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Transcription Factor ADS-4 Regulates Adaptive Responses and Resistance to Antifungal Azole Stress

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Azoles are commonly used as antifungal drugs or pesticides to control fungal infections in medicine and agriculture. Fungi adapt to azole stress by rapidly activating the transcription of a number of genes, and transcriptional increases in some azole-responsive genes can elevate azole resistance. The regulatory mechanisms that control transcriptional responses to azole stress in filamentous fungi are not well understood. This study identified a bZIP transcription factor, ADS-4 (*a*ntifungal *d*rug *s*ensitive-4), as a new regulator of adaptive responses and resistance to antifungal azoles. Transcription of *ads-4* in *Neurospora crassa* cells increased when they were subjected to ketoconazole treatment, whereas the deletion of *ads-4* resulted in hypersensitivity to ketoconazole and fluconazole. In contrast, the overexpression of *ads-4* increased resistance to fluconazole and ketoconazole in *N. crassa*. Transcriptome sequencing (RNA-seq) analysis, followed by quantitative reverse transcription (qRT)-PCR confirmation, showed that ADS-4 positively regulated the transcriptional responses of at least six genes to ketoconazole stress in *N. crassa*. The gene products of four ADS-4-regulated genes are known contributors to azole resistance, including the major efflux pump CDR4 (Pdr5p ortholog), an ABC multidrug transporter (NcAbcB), sterol C-22 desaturase (ERG5), and a lipid transporter (NcRTA2) that is involved in calcineurin-mediated azole resistance. Deletion of the *ads-4*-homologous gene Af*ads-4* in *Aspergillus fumigatus* caused hypersensitivity to itraconazole and ketoconazole, which suggested that ADS-4 is a functionally conserved regulator of adaptive responses to azoles. This study provides important information on a new azole resistance factor that could be targeted by a new range of antifungal pesticides and drugs.

Filamentous fungi cause over 70% of plant diseases, and some can cause deadly infections in humans (1–5). Azoles (e.g., itraconazole [ITA], fluconazole [FLC], and ketoconazole [KTC]) are the most commonly used antifungal drugs in medicine, and some azoles, such as triadimenol and propiconazole, are also used to control fungal diseases in plants (6). Antifungal azoles inhibit 14α-methyl sterol demethylase (encoded by *ERG11*), a key enzyme involved in fungal ergosterol biosynthesis. This leads to changes in membrane consistency (7, 8). In addition to blocking ergosterol production, the inhibition of ERG11 by azoles results in the accumulation of toxic 14α-methylated sterol intermediates (9, 10).

Fungi can adapt to azole stress by rapidly increasing the expression of a number of genes. Increased expression of some azole-responsive genes, such as genes encoding azole efflux pumps and genes involved in ergosterol biosynthesis, can increase resistance to azoles (11, 12). Previous studies in *Saccharomyces cerevisiae* and *Candida albicans* identified a number of regulatory genes that mediate azole responses. The transcription factors Pdr1p and Pdr3p in *S. cerevisiae* and their homologs in *C. albicans* regulate azole responses by controlling multidrug efflux pump genes (13–15). However, filamentous fungi do not have such homologs. The transcription factor Upc2p in *C. albicans* and its ortholog Ecm22p in *S. cerevisiae* regulate azole responses by upregulating ergosterol synthesis genes and multidrug efflux pump genes (16–19). Although Upc2p homologs are present in filamentous fungi, a deletion mutant (FGSC 11076) of the *Neurospora crassa* Upc2p homolog gene (NCU03686) was not hypersensitive to ketoconazole (20). To date, only one transcription factor, AP-1, is known to play a role in the azole responses of both yeasts and filamentous fungi (21–23). It is possible that filamentous fungi have azole response regulation mechanisms that are different from those found in yeasts.

Neurospora crassa, which has knockout mutants for over 6,000 genes, was recently used as a model to study how filamentous fungi respond to azole stress. This led to the discovery of a number of new azole-responsive genes that play important roles in azole resistance (20, 24, 25). One of the newly identified genes encodes a transcription factor (CCG-8) in *N. crassa* that regulates the transcriptional responses to ketoconazole of 78 genes, including the azole-target-coding gene *erg11* and the Pdr5p-like ABC-transporter-coding gene *cdr4*. Its homolog in another filamentous fungus, *Fusarium verticillioides*, has a similar role (20).

Using *N. crassa* as a model, this study identified another new transcription factor, ADS-4, which is essential for normal azole resistance in both *N. crassa* and *Aspergillus fumigatus*. ADS-4 regulated the transcriptional responses to ketoconazole of at least six genes.

MATERIALS AND METHODS

Strain cultivation. The *Neurospora crassa* wild-type (WT) strain and knockout mutants used in this study were purchased from the Fungal

Received 6 March 2015 Returned for modification 29 March 2015
Accepted 13 June 2015

Accepted manuscript posted online 22 June 2015

Citation Wang K, Zhang Z, Chen X, Sun X, Jin C, Liu H, Li S. 2015. Transcription factor ADS-4 regulates adaptive responses and resistance to antifungal azole stress. Antimicrob Agents Chemother 59:5396–5404. doi:10.1128/AAC.00542-15.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00542-15.

doi:10.1128/AAC.00542-15

TABLE 1 Gene-specific primer pairs

Process and gene	Primer name	Nucleotide sequence (5′ to 3′)
Complementation		
ads-4	ads4-F	TGGTTACGTGTTCTGCGTCAGTATC
	ads4-R	TCAGTCCCTATAACGAACACCTCAC
N. crassa transformant and double-mutant screening		
ads-4	ads4v-F	CTTTCCAACCCAACCATC
	ads4v-R	GTCCGCTATACTGCTGTCC
Construction of *ads-4* overexpression strain		
cfp	cfp-F	CGACCTCAAACCTCAACAAAC
	cfp-R	ATATCAGATCCGATGCTCTCTCTTTAGGGTGAG
ads-4	hismycads4-F	GAGAGAGCATCGGATCTGATATCATCGATTTAAAGC
	hismycads4-R	TTTGCCCTCGCGAGCACTAACGTGGAAAATC
trpc	trpc-F	TTAGTGCTCGCGAGGGCAAAGGAATAGAGTAG
	trpc-R	AAGCAGCCCAGTAGTAGGTTGA
Construction of *Afu1g16460*-knockout strain		
Afu1g16460 upstream	Afu1g16460U-XhoI-F	CCGCTCGAGGCGGCCGCGATGTCCGAAAAAAGGCAGAGG
	Afu1g16460U-ClaI-SmaI-R	CCATCGATCCCGGGGATGTGCGTATGCACGAGGTTC
Afu1g16460 downstream	Afu1g16460D-ClaI-F	CCATCGATCCCCCCTCTTTCCTTTGTTCATG
	Afu1g16460D-BamHI-R	CGGGATCCCGTAGGTTTTCCCTCGTCTGAA
A. fumigatus transformant screening		
Afu1g16460	Afu1g16460V-F	GGCTTCATTGGTCCGTGC
	Afu1g16460V-R	AAGCCATTCTCGCAAGCC
qRT-PCR analysis		
ads-4	Qncu08744-F	TCGAACTCTTGGGACTGCCAGAAA
	Qncu08744-R	AAGGCATTCCGATTGAGTCCGCTA
N*cmmt2*	Qncu07879-F	ACACCGTTTCTGCCCTCT
	Qncu07879-R	CCGCCAGCTCTATATCCA
erg5	Qncu05278-F	TTTCACCTTCCTCