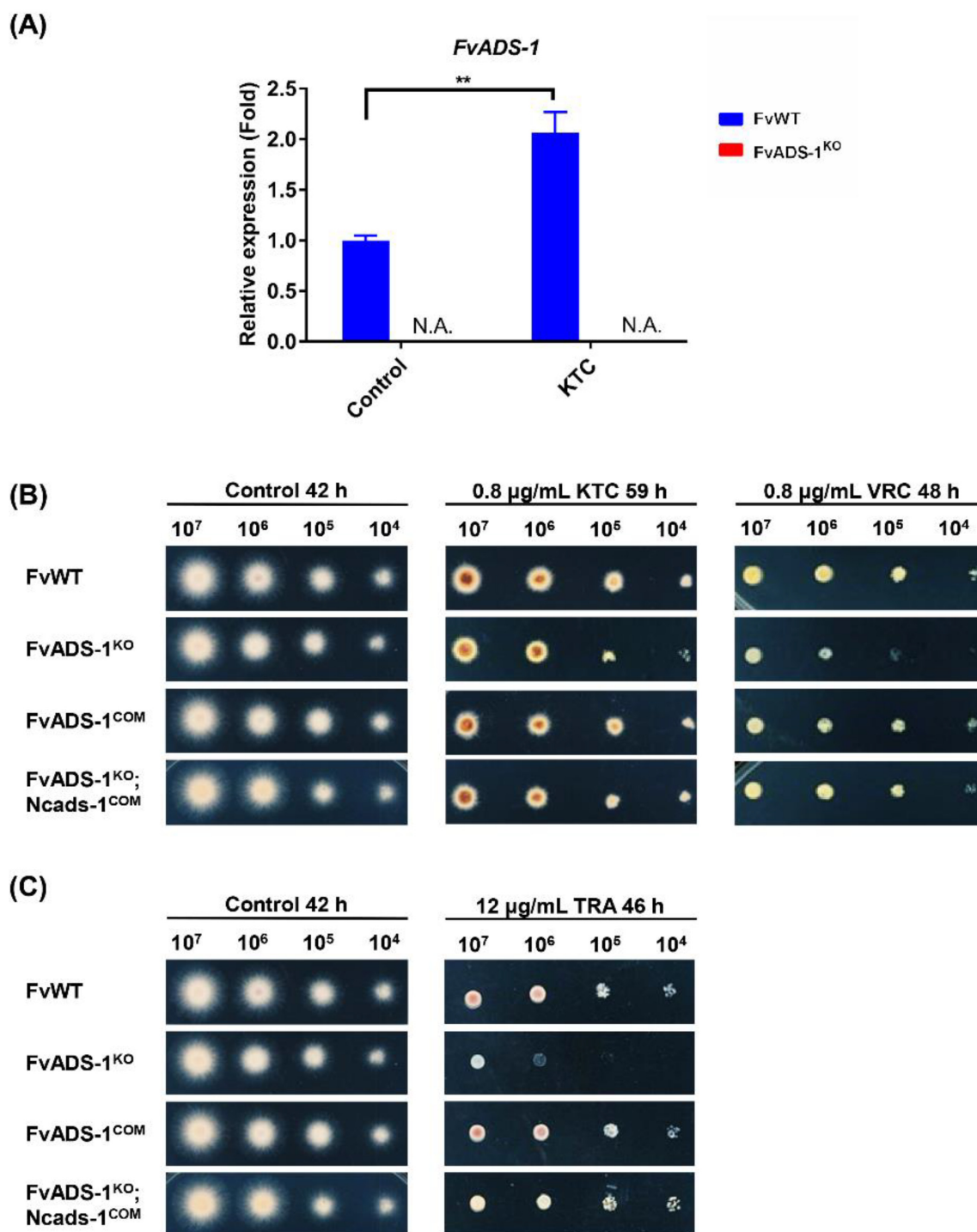


Figure 2. Functional analysis of *FvADS-1* in the azole stress response of *F. verticillioides*. (A) Transcriptional response of *FvADS-1* to KTC stress. The expression of *FvADS-1* in FvWT and FvADS-1^{KO} strains was quantified by qRT-PCR after incubation with or without 2 µg/mL KTC for 16 h. Expression levels were normalized to β -tubulin and calculated using the $2^{-\Delta\Delta C_t}$ method. Data represent the mean $\pm$ SD of three biological replicates. N.A. indicates Not Available. Statistical significance was determined by *t*-test (** $p < 0.01$). Blue bars, FvWT strain; red bars, FvADS-1^{KO} strain. (B,C) Azole susceptibility assays. Ten-fold serial dilutions of conidial suspensions from the indicated strains—FvWT, FvADS-1^{KO}, the complemented strain (FvADS-1^{COM}), and the heterologous complemented strain (FvADS-1^{KO}; Ncads-1^{COM})—were spotted onto PDA plates containing either no drug (Control), the clinical azole KTC and voriconazole (VRC) (B), or the agricultural azole triadimefon (TRA) (C). Plates were incubated at 28 °C in the dark. All experiments were performed in three independent biological replicates.

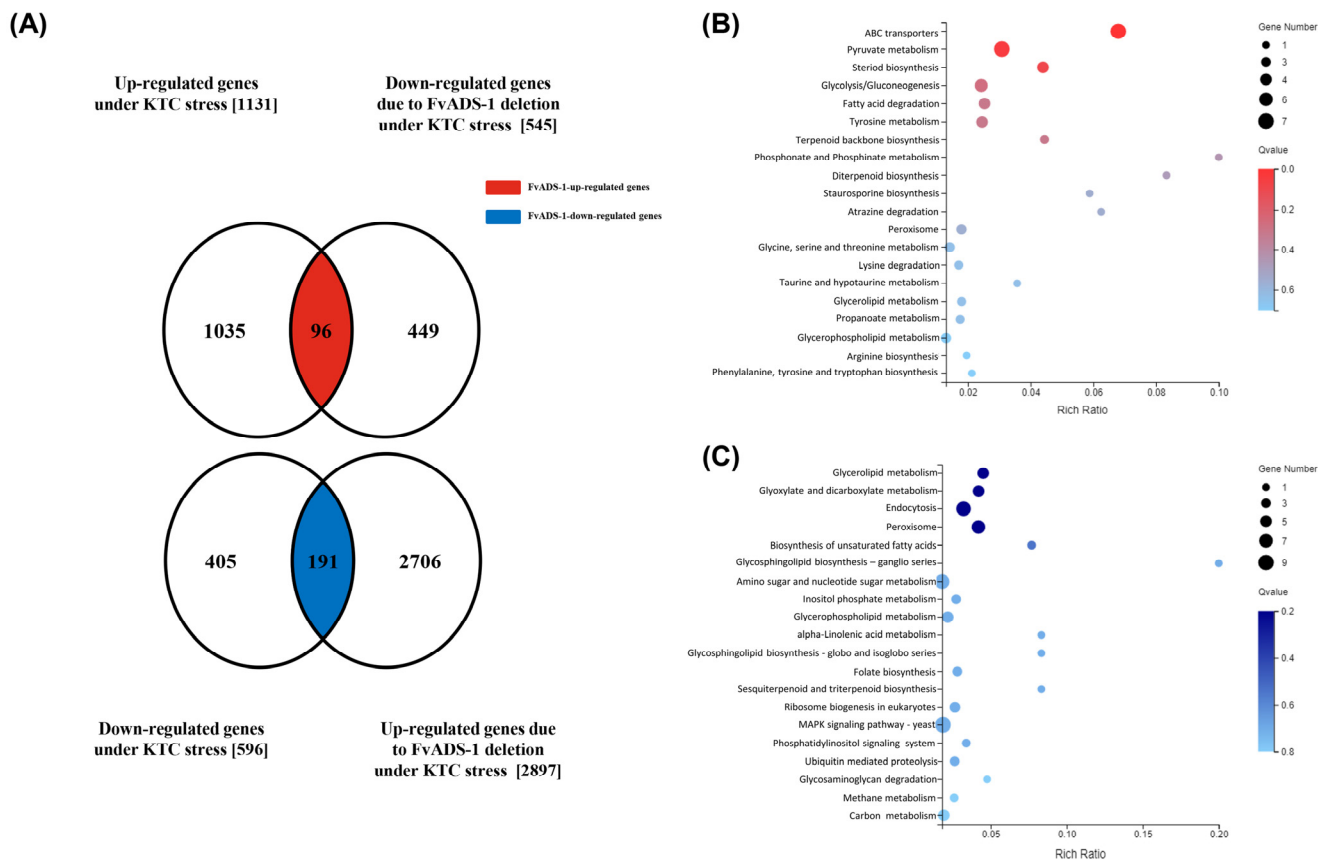


Figure 3. Identification of FvADS-1-dependent genes under KTC stress. (A) Venn diagram showing the overlap of genes regulated by KTC in FvWT and FvADS-1^{KO} strains, based on comparative RNA-seq analysis for FvWT and FvADS-1^{KO} strains. KEGG pathway enrichment analysis of FvADS-1-up-regulated genes (B) and FvADS-1-down-regulated genes (C). The 20 most significantly enriched pathways (smallest Q-value) are shown. Dot size corresponds to the number of enriched genes per pathway; color intensity represents the significance level (Q-value).

To gain insights into the biological processes regulated by FvADS-1 under azole stress, KEGG pathway analysis was performed on above identified FvADS-1-dependent genes. The results showed that FvADS-1-upregulated genes were primarily enriched in “ABC transporters”, “Steroid biosynthesis” and “Peroxisome” (Figure 3B). These results indicate that FvADS-1 may mediate resistance to azoles by promoting the activity of drug efflux pumps, regulating sterol homeostasis and countering drug-induced oxidative stress. FvADS-1-downregulated genes were primarily enriched into “Metabolic processes” and “Protein homeostasis” (e.g., ribosome biogenesis in eukaryotes, ubiquitin mediated proteolysis) (Figure 3C). These results indicated that FvADS-1 potentially supported survival under drug pressure through reprogramming metabolic processes, maintaining protein homeostasis.

3.5. FvADS-1 Positively Regulates the Transcriptional Expression of ABC Transporters and Intracellular KTC Accumulation in *F. verticillioides*

Among the FvADS-1-upregulated genes, seven genes were enriched in the category of “ABC transporters” (Figure 3B, Table 2). Their KTC-induced expression was significantly diminished due to the deletion of *FvADS-1* (Figure 4A). The genes *FVEG_01835*, *FVEG_11089*, *FVEG_16559*, and *FVEG_13230* encode proteins that exhibit high sequence similarity to CDR4, the major Pdr5p-like ABC transporter in *N. crassa*. [51]. The protein encoded by *FVEG_13167* exhibited the greatest similarity to AtrF of *A. fumigatus* [52]. These genes were designated *FvCDR4A*, *FvCDR4B*, *FvCDR4C*, *FvCDR4D* and *FvAtrF* in this study,

respectively. The remaining two genes (*FVEG_04890*, *FVEG_07622*) have greatest protein similarity to the ABC multidrug transporters (XP_748598.1, XP_752405.2) of *A. fumigatus*, respectively, with unknown functions. Therefore, they were accordingly designated *FvABC1* and *FvABC2*. The responses of all these ABC transporters encoding genes to azoles were further verified in *FvWT*, *FvADS-1^{KO}* and *FvADS-1^{COM}* strain by qRT-PCR. After KTC treatment, the transcript levels of *FvCDR4A*, *FvCDR4B*, *FvCDR4C* and *FvCDR4D* increased by 2.39-, 4.70-, 3.49-, and 8.68-fold in *FvWT*, respectively, but in *FvADS-1^{KO}* strain, the increases were only 51.01%, 44.49%, 0.02% and 60.07% of those observed in *FvWT* (Figure 4B). Similar results were obtained for *FvAtrF*, *FvABC1* and *FvABC2* (Figure 4B). Gene expression in *FvADS-1^{COM}* strain was restored to wild-type levels (Figure 4B). Therefore, ADS-1 positively regulates the expression of ABC transporter genes in response to azole stress.

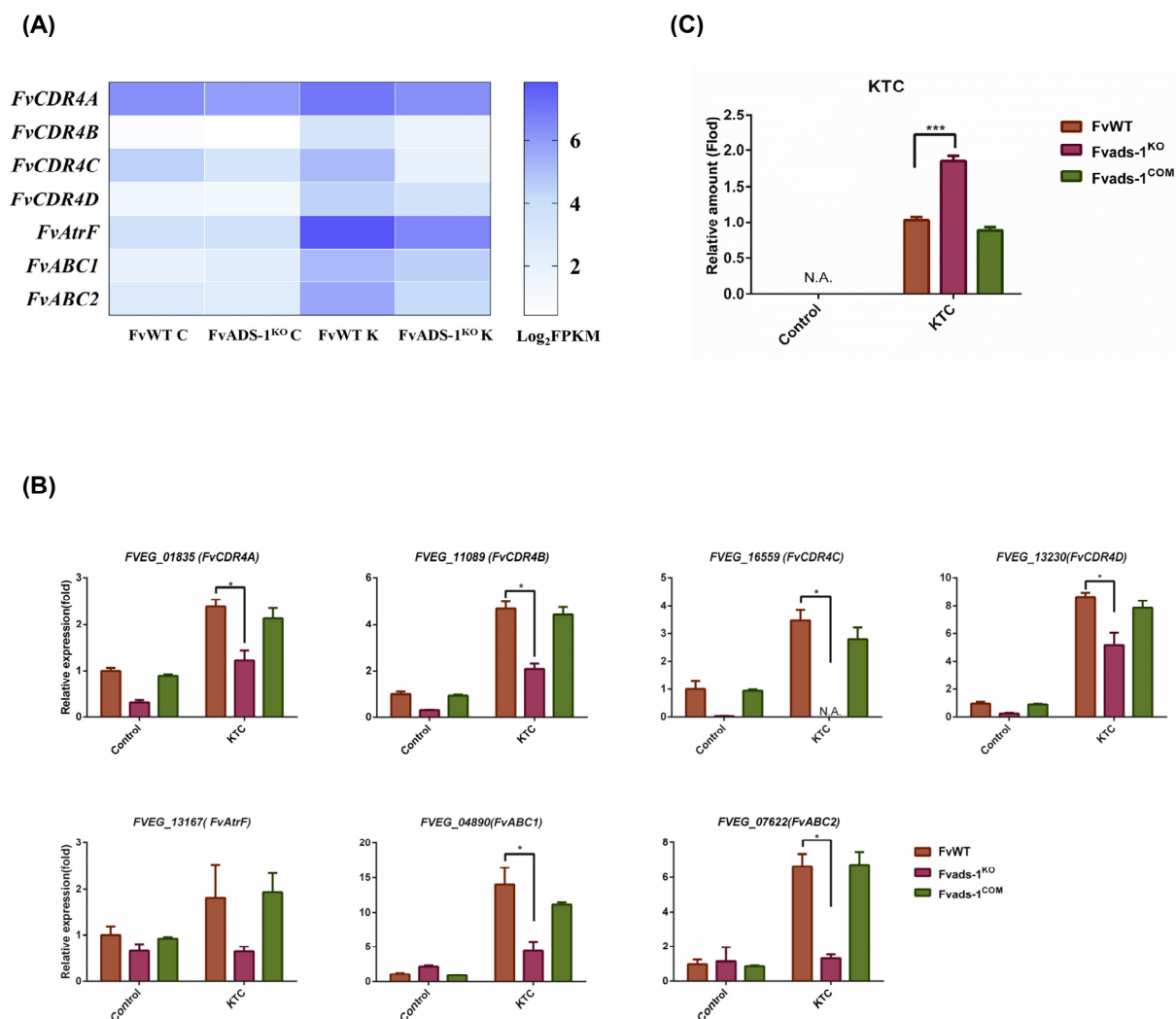


Figure 4. *FvADS-1* regulates ABC transporter expression and modulates intracellular ketoconazole accumulation in *F. verticillioides*. (A) Heatmap comparing the transcriptional response of seven *FvADS-1*-dependent ABC transporter genes to KTC. Log-transformed and scaled average FPKM values were used for visualization. (B) qRT-PCR validation of ABC transporter gene expression. Transcript levels in *FvWT*, *FvADS-1^{KO}* and *FvADS-1^{COM}* strains, treated with or without 2 $\mu\text{g/mL}$ KTC for 16 h, were normalized to $\beta\text{-tubulin}$. Expression in untreated *FvWT* was set to 1. Data represent mean $\pm$ SD of three biological replicates. Significance was determined by *t*-test (* $p < 0.05$). (C) Intracellular KTC accumulation. The *FvWT*, *FvADS-1^{KO}* and *FvADS-1^{COM}* strains were treated with 2 $\mu\text{g/mL}$ KTC for 16 h; untreated samples served as controls. The KTC level in treated *FvWT* was set to 1. Values are mean $\pm$ SD from two independent experiments, each with two technical replicates. Significance is indicated (***) $p < 0.001$.

Table 2. Differentially expressed genes in FvWT and FvADS-1^{KO} strains under KTC stress, identified by RNA-seq analysis.

Locus ^a	Gene Name	Fun-Anno ^b	FPKM ^c FvWT	FPKM ^c FvWTK	Fold ^d (FvWTK/FvWT)	FPKM ^c FvADS-1 ^{KO}	FPKM ^c FvADS-1 ^{KO} K	Fold ^d (FvADS-1 ^{KO} K/FvWTK)
FVEG_01835	<i>FvCDR4A</i>	ABC1 transport protein	80.43	124.57	1.55	59.96	76.93	0.62
FVEG_11089	<i>FvCDR4B</i>	ATPase	1.62	9.89	6.12	1.34	3.25	0.33
FVEG_16559	<i>FvCDR4C</i>	ATPase	22.3	34.44	1.54	10	3.68	0.11
FVEG_13230	<i>FvCDR4D</i>	hypothetical protein	2.67	19.94	7.48	2.31	12.58	0.63
FVEG_13167	<i>FvAtrF</i>	ATP-binding protein multidrug cassette transport	12.67	231.21	18.26	12.32	93.07	0.40
FVEG_07622	<i>FvABC1</i>	hypothetical protein	3.73	36.04	9.68	5.05	22.87	0.63
FVEG_04890	<i>FvABC2</i>	hypothetical protein	6.48	50.9	7.85	5.82	18.16	0.36
FVEG_09178	<i>FvERG3</i>	C-5 sterol desaturase	254.9	4271.94	16.76	279.94	2065.65	0.48
FVEG_04521	<i>FvERG12</i>	mevalonate kinase	121.86	787.59	6.46	121.19	512.26	0.65
FVEG_06448	<i>FvERG25</i>	methylsterol monooxygenase	579.09	9199.47	15.89	617.42	5221.27	0.57
FVEG_13168		hypothetical protein	4.05	13.07	3.23	3.01	7.61	0.58
FVEG_01123	<i>FvCYP51A</i>	cytochrome P450, family 51 (sterol 14-demethylase)	165.69	2074.84	12.52	162.60	1507.37	0.73
FVEG_10277	<i>FvCYP51B</i>	cytochrome P450, family 51 (sterol 14-demethylase)	0.17	138.38	838.64	0.1	609.77	4.41
FVEG_12391	<i>FvCYP51C</i>	cytochrome P450, family 51 (sterol 14-demethylase)	41.31	54.48	1.32	36.77	55.05	1.01
FVEG_10782		hypothetical protein	10.56	17.14	1.62	11.30	6.61	0.39
FVEG_10722		hypothetical protein	42.68	92.37	2.16	53.45	58.58	0.63

NOTE: FvWT: *F. verticillioides* wild type; FvADS-1^{KO}: *FvADS-1* knockout mutant; “K” denotes treatment with ketoconazole. ^a: gene ID of FvADS-1-dependent genes involved in azoles adaptation. ^b: Function annotations were obtained from the Fungi Functional Genomics Database (<http://fungidb.org>). ^c: FPKM: Fragments Per Kilobase of transcript per Million fragments mapped; values represent the mean of two biological replicates. ^d: Fold change in expression.

ABC transporters are able to pump azoles out of cells by hydrolyzing ATP, thus conferring fungal tolerance to azoles. We then examined whether intracellular KTC accumulation is affected by FvADS-1. Consistent with impaired efflux, intracellular KTC accumulation in FvADS-1^{KO} strain was 194.6% of that in FvWT under KTC treatment ($p < 0.0001$, Figure 4C), whereas accumulation in FvADS-1^{COM} strain was comparable to the wild type. These results demonstrate that FvADS-1 enhances azole efflux by transcriptional activation of ABC transporters.

3.6. FvADS-1 Positively Regulates the Transcriptional Expression of Ergosterol Biosynthesis Genes and Maintains Sterol Homeostasis in *F. verticillioides*

Among the FvADS-1-upregulated genes, four genes involved in sterol biosynthesis were enriched (Figure 3B, Table 2). Their induction by KTC was attenuated due to the deletion of FvADS-1 (Figure 5A). Based on homology, the proteins encoded by FVEG_09178, FVEG_04521 and FVEG_06448 were named as FvERG3, FvERG12 and FvERG25 in this study, respectively. FVEG_13168 encodes an uncharacterized protein. qRT-PCR validated that KTC-mediated induction of all four ergosterol biosynthesis genes was markedly reduced in FvADS-1^{KO} strain and restored to wild-type levels in FvADS-1^{COM} strain (Figure 5B).

The *Fusarium* possesses three azole target genes, which encode enzymes that are crucial for ergosterol biosynthesis [53]. In *F. verticillioides*, the three CYP51s are encoded by FvCYP51A (FVEG_01123), FvCYP51B (FVEG_10277), and FvCYP51C (FVEG_12391). The transcriptome analysis revealed that three azole target genes were induced 12.52-, 838.64-, and 1.32-fold, respectively, upon KTC treatment (Figure 5A, Table 2). qRT-PCR confirmed strong induction of FvCYP51A and FvCYP51B in FvWT, which was differentially affected in FvADS-1^{KO} strain: FvCYP51A induction was reduced to 28.6% of wild-type levels, while FvCYP51B expression was 1.87-fold higher. FvCYP51C expression was similar with and without drug treatment (Figure 5C). These results collectively demonstrate that FvADS-1 positively regulates the transcription of azole target gene FvCYP51A, while the upregulation of FvCYP51B compensated for impairment of FvADS-1-mediated regulation, and FvCYP51C exhibited the weakest responsiveness to KTC stress.

ERG12 is a key enzyme in early ergosterol pathway, which can channel products into different biosynthetic pathways. ERG11 represents a rate-limiting step in the late ergosterol pathway, meanwhile ERG3 and ERG25 are also important enzymes in the late pathway [54]. Therefore, we further examined whether intracellular ergosterol profiles were affected by FvADS-1 deletion. While basal ergosterol levels were similar between strains, KTC treatment resulted in a dramatic reduction in intracellular ergosterol in FvADS-1^{KO} strain but not in the wild-type or complemented strains ($p < 0.01$, Figure 5D). No significant differences were observed in the levels of lanosterol, or alternate pathway sterols (eburicol and toxic sterol) (Figure 5D), indicating that FvADS-1 maintains ergosterol biosynthesis without shifting flux to the alternative pathway.

3.7. FvADS-1 Positively Regulates the Transcriptional Expression of Peroxisomal Genes and Confers Resistance to Oxidative Stress in *F. verticillioides*

Among the FvADS-1-upregulated genes, two peroxisomal genes (FVEG_10782, FVEG_10722) were significantly enriched (Figure 3B, Table 2), and their induction was FvADS-1-dependent (Figure 6A). qRT-PCR confirmed that expression of both peroxisomal genes was significantly attenuated in FvADS-1^{KO} strain and restored to wild-type levels in FvADS-1^{COM} strain. (Figure 6B). Given the role of peroxisomes in redox homeostasis, we measured the susceptibility of FvWT, FvADS-1^{KO} and FvADS-1^{COM} strains to oxidative stress. The FvADS-1^{KO} strain exhibited significantly increased sensitivity to 2 mM H₂O₂ compared to FvWT, a phenotype rescued by genetic complementation (Figure 6C). This

indicates that FvADS-1 contributes to oxidative stress tolerance, likely through regulating peroxisomal function in *F. verticillioides*.

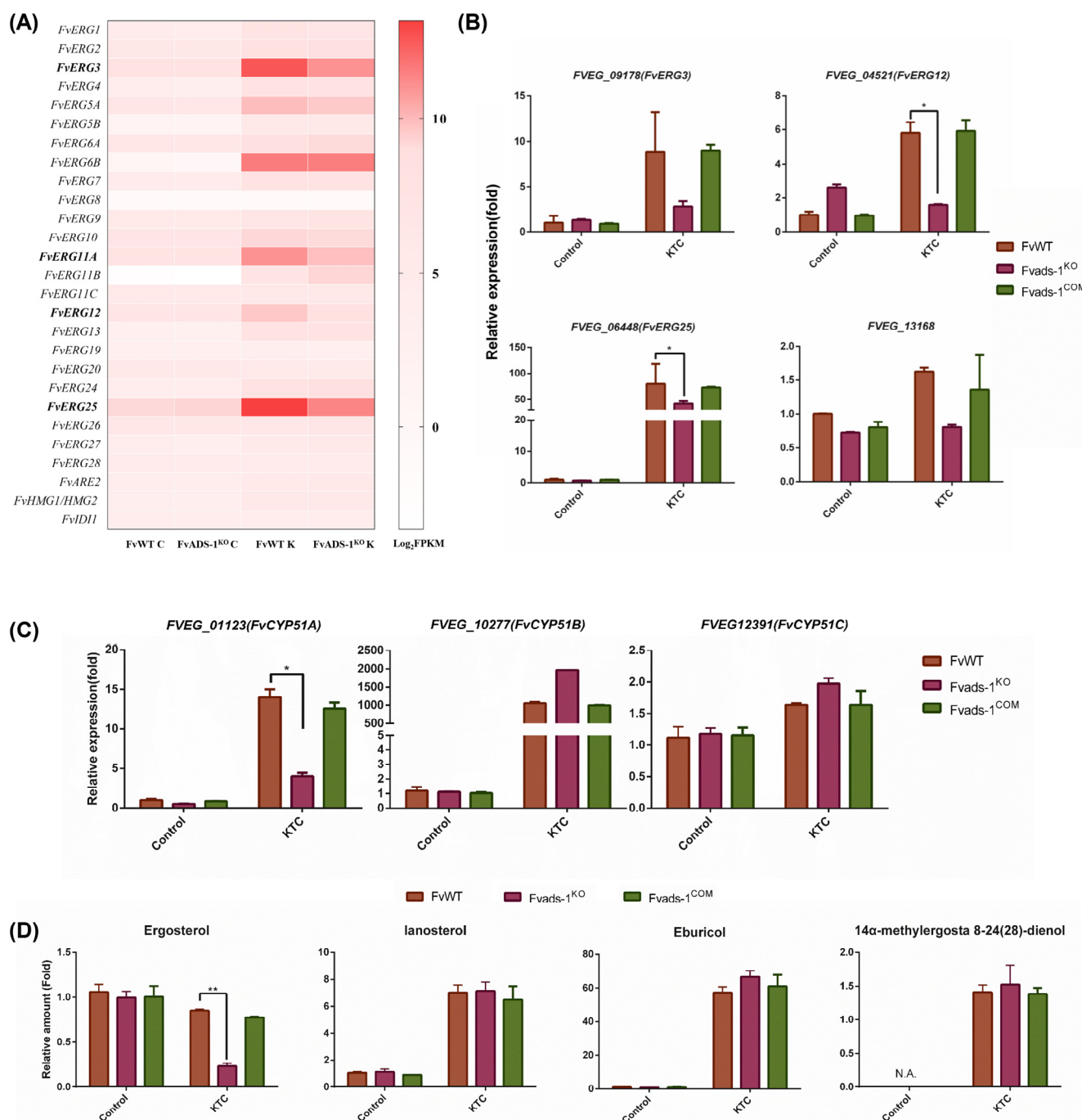


Figure 5. FvADS-1 regulates ergosterol biosynthesis pathway genes and sterol homeostasis in *F. verticillioides*. **(A)** Heatmap comparing the transcriptional responses of ergosterol biosynthesis pathway genes to KTC. Log-transformed and scaled average FPKM values were used for visualization. **(B,C)** qRT-PCR analysis of ergosterol biosynthesis gene expression. Transcript levels in FvWT, FvADS-1^{KO} and FvADS-1^{COM} strains, treated with or without 2 µg/mL KTC for 16 h, were normalized to β -tubulin. Expression in untreated FvWT was set to 1. Data represent mean $\pm$ SD of three biological replicates. Significance was determined by *t*-test (* *p* < 0.05). **(D)** Intracellular levels of ergosterol and key metabolic intermediates. The FvWT, FvADS-1^{KO} and FvADS-1^{COM} strains were treated with 2 µg/mL KTC for 16 h; untreated samples served as controls. Sterol content in treated FvWT was set to 1. Values are mean $\pm$ SD from two independent experiments, each with two technical replicates. N.A. indicates Not Available. Significance is indicated (** *p* < 0.01).

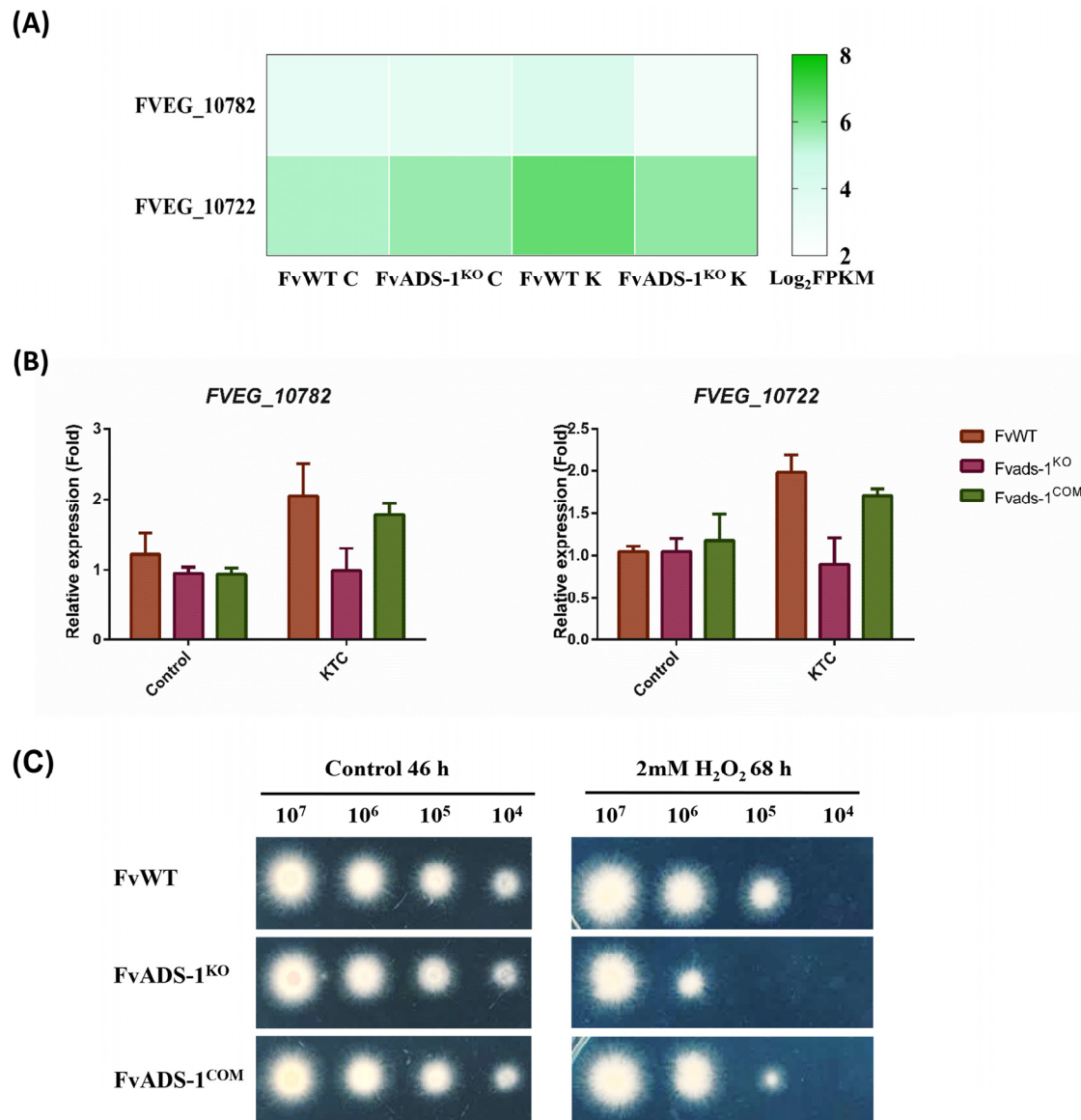


Figure 6. FvADS-1 regulates peroxisomal genes and confers tolerance to oxidative stress in *F. verticillioides*. (A) Heatmap comparing the transcriptional responses of two FvADS-1-dependent peroxisomal genes to KTC. Log-transformed and scaled average FPKM values were used for visualization. (B) qRT-PCR analysis of peroxisomal genes expression. Transcript levels in FvWT, FvADS-1^{KO} and FvADS-1^{COM} strains, treated with or without 2 µg/mL KTC for 16 h, were normalized to β -tubulin. Expression in untreated FvWT was set to 1. Data represent mean $\pm$ SD of three biological replicates. (C) Oxidative stress susceptibility assay. Ten-fold serial dilutions of conidial suspensions from the indicated strains—FvWT, FvADS-1^{KO} and FvADS-1^{COM}—were spotted onto PDA plates containing either no stressor (Control) or 2 mM H₂O₂. Plates were incubated at 28 °C in the dark. All experiments were performed in three independent biological replicates.

3.8. FvADS-1 Is Essential for *F. verticillioides* Virulence in Maize

Previous studies have demonstrated a strong correlation of ABC transporter activity, ergosterol biosynthesis, tolerance to oxidative stress with *F. graminearum* virulence [26,55–57]. Given the regulatory role of FvADS-1 in ABC transporters, ergosterol biosynthesis, and peroxisomal function, we further evaluated its impact on fungal virulence in maize. Seedlings inoculated with FvWT and FvADS-1^{COM} developed severe stunting and reduced biomass compared to water-treated controls 10 days post-inoculation. In contrast, plants inoculated with the FvADS-1^{KO} strain were phenotypically similar to controls, showing no significant

differences in seedling weight (fresh/dry), stalk diameter, or plant height (Figure 7A,B). No visible root symptoms were observed at this stage. These results demonstrate that FvADS-1 is essential for full virulence of *F. verticillioides* on maize.

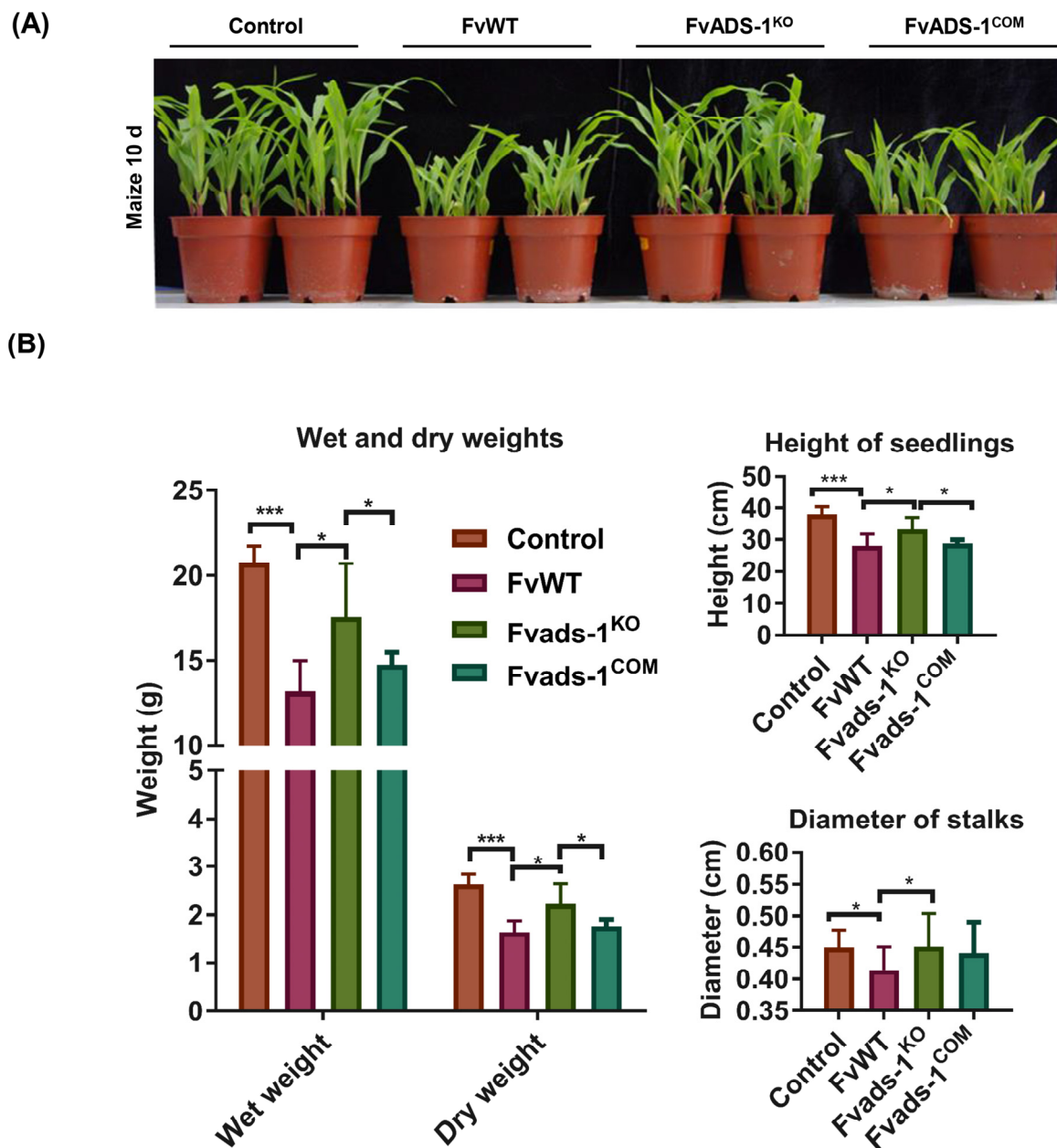


Figure 7. Virulence analysis of FvADS-1 in maize infection. (A) Pathogenicity assay of FvWT, FvADS-1^{KO} and FvADS-1^{COM} on maize seedlings. Seedlings at the one-true-leaf stage with 10–15 cm taproots were inoculated by root immersion in a suspension of 10^4 conidia/mL for 30 min. Water-treated seedlings served as the control. Seedlings were then transferred to pots containing twice-autoclaved soil (8 seedlings per pot; 5 replicate pots per treatment). Plants were maintained under a 14 h/10 h light/dark cycle at 30 °C/20 °C and watered as needed. (B) Disease-related parameters measured 10 days post-inoculation: seedling survival, fresh and dry shoot weight, stalk diameter, and plant height. Data are presented as mean $\pm$ standard deviation. Significance was determined by *t*-test (* $p < 0.05$, *** $p < 0.001$).

3.9. Deletion of FoADS-1 Increases Azole Susceptibility in *F. oxysporum*

We next extended our observations to *F. oxysporum*, a pathogenic fungus causing *Fusarium* Wilt Syndrome. In *F. oxysporum*, the protein encoded by FOXG_09079 shared the

greatest similarity with FvADS-1 (98% sequence identical [697/709]; 98% sequence positive [701/709]) and was named as FoADS-1 in this study.

To investigate FvADS-1 functional conservation, we constructed a *FoADS-1* knockout mutant (*FoADS-1*^{KO}) by protoplast transformation as previously described. The *FoADS-1*^{KO} strain exhibited wild-type growth on drug-free PDA but showed significantly increased sensitivity to KTC (20 µg/mL), VRC (2 µg/mL), and TRA (30 µg/mL) (Figure 8A,B). The above data confirm that ADS-1-mediated azole resistance is a conserved function within the *Fusarium* genus.

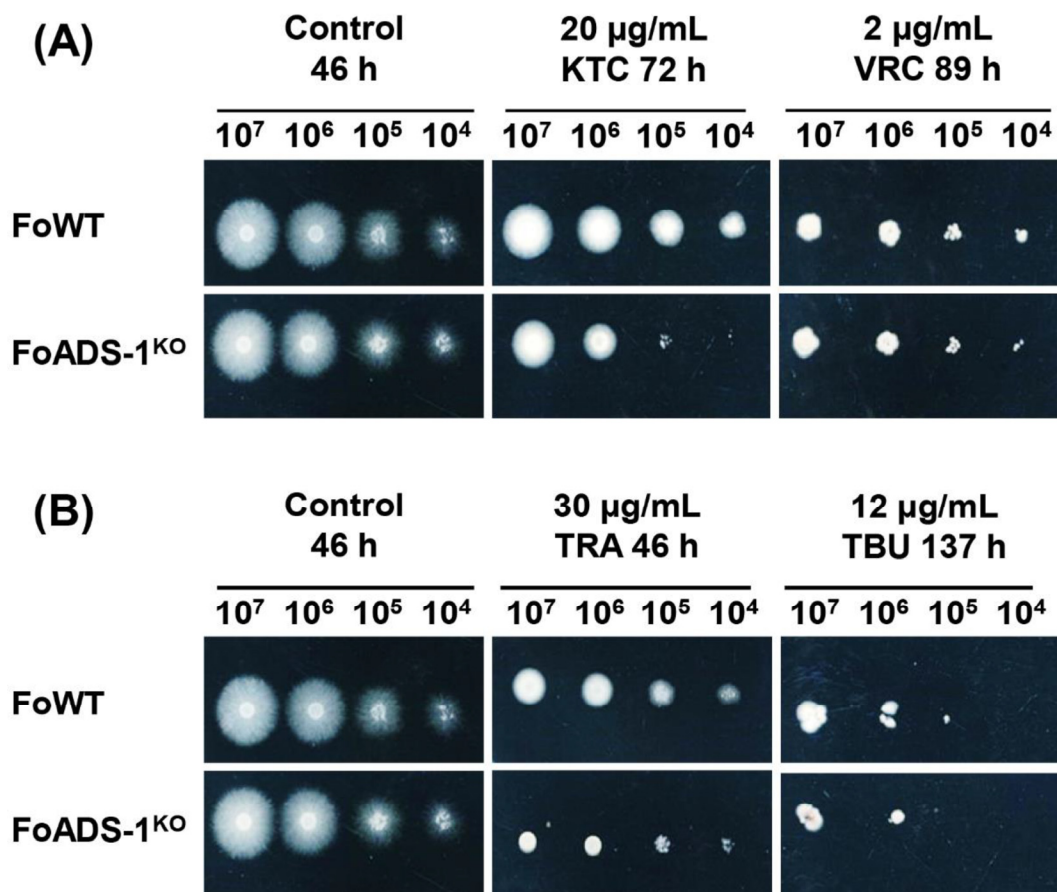


Figure 8. Functional analysis of FoADS-1 in the azole stress response of *Fusarium oxysporum*. Azole susceptibility assays of *F. oxysporum* wild-type strain (FoWT) and the *FoADS-1* knockout mutant (*FoADS-1*^{KO}). Ten-fold serial dilutions of conidial suspensions were spotted onto PDA plates containing (A) the clinical azole KTC or VRC, or (B) the agricultural azole TRA. Untreated PDA plates served as controls. Plates were incubated at 28 °C in the dark. Experiments were performed in three independent biological replicates.

4. Discussion

Although azole antifungals are widely used in clinic and agriculture [12,13,16], fungi can adapt to azole stress by altering the transcriptional expression of a variety of genes [37,49,58–60]. Therefore, understanding the mechanisms by which fungi mediate the transcriptional responses to azole stress, is valuable for decoding the mechanisms of azole resistance and developing effective treatment strategies. However, this is largely unknown in pathogenic *Fusarium* species, especially the dual pathogen *F. verticillioides*, which causes infections in both maize and humans [6,61–63]. In this study, by characterizing the transcriptional response to azoles in *F. verticillioides*, we identified the transcription factor FvADS-1 with a positive role in azole resistance by co-regulating drug efflux and ergosterol

synthesis. FoADS-1, the *F. oxysporum* homolog of FvADS-1, is essential for azole resistance. Its widespread evolutionary conservation across *Fusarium* genomes indicates an important conserved function within the *Fusarium* genus, including in other clinically important *Fusarium* species. Interestingly, FvADS-1 is homologous to *N. crassa* ADS-1, which similarly responds transcriptionally to azoles and contributes to resistance by regulating drug efflux pumps and azole target genes. This suggests that divergent fungi, particularly filamentous species, may employ a similar transcriptional regulatory strategy to cope with azole stress. More importantly, FvADS-1 also positively regulates the virulence of *F. verticillioides*, potentially through its influence on ABC transporters, sterol biosynthesis, and oxidative stress responses. Thus, our study identifies FvADS-1 as a key regulator of the azole stress response and virulence in *F. verticillioides*, providing novel insights into azole resistance and virulence in *Fusarium*.

Azoles are synthetic antifungal agents not encountered in natural environments [64,65]. Crystallographic studies have shown that the nitrogen atom in the azole ring coordinates the heme iron of CYP51, occupying the same position as the oxygen atom in the sterol substrate during catalysis [66,67]. This molecular mimicry underlies their competitive inhibition [64]. Fungi have likely evolved responsive networks to handle naturally occurring sterol-like inhibitors, such as plant saponins [68], phytosterols [69], and isatin derivatives [70,71], which compete for CYP51 binding or disrupt membrane integrity. Moreover, natural physiological stresses—including hypoxia and iron limitation—can also compromise ergosterol biosynthesis, suggesting that the regulatory circuitry governing sterol homeostasis is part of an ancient, integrated system for coping with diverse forms of sterol stress. Upon azole treatment, the expression of genes involved in diverse cellular processes is altered in *F. verticillioides*, reflecting the activation of an ancient, pre-adapted stress-response program. As an essential component of the fungal cell membranes, inhibition of ergosterol biosynthesis leads to widespread cellular impairments including cell membrane fluidity and integrity, vacuolar acidification, mitochondrial defects, etc. [72–74]. Consequently, it is not surprising that sterol biosynthesis inhibition triggers widespread transcriptional reprogramming to maintain cellular homeostasis. Among them, genes encoding drug efflux pumps (ABC transporters) and drug targets (ergosterol biosynthesis enzymes) are commonly induced and represent the most common mechanisms of azole resistance in fungi [75]. ABC transporters mediate azole efflux via ATP hydrolysis [76], while drug targets upregulation buffers the toxicity of azole inhibition [77,78]. Consistent with this, our study demonstrates activated expression of ABC transporters and ergosterol biosynthetic genes under azole stress (Figure 1B). Additionally, genes involved in fatty acid, amino acid, and heme metabolism were induced, while those involved in translation and mitochondrial function were repressed (Figure 1B,C), mirroring responses observed in other fungi [18,19,50,79]. These processes have been demonstrated to be functionally associated with sterol biosynthesis, such as lipid metabolism [80], heme biosynthesis [81,82], translation [50,79] and mitochondrial function [83]. Thus, our results regarding the responses to azole stress in *F. verticillioides* highlight a conserved fungal strategy for coping with sterol biosynthesis inhibition.

Despite similar overall responses to azole stress, the underlying transcriptional regulatory mechanisms differ among fungi. For instance, sterol biosynthesis is regulated by SREBP in *S. pombe*, Upc2p in *S. cerevisiae*, and multiple transcription factors in *N. crassa* in response to ergosterol depletion [37,84–88]. Regulation of efflux pumps is even more complex, involving Pdr5p in *S. cerevisiae*, Tac1p in *Candida* species, AtrR in *A. fumigatus*, and ADS-1, CCG-8, and CSP-1 in *N. crassa*. This diversity likely stems from the lower conservation and functional rewiring of transcription factors across fungi [37,88–92]. Interestingly, our study reveals that FvADS-1, a conserved transcription factor among filamentous fungi,

regulates both sterol biosynthesis genes and drug efflux pumps under azole stress, similar to its homologs in *N. crassa* and *Aspergillus flavus*. Thus, ADS-1 represents a conserved regulator of the azole stress response in filamentous fungi. Azoles transcriptionally activate *ADS-1*, thereby modulating the fungal stress response. However, the function of *ADS-1* may also diverge; while *N. crassa ads-1* deletion does not affect oxidative stress resistance, the *F. verticillioides FvADS-1* deletion mutant exhibits increased H₂O₂ sensitivity, potentially via peroxisomal regulation. This suggests functional adaptation of transcription factors among fungi, warranting further investigation.

The mechanisms by which *FvADS-1* regulates the azole stress response involve multiple pathways (summarized in Figure 9). First, *FvADS-1* positively regulates the transcriptional expression of ABC transporters in *F. verticillioides*. Drug efflux pumps play a crucial role in azole resistance [51]. ABC transporters are one of the main azole efflux pumps in fungi. In this study, we found that *FvADS-1* positively regulates the transcriptional expression of 7 ABC transporter genes to KTC stress (Table 2). Consistent with this, deletion of *FvADS-1* resulted in increased intracellular KTC accumulation (Figure 4C). Thus, *FvADS-1* enhances azole efflux by activating ABC transporter expression, contributing to resistance. Second, *FvADS-1* positively regulates the transcriptional expression of ergosterol biosynthesis genes under KTC stress. Upregulation of these genes enhances azole resistance in many fungi, including *Candida albicans*, *Saccharomyces cerevisiae*, and *N. crassa* [18,38,93,94]. We found that *FvADS-1* positively regulates the transcription of *FvERG3*, *FvERG12*, *FvERG25*, and the azole target gene *FvCYP51A* in *F. verticillioides* (Table 2). Accordingly, deletion of *FvADS-1* significantly reduced intracellular ergosterol content under azole stress (Figure 5D). Therefore, *FvADS-1* plays a key role in maintaining ergosterol biosynthesis under KTC stress. Third, *FvADS-1* positively regulates fungal response to oxidative stress. Antifungals such as ketoconazole can induce oxidative stress [48,73,95,96]. We found that deletion of *FvADS-1* alters peroxisomal gene expression and increases sensitivity to H₂O₂ (Figure 6). Therefore, *FvADS-1* potentiates the ability of *F. verticillioides* to cope with oxidative stress, providing a coordinated defense in which cells increase drug efflux and promote steroid biosynthesis together, thereby enabling survival under azole pressure. Taken together, these findings demonstrate that *FvADS-1* regulates azole resistance through multiple, integrated mechanisms. Based on our transcriptomic and genetic evidence, we propose that *FvADS-1* likely functions as a direct transcriptional activator of azole-responsive genes, an inference supported by its conserved Zn(II)₂Cys₆ DNA-binding domain and the rapid, coordinated induction of target genes. However, direct evidence of promoter binding, such as through ChIP-seq, ChIP-qPCR, or EMSA, is not included in the present study. Definitive validation of its DNA-binding activity and genome-wide target mapping therefore remains a key objective for future research.

In addition to modulating azole sensitivity, *FvADS-1* is also critical for the virulence of *F. verticillioides* to maize (Figure 7). Previous studies have shown that alterations in ergosterol biosynthesis affect the virulence of *F. graminearum*. For example, silencing lanosterol C14 α -demethylase-encoding genes (*CYP51*) attenuated infection symptoms on *Arabidopsis* leaves [97]. Deletion of *FgERG3* and *FgERG4* also dramatically impaired virulence on wheat [56,57]. ABC transporters have also been implicated in the virulence of *C. albicans*. *Cdr1p* and *Cdr2p* (ABC transporters) export phospholipids to maintain membrane stability, evading host immune recognition [98,99]. ABC transporters contribute to biofilm formation and pathogenicity [100,101]. Moreover, reactive oxygen species (ROS) play a crucial role in pathogen-host interactions. Plants employ oxidative bursts as an early defense mechanism against pathogens [56,57]. Fumonisin are virulence-associated metabolites whose biosynthetic genes (*WDR1*, *PNG1*, *ORF21*) are upregulated under azole stress in the *FvADS-1* deletion mutant (Table S4), indicating that *FvADS-1* participates in the drug-

responsive regulation of the fumonisin pathway. However, whether this transcriptional change leads to altered fumonisin production remains an important open question. Taken together, FvADS-1 has roles in regulating the above virulence-associated processes, thus indicating that FvADS-1 affects *F. verticillioides* infections on maize by integrating multiple physiological responses critical for both azole survival and host pathogenicity. However, the exact mechanism involved remains to be further explored.

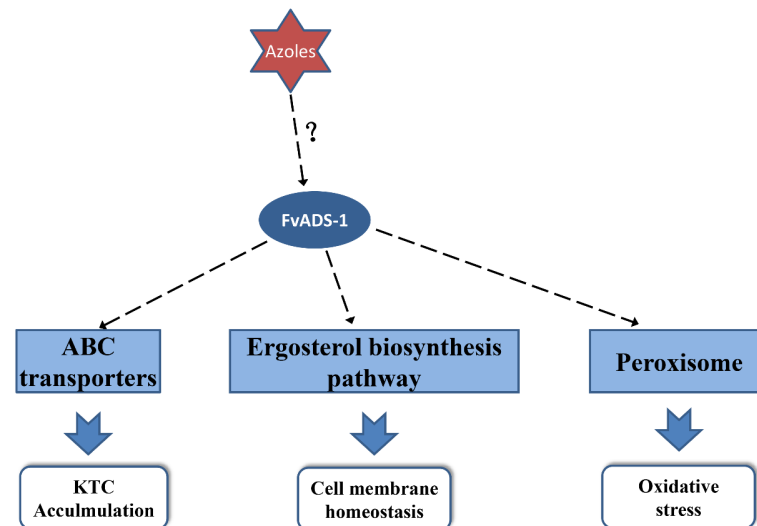


Figure 9. Model of the regulatory mechanism of FvADS-1 in response to azole stress. Under azole stress, *FvADS-1* transcription is activated. The FvADS-1 protein subsequently promotes the expression of downstream target genes, including those encoding ABC transporters, ergosterol biosynthesis pathway enzymes, and peroxisomal components, thereby coordinating adaptive responses to azole exposure. Question marks (?) indicate that the transcription of *FvADS-1* is activated by an unknown factor under azole stress. Dashed arrows denote an unidentified type of regulation, while solid blue arrows represent the affected biological processes.

In summary, we identified a transcription factor FvADS-1 in *F. verticillioides*, as well as its homolog FoADS-1 in *F. oxysporum*. Under azole stress, the transcription level of *FvADS-1* was significantly increased and FvADS-1 positively regulated KTC efflux, ergosterol biosynthesis and the oxidative stress response, thereby promoting azole resistance. Our study, along with our previous studies, indicates that the transcription factor ADS-1 is a conserved regulator of ABC transporters and sterol biosynthesis in fungi belonging to Pezizomycotina, and exhibits a conserved regulatory role similar to that of AtrR in *Aspergillus* [58,91], while also likely operating within a broader interactive network. More importantly, we revealed a role of FvADS-1 in the virulence of *F. verticillioides*, highlighting the important role of ADS-1 in pathogenic fungi. Collectively, our study identified a conserved transcription factor governing both azole stress response and the virulence of *F. verticillioides*, providing new insights in azole stress response and virulence in pathogenic fungi and giving comprehensive theoretical guidance for the formulation of therapeutic regimens for pathogenic filamentous fungi clinically and agriculturally.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jof12020157/s1>, Figure S1: KEGG enrichment analysis of azole-responsive genes in *Fusarium verticillioides* wild-type strain (FvWT); Figure S2: The transcription factor FvADS-1 is highly conserved among filamentous fungi; Figure S3: Roles of FvADS-1 in osmotic and temperature stress responses and conidial production; Figure S4: Correlation analysis of transcriptomic profiles across samples; Table S1: Strains used in this study; Table S2: Primers used for strain and plasmid construction in this study; Table S3: Primers used for qRT-PCR; Table S4: Transcriptional

profiling of fumonisin biosynthetic genes across *Fusarium* strains by RNA-seq analysis; Supplemental File S1: All genes identified after the removal of low-abundance transcripts; Supplemental File S2: KTC upregulated genes; Supplemental File S3: KTC downregulated genes; Supplemental File S4: FvADS-1 dependent KTC upregulated genes; Supplemental File S5: FvADS-1 dependent KTC downregulated genes.

Author Contributions: Conceptualization, Y.Y. and C.H.; methodology, Y.Y., H.Z. and Z.Z.; validation, Y.Y., M.Z. and C.H.; data curation, H.Z., Z.Z. and M.Z.; writing—original draft preparation, Y.Y. and C.H.; writing—review and editing, Y.Y., H.Z., Z.Z., M.Z. and C.H.; supervision, S.L. and C.H.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This project was supported by grants from the Natural Science Foundation of Tianjin, China (23JCQNJC00780), the Tianjin Municipal Education Commission Project (2023KJ209).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The RNA-seq data have been deposited in the GEO database under accession number GSE315078. Data are included in the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Bacon, C.W.; Nelson, P.E. Fumonisin production in corn by toxigenic strains of *Fusarium moniliforme* and *Fusarium proliferatum*. *J. Food Prot.* **1994**, *57*, 514–521. [\[CrossRef\]](#)
- Li, W.; He, P.; Jin, J. Effect of potassium on ultrastructure of maize stalk pith and young root and their relation to stalk rot resistance. *Agric. Sci. China* **2010**, *9*, 1467–1474. [\[CrossRef\]](#)
- Shim, W.B.; Flaherty, J.E.; Woloshuk, C.P. Comparison of fumonisin B1 biosynthesis in maize germ and degermed kernels by *Fusarium verticillioides*. *J. Food Prot.* **2003**, *66*, 2116–2122. [\[CrossRef\]](#)
- Bush, B.J.; Carson, M.L.; Cubeta, M.A.; Hagler, W.M.; Payne, G.A. Infection and fumonisin production by *Fusarium verticillioides* in developing maize kernels. *Phytopathology* **2004**, *94*, 88–93. [\[CrossRef\]](#) [\[PubMed\]](#)
- Silva, J.J.; Viaro, H.P.; Ferranti, L.S.; Oliveira, A.L.M.; Ferreira, J.M.; Ruas, C.F.; Ono, E.Y.S.; Fungaro, M.H.P. Genetic structure of *Fusarium verticillioides* populations and occurrence of fumonisins in maize grown in Southern Brazil. *Crop Prot.* **2017**, *99*, 160–167. [\[CrossRef\]](#)
- Blacutt, A.A.; Gold, S.E.; Voss, K.A.; Gao, M.; Glenn, A.E. *Fusarium verticillioides*: Advancements in understanding the toxicity, virulence, and niche adaptations of a model mycotoxigenic pathogen of maize. *Phytopathology* **2018**, *108*, 312–326. [\[CrossRef\]](#)
- Dignani, M.C.; Anaissie, E. Human fusariosis. *Clin. Microbiol. Infect. Dis.* **2004**, *10*, 67–75. [\[CrossRef\]](#) [\[PubMed\]](#)
- Tortorano, A.M.; Prigitano, A.; Esposto, M.C.; Arsic Arsenijevic, V.; Kolarovic, J.; Ivanovic, D.; Paripovic, L.; Klingspor, L.; Nordoy, I.; Hamal, P.; et al. European Confederation of Medical Mycology (ECMM) epidemiological survey on invasive infections due to *Fusarium* species in Europe. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 1623–1630. [\[CrossRef\]](#)
- Nucci, M.; Anaissie, E. *Fusarium* infections in immunocompromised patients. *Clin. Microbiol. Rev.* **2007**, *20*, 695–704. [\[CrossRef\]](#)
- Fanci, R.; Pini, G.; Bartolesi, A.M.; Pecile, P. Refractory disseminated fusariosis by *Fusarium verticillioides* in a patient with acute myeloid leukaemia relapsed after allogeneic hematopoietic stem cell transplantation: A case report and literature review. *Rev. Iberoam. Micol.* **2013**, *30*, 51–53. [\[CrossRef\]](#)
- Rosa, P.D.d.; Ramirez-Castrillon, M.; Borges, R.; Aquino, V.; Meneghello Fuentesfria, A.; Zubaran Goldani, L. Epidemiological aspects and characterization of the resistance profile of *Fusarium* spp. in patients with invasive fusariosis. *J. Med. Microbiol.* **2019**, *68*, 1489–1496. [\[CrossRef\]](#)
- Perfect, J.R.; Marr, K.A.; Walsh, T.J.; Greenberg, R.N.; DuPont, B.; De La Torre-Cisneros, J.; Just-Nübling, G.; Schlamm, H.T.; Lutsar, I.; Espinel-Ingroff, A.; et al. Voriconazole treatment for less-common, emerging, or refractory fungal infections. *Clin. Infect. Dis.* **2003**, *36*, 1122–1131. [\[CrossRef\]](#) [\[PubMed\]](#)
- Reis, A.; Sundmacher, R.; Tinteln, K.; Agostini, H.; Jensen, H.; Althaus, C. Successful treatment of ocular invasive mould infection (fusariosis) with the new antifungal agent voriconazole. *Br. J. Ophthalmol.* **2000**, *84*, 932–933. [\[CrossRef\]](#)
- Shapiro, R.S.; Robbins, N.; Cowen, L.E. Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol. Mol. Biol. Rev.* **2011**, *75*, 213–267. [\[CrossRef\]](#)
- Li, D.; Amburgey-Crovetti, K.; Applebach, E.; Steen, T.Y.; Calderone, R. The dual pathogen *Fusarium*: Diseases, incidence, azole resistance, and biofilms. *J. Fungi* **2025**, *11*, 294. [\[CrossRef\]](#)

16. Raad, I.I.; Hachem, R.Y.; Herbrecht, R.; Graybill, J.R.; Hare, R.; Corcoran, G.; Kontoyiannis, D.P. Posaconazole as salvage treatment for invasive fusariosis in patients with underlying hematologic malignancy and other conditions. *Clin. Infect. Dis.* **2006**, *42*, 1398–1403. [[CrossRef](#)] [[PubMed](#)]
17. Muhammed, M.; Coleman, J.J.; Carneiro, H.A.; Mylonakis, E. The challenge of managing fusariosis. *Virulence* **2011**, *2*, 91–96. [[CrossRef](#)]
18. Agarwal, A.K.; Rogers, P.D.; Baerson, S.R.; Jacob, M.R.; Barker, K.S.; Cleary, J.D.; Walker, L.A.; Nagle, D.G.; Clark, A.M. Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **2003**, *278*, 34998–35015. [[CrossRef](#)]
19. Liu, T.T.; Lee, R.E.B.; Barker, K.S.; Lee, R.E.; Wei, L.; Homayouni, R.; Rogers, P.D. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* **2005**, *49*, 2226–2236. [[CrossRef](#)] [[PubMed](#)]
20. Hu, C.; Zhou, M.; Cao, X.; Xue, W.; Zhang, Z.; Li, S.; Sun, X. Coordinated regulation of membrane homeostasis and drug accumulation by novel kinase STK-17 in response to antifungal azole treatment. *Microbiol. Spectr.* **2022**, *10*, e00127-22. [[CrossRef](#)]
21. Klimova, N.; Yeung, R.; Kachurina, N.; Turcotte, B. Phenotypic analysis of a family of transcriptional regulators, the zinc cluster proteins, in the human fungal pathogen *Candida glabrata*. *G3 Genes Genomes Genet.* **2014**, *4*, 931–940. [[CrossRef](#)]
22. MacPherson, S.; Larochelle, M.; Turcotte, B. A fungal family of transcriptional regulators: The zinc cluster proteins. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 583–604. [[CrossRef](#)] [[PubMed](#)]
23. Zuriegat, Q.; Zheng, Y.; Liu, H.; Wang, Z.; Yun, Y. Current progress on pathogenicity-related transcription factors in *Fusarium oxysporum*. *Mol. Plant Pathol.* **2021**, *22*, 882–895. [[CrossRef](#)]
24. Zhang, Q.; Gao, M.; Wu, L.; Wu, H.; Chen, Y.; Wang, Y. Expression network of transcription factors in resistant and susceptible tung trees responding to *Fusarium* wilt disease. *Ind. Crops Prod.* **2018**, *122*, 10. [[CrossRef](#)]
25. Liu, Z.; Jian, Y.; Chen, Y.; Kistler, H.C.; He, P.; Ma, Z.; Yin, Y. A phosphorylated transcription factor regulates sterol biosynthesis in *Fusarium graminearum*. *Nat. Commun.* **2019**, *10*, 1228. [[CrossRef](#)] [[PubMed](#)]
26. Zhao, Y.; Sun, H.; Li, J.; Ju, C.; Huang, J. The transcription factor FgAtrR regulates asexual and sexual development, virulence, and DON production and contributes to intrinsic resistance to azole fungicides in *Fusarium graminearum*. *Biology* **2022**, *11*, 326. [[CrossRef](#)]
27. Wang, C.; Li, P.; Cong, W.; Ma, N.; Zhou, M.; Hou, Y. The transcription factor FgCreA modulates ergosterol biosynthesis and sensitivity to DMI fungicides by regulating transcription of *FgCyp51A* and *FgErg6A* in *Fusarium graminearum*. *Int. J. Biol. Macromol.* **2025**, *284*, 137903. [[CrossRef](#)]
28. Wang, H.; Gai, Y.; Zhao, Y.; Wang, M.; Ma, Z. The calcium-calcineurin and high-osmolarity glycerol pathways co-regulate tebuconazole sensitivity and pathogenicity in *Fusarium graminearum*. *Pestic. Biochem. Physiol.* **2023**, *190*, 105311. [[CrossRef](#)]
29. Lan, N.; Zhang, H.; Hu, C.; Wang, W.; Calvo, A.M.; Harris, S.D.; Chen, S.; Lia, S. Coordinated and Distinct Functions of Velvet Proteins in *Fusarium verticillioides*. *Eukaryot. Cell* **2014**, *13*, 909–918. [[CrossRef](#)]
30. Kück, U.; Hoff, B. New tools for the genetic manipulation of filamentous fungi. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 51–62. [[CrossRef](#)]
31. Keeler, C.L.; Kingsley, D.H.; Burton, C.R.A. Identification of the thymidine kinase gene of infectious laryngotracheitis virus. *Avian Dis.* **1991**, *35*, 920–929. [[CrossRef](#)]
32. Yoruk, E.; Albayrak, G. Geneticin (G418) resistance and electroporation-mediated transformation of *Fusarium graminearum* and *F. culmorum*. *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 268–273. [[CrossRef](#)]
33. Gu, X.; Xue, W.; Yin, Y.; Liu, H.; Li, S.; Sun, X. The Hsp90 co-chaperones Sti1, Aha1, and P23 regulate adaptive responses to antifungal azoles. *Front. Microbiol.* **2016**, *7*, 1571. [[CrossRef](#)]
34. Azor, M.; Gene, J.; Cano, J.; Sutton, D.A.; Fothergill, A.W.; Rinaldi, M.G.; Guarro, J. In vitro antifungal susceptibility and molecular characterization of clinical isolates of *Fusarium verticillioides* (*F. moniliforme*) and *Fusarium thapsinum*. *Antimicrob. Agents Chemother.* **2008**, *52*, 2228–2231. [[CrossRef](#)]
35. Zhou, M.; Yu, P.; Hu, C.; Fang, W.; Jin, C.; Li, S.; Sun, X. Suppressed Protein Translation Caused by MSP-8 Deficiency Determines Fungal Multidrug Resistance with Fitness Cost. *Adv. Sci.* **2025**, *12*, e2412514. [[CrossRef](#)]
36. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]
37. Yin, Y.; Zhang, H.; Zhang, Y.; Hu, C.; Sun, X.; Liu, W.; Li, S. Fungal Zn(II)2Cys6 transcription factor ADS-1 regulates drug efflux and ergosterol metabolism under antifungal azole stress. *Antimicrob. Agents Chemother.* **2021**, *65*, e01316-20. [[CrossRef](#)] [[PubMed](#)]
38. Hu, C.; Zhou, M.; Wang, W.; Sun, X.; Yarden, O.; Li, S. Abnormal ergosterol biosynthesis activates transcriptional responses to antifungal azoles. *Front. Microbiol.* **2018**, *9*, 9. [[CrossRef](#)] [[PubMed](#)]
39. Glenn, A.E.; Gold, S.E.; Bacon, C.W. *Fdb1* and *Fdb2*, *Fusarium verticillioides* loci necessary for detoxification of preformed antimicrobials from corn. *Mol. Plant-Microbe Interact.* **2002**, *15*, 91–101. [[CrossRef](#)] [[PubMed](#)]

40. Bacon, C.W.; Hinton, D.M.; Richardson, M.D. A corn seedling assay for resistance to *Fusarium moniliforme*. *Plant Dis.* **1994**, *78*, 302–305. [\[CrossRef\]](#)
41. Nirmaladevi, D.; Venkataramana, M.; Srivastava, R.K.; Uppalapati, S.R.; Gupta, V.K.; Yli-Mattila, T.; Clement Tsui, K.M.; Srinivas, C.; Niranjana, S.R.; Chandra, N.S. Molecular phylogeny, pathogenicity and toxigenicity of *Fusarium oxysporum* f. sp. *lycopersici*. *Sci. Rep.* **2016**, *6*, 21367. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Xu, Y.; Zhou, H.; Zhao, G.; Yang, J.; Luo, Y.; Sun, S.; Wang, Z.; Li, S.; Jin, C. Genetical and O-glycoproteomic analyses reveal the roles of three protein O-mannosyltransferases in phytopathogen *Fusarium oxysporum* f.sp. *cucumerinum*. *Fungal Genet. Biol.* **2020**, *134*, 103285. [\[CrossRef\]](#)
43. Du, W.; Zhai, P.; Wang, T.; Bromley, M.J.; Zhang, Y.; Lu, L. The C₂H₂ transcription factor SltA contributes to azole resistance by coregulating the expression of the drug target Erg11A and the drug efflux pump Mdr1 in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2021**, *65*, e01839-20. [\[CrossRef\]](#)
44. Zeng, M.; Zhou, X.; Yang, C.; Liu, Y.; Zhang, J.; Xin, C.; Qin, G.; Liu, F.; Song, Z. Comparative analysis of the biological characteristics and mechanisms of azole resistance of clinical *Aspergillus fumigatus* strains. *Front. Microbiol.* **2023**, *14*, 1253197. [\[CrossRef\]](#)
45. Jorda, T.; Puig, S. Regulation of ergosterol biosynthesis in *Saccharomyces cerevisiae*. *Genes* **2020**, *11*, 795. [\[CrossRef\]](#)
46. Czajka, K.M.; Venkataraman, K.; Brabant-Kirwan, D.; Santi, S.A.; Verschoor, C.; Appanna, V.D.; Singh, R.; Saunders, D.P.; Tharmalingam, S. Molecular mechanisms associated with antifungal resistance in pathogenic *Candida* species. *Cells* **2023**, *12*, 2655. [\[CrossRef\]](#)
47. Kolaczowska, A.; Kolaczowski, M.; Delahodde, A.; Goffeau, A. Functional dissection of Pdr1p, a regulator of multidrug resistance in *Saccharomyces cerevisiae*. *Mol. Genet. Genom.* **2002**, *267*, 96–106. [\[CrossRef\]](#)
48. Sasse, C.; Bastakis, E.; Bakti, F.; Höfer, A.M.; Zangl, I.; Schüller, C.; Köhler, A.M.; Gerke, J.; Krappmann, S.; Finkernagel, F.; et al. Induction of *Aspergillus fumigatus* zinc cluster transcription factor OdrA/Mdu2 provides combined cellular responses for oxidative stress protection and multiple antifungal drug resistance. *mBio* **2023**, *14*, e0262823. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Li, Y.; Dai, M.; Lu, L.; Zhang, Y. The C₂H₂-type transcription factor ZfpA, coordinately with CrzA, affects azole susceptibility by regulating the multidrug transporter gene *atrF* in *Aspergillus fumigatus*. *Microbiol. Spectr.* **2023**, *11*, 00325-23.
50. Liu, X.; Jiang, J.; Shao, J.; Yin, Y.; Ma, Z. Gene transcription profiling of *Fusarium graminearum* treated with an azole fungicide tebuconazole. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1105–1114. [\[CrossRef\]](#)
51. Zhang, Y.; Zhang, Z.; Zhang, X.; Zhang, H.; Sun, X.; Hu, C.; Li, S. CDR4 is the major contributor to azole resistance among four Pdr5p-like ABC transporters in *Neurospora crassa*. *Fungal Biol.* **2012**, *116*, 848–854. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Slaven, J.W.; Anderson, M.J.; Sanglard, D.; Dixon, G.K.; Bille, J.; Roberts, I.S.; Denning, D.W. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal Genet. Biol.* **2002**, *36*, 199–206. [\[CrossRef\]](#)
53. Becher, R.; Weihmann, F.; Deising, H.B.; Wirsal, S.G. Development of a novel multiplex DNA microarray for *Fusarium graminearum* and analysis of azole fungicide responses. *BMC Genom.* **2011**, *12*, 52. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Bhattacharya, S.; Esquivel, B.D.; White, T.C. Overexpression or deletion of ergosterol biosynthesis genes alters doubling time, response to stress agents, and drug susceptibility in *Saccharomyces cerevisiae*. *Am. Soc. Microbiol.* **2018**, *9*, e01291-18. [\[CrossRef\]](#)
55. Abou Ammar, G.; Tryono, R.; Doll, K.; Karlovsky, P.; Deising, H.B.; Wirsal, S.G. Identification of ABC transporter genes of *Fusarium graminearum* with roles in azole tolerance and/or virulence. *PLoS ONE* **2013**, *8*, e79042. [\[CrossRef\]](#)
56. Yun, Y.; Yin, D.; Dawood, D.H.; Liu, X.; Chen, Y.; Ma, Z. Functional characterization of FgERG3 and FgERG5 associated with ergosterol biosynthesis, vegetative differentiation and virulence of *Fusarium graminearum*. *Fungal Genet. Biol.* **2014**, *68*, 60–70. [\[CrossRef\]](#)
57. Liu, X.; Jiang, J.; Yin, Y.; Ma, Z. Involvement of FgERG4 in ergosterol biosynthesis, vegetative differentiation and virulence in *Fusarium graminearum*. *Mol. Plant Pathol.* **2013**, *14*, 71–83. [\[CrossRef\]](#)
58. Paul, S.; Stamnes, M.; Thomas, G.H.; Liu, H.; Hagiwara, D.; Gomi, K.; Filler, S.G.; Moye-Rowley, W.S. AtrR is an essential determinant of azole resistance in *Aspergillus fumigatus*. *Mol. Biol. Physiol.* **2019**, *10*, e02563-18. [\[CrossRef\]](#)
59. Shrivastava, M.; Kouyoumdjian, G.S.; Kirbizakis, E.; Ruiz, D.; Henry, M.; Vincent, A.T.; Sellam, A.; Whiteway, M. The Atr1 transcription factor directs regulation of the ergosterol pathway and azole resistance in *Candida albicans*. *mBio* **2023**, *14*, 01807-23. [\[CrossRef\]](#)
60. Degradi, L.; Tava, V.; Esposto, M.C.; Prigitano, A.; Bulgari, D.; Kunova, A.; Saracchi, M.; Cortesi, P.; Pasquali, M. Genomic Insights into *Fusarium verticillioides* diversity: The genome of two clinical isolates and their demethylase inhibitor fungicides susceptibility. *Pathogens* **2024**, *13*, 1062. [\[CrossRef\]](#) [\[PubMed\]](#)
61. Tezcan, G.; Ozhak-Baysan, B.; Alastruey-Izquierdo, A.; Ogunc, D.; Ongut, G.; Yildiran, S.T.; Hazar, V.; Cuenca-Estrella, M.; Rodriguez-Tudela, J.L. Disseminated fusariosis caused by *Fusarium verticillioides* in an acute lymphoblastic leukemia patient after allogeneic hematopoietic stem cell transplantation. *J. Clin. Microbiol.* **2009**, *47*, 278–281. [\[CrossRef\]](#)

62. Guarro, J. Fusariosis, a complex infection caused by a high diversity of fungal species refractory to treatment. *Eur. J. Clin. Microbiol. Infect. Dis.* **2013**, *32*, 1491–1500. [[CrossRef](#)]
63. Batista, B.G.; Chaves, M.A.; Reginatto, P.; Saraiva, O.J.; Fuentefria, A.M. Human fusariosis: An emerging infection that is difficult to treat. *Rev. Soc. Bras. Med. Trop.* **2020**, *53*, e20200013. [[CrossRef](#)]
64. Warrilow, A.G.; Martel, C.M.; Parker, J.E.; Melo, N.; Lamb, D.C.; Nes, W.D.; Kelly, D.E.; Kelly, S.L. Azole binding properties of *Candida albicans* sterol 14-alpha demethylase (CaCYP51). *Antimicrob. Agents Chemother.* **2010**, *54*, 4235–4245. [[CrossRef](#)] [[PubMed](#)]
65. Lepesheva, G.I.; Waterman, M.R. Sterol 14 α -demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *Biochim. Biophys. Acta* **2007**, *1770*, 467–477. [[CrossRef](#)]
66. Monk, B.C.; Tomasiak, T.M.; Keniya, M.V.; Huschmann, F.U.; Tyndall, J.D.; O'Connell, J.D., 3rd; Cannon, R.D.; McDonald, J.G.; Rodriguez, A.; Finer-Moore, J.S.; et al. Architecture of a single membrane spanning cytochrome P450 suggests constraints that orient the catalytic domain relative to a bilayer. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3865–3870. [[CrossRef](#)]
67. Hargrove, T.Y.; Friggeri, L.; Wawrzak, Z.; Qi, A.; Hoekstra, W.J.; Schotzinger, R.J.; York, J.D.; Guengerich, F.P.; Lepesheva, G.I. Structural analyses of *Candida albicans* sterol 14alpha-demethylase complexed with azole drugs address the molecular basis of azole-mediated inhibition of fungal sterol biosynthesis. *J. Biol. Chem.* **2017**, *292*, 6728–6743. [[CrossRef](#)] [[PubMed](#)]
68. Osbourn, A.E. Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell* **1996**, *8*, 1821–1831. [[CrossRef](#)] [[PubMed](#)]
69. Weete, J.D.; Gandhi, S.R. Sterols of the phylum Zygomycota: Phylogenetic implications. *Lipids* **1997**, *32*, 1309–1316. [[CrossRef](#)]
70. Nain, S.; Mathur, G.; Anthwal, T.; Sharma, S.; Paliwal, S. Synthesis, characterization, and antibacterial activity of new isatin derivatives. *Pharm. Chem. J.* **2023**, *57*, 196–203. [[CrossRef](#)]
71. Tangadanchu, V.K.R.; Sui, Y.F.; Zhou, C.H. Isatin-derived azoles as new potential antimicrobial agents: Design, synthesis and biological evaluation. *Bioorganic Med. Chem. Lett.* **2021**, *41*, 128030. [[CrossRef](#)]
72. Kato, M.; Wickner, W. Ergosterol is required for the Sec18/ATP-dependent priming step of homotypic vacuole fusion. *EMBO J.* **2001**, *20*, 4035–4040. [[CrossRef](#)]
73. Yang, S.; Yan, D.; Li, M.; Li, D.; Zhang, S.; Fan, G.; Peng, L.; Pan, S. Ergosterol depletion under bifonazole treatment induces cell membrane damage and triggers a ROS-mediated mitochondrial apoptosis in *Penicillium expansum*. *Fungal Biol.* **2022**, *126*, 1–10. [[CrossRef](#)]
74. Chen, C.; Xie, Z.; Cong, L.; Mao, S.; Wang, L.; Wu, Y.; Zhang, Y.; Zhou, Q.; Ahmad, A.; Chang, W.; et al. Antifungal activity of a maleimide derivative: Disruption of cell membranes and interference with iron ion homeostasis. *Mycology* **2025**, *16*, 382–401. [[CrossRef](#)] [[PubMed](#)]
75. Perlin, D.S.; Rautemaa-Richardson, R.; Alastruey-Izquierdo, A. The global problem of antifungal resistance: Prevalence, mechanisms, and management. *Lancet Infect. Dis.* **2017**, *17*, e383–e392. [[CrossRef](#)] [[PubMed](#)]
76. Revie, N.M.; Iyer, K.R.; Robbins, N.; Cowen, L.E. Antifungal drug resistance: Evolution, mechanisms and impact. *Curr. Opin. Microbiol.* **2018**, *45*, 70–76. [[CrossRef](#)]
77. Sanglard, D.; Ischer, F.; Monod, M.; Bille, J. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: Characterization of CDR2, a new multidrug ABC transporter gene. *Microbiology* **1997**, *143*, 405–416. [[CrossRef](#)]
78. Perea, S.; López-Ribot, J.L.; Kirkpatrick, W.R.; McAtee, R.K.; Santillán, R.A.; Martínez, M.; Calabrese, D.; Sanglard, D.; Patterson, T.F. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **2001**, *45*, 2676–2684. [[CrossRef](#)] [[PubMed](#)]
79. da Silva Ferreira, M.E.; Malavazi, I.; Savoldi, M.; Brakhage, A.A.; Goldman, M.H.; Kim, H.S.; Nierman, W.C.; Goldman, G.H. Transcriptome analysis of *Aspergillus fumigatus* exposed to voriconazole. *Curr. Genet.* **2006**, *50*, 32–44. [[CrossRef](#)]
80. Swain, E.; Baudry, K.; Stuke, J.; McDonough, V.; Germann, M.; Nickels, J.T., Jr. Sterol-dependent regulation of sphingolipid metabolism in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **2002**, *277*, 26177–26184. [[CrossRef](#)]
81. Parks, L.W.; Casey, W.M. Physiological implications of sterol biosynthesis in yeast. *Annu. Rev. Microbiol.* **1995**, *49*, 95–116. [[CrossRef](#)]
82. Bammert, G.F.; Fostel, J.M. Genome-wide expression patterns in *Saccharomyces cerevisiae*: Comparison of drug treatments and genetic alterations affecting biosynthesis of ergosterol. *Antimicrob. Agents Chemother.* **2000**, *44*, 1255–1265. [[CrossRef](#)]
83. Shekhova, E.; Knemeyer, O.; Brakhage, A.A. Induction of mitochondrial reactive oxygen species production by itraconazole, terbinafine, and amphotericin b as a mode of action against *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2017**, *61*, e00978-17. [[CrossRef](#)]
84. Robichon, C.; Dugail, I. De novo cholesterol synthesis at the crossroads of adaptive response to extracellular stress through SREBP. *Biochimie* **2007**, *89*, 260–264. [[CrossRef](#)] [[PubMed](#)]
85. Vik, A.; Rine, J. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **2001**, *21*, 6395–6405. [[CrossRef](#)] [[PubMed](#)]

86. Sun, X.; Wang, K.; Yu, X.; Liu, J.; Zhang, H.; Zhou, F.; Xie, B.; Li, S. Transcription factor CCG-8 as a new regulator in the adaptation to antifungal azole stress. *Antimicrob. Agents Chemother.* **2014**, *58*, 1434–1442. [[CrossRef](#)]
87. Wang, K.; Zhang, Z.; Chen, X.; Sun, X.; Jin, C.; Liu, H.; Li, S. Transcription factor ADS-4 regulates adaptive responses and resistance to antifungal azole stress. *Antimicrob. Agents Chemother.* **2015**, *59*, 5396–5404. [[CrossRef](#)] [[PubMed](#)]
88. Chen, X.; Xue, W.; Zhou, J.; Zhang, Z.; Wei, S.; Liu, X.; Sun, X.; Wang, W.; Li, S. De-repression of CSP-1 activates adaptive responses to antifungal azoles. *Sci. Rep.* **2016**, *6*, 19447. [[CrossRef](#)]
89. Kolaczowska, A.; Kolaczowski, M.; Goffeau, A.; Moye-Rowley, W.S. Compensatory activation of the multidrug transporters Pdr5p, Snq2p, and Yor1p by Pdr1p in *Saccharomyces cerevisiae*. *FEBS Lett.* **2008**, *582*, 977–983. [[CrossRef](#)]
90. Coste, A.; Turner, V.; Ischer, F.; Morschhäuser, J.; Forche, A.; Selmecki, A.; Berman, J.; Bille, J.; Sanglard, D. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics* **2006**, *172*, 2139–2156. [[CrossRef](#)]
91. Hagiwara, D.; Miura, D.; Shimizu, K.; Paul, S.; Ohba, A.; Gono, T.; Watanabe, A.; Kamei, K.; Shintani, T.; MoyeRowley, W.S.; et al. A novel Zn²⁺-Cys⁶ transcription factor AtrR plays a key role in an azole resistance mechanism of *Aspergillus fumigatus* by co-regulating *cyp51A* and *cdr1B* expressions. *PLoS Pathog.* **2017**, *13*, e1006096. [[CrossRef](#)]
92. Xue, W.; Yin, Y.; Ismail, F.; Hu, C.; Zhou, M.; Cao, X.; Li, S.; Sun, X. Transcription factor CCG-8 plays a pivotal role in azole adaptive responses of *Neurospora crassa* by regulating intracellular azole accumulation. *Curr. Genet.* **2019**, *65*, 735–745. [[CrossRef](#)]
93. Heilmann, C.J.; Schneider, S.; Barker, K.S.; Rogers, P.D.; Morschhäuser, J. An A643T mutation in the transcription factor Upc2p causes constitutive *ERG11* upregulation and increased fluconazole resistance in *Candida albicans*. *Antimicrob. Agents Chemother.* **2010**, *54*, 353–359. [[CrossRef](#)]
94. Sun, X.; Wang, W.; Wang, K.; Yu, X.; Liu, J.; Zhou, F.; Xie, B.; Li, S. Sterol C-22 desaturase ERG5 mediates the sensitivity to antifungal azoles in *Neurospora crassa* and *Fusarium verticillioides*. *Front. Microbiol.* **2013**, *4*, 127. [[CrossRef](#)] [[PubMed](#)]
95. Munoz-Megias, M.L.; Sanchez-Fresneda, R.; Solano, F.; Maicas, S.; Martinez-Esparza, M.; Arguelles, J.C. The antifungal effect induced by itraconazole in *Candida parapsilosis* largely depends on the oxidative stress generated at the mitochondria. *Curr. Genet.* **2023**, *69*, 165–173. [[CrossRef](#)]
96. Khan, A.; Ahmad, A.; Akhtar, F.; Yousuf, S.; Xess, I.; Khan, L.A.; Manzoor, N. Induction of oxidative stress as a possible mechanism of the antifungal action of three phenylpropanoids. *FEMS Yeast Res.* **2011**, *11*, 114–122. [[CrossRef](#)] [[PubMed](#)]
97. Kocha, A.; Kumara, N.; Weber, L.; Kellerc, H.; Imania, J.; Kogel, K.-H. Host-induced gene silencing of cytochrome P450 lanosterol C14 α -demethylase-encoding genes confers strong resistance to *Fusarium* species. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 19324–19329. [[CrossRef](#)] [[PubMed](#)]
98. Cavalheiro, M.; Pais, P.; Galocha, M.; Teixeira, M.C. Host-pathogen interactions mediated by *mdr* transporters in fungi: As pleiotropic as it gets! *Genes* **2018**, *9*, 332. [[CrossRef](#)]
99. Smriti; Krishnamurthy, S.; Dixit, B.L.; Gupta, C.M.; Milewski, S.; Prasad, R. ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. *Yeast* **2002**, *19*, 303–318. [[CrossRef](#)]
100. Mukherjee, P.K.; Chandra, J.; Kuhn, D.M.; Ghannoum, M.A. Mechanism of fluconazole resistance in *Candida albicans* biofilms: Phase-specific role of efflux pumps and membrane sterols. *Infect. Immun.* **2003**, *71*, 4333–4340. [[CrossRef](#)]
101. Suchodolski, J.; Muraszko, J.; Korba, A.; Bernat, P.; Krasowska, A. Lipid composition and cell surface hydrophobicity of *Candida albicans* influence the efficacy of fluconazole-gentamicin treatment. *Yeast* **2020**, *37*, 117–129. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.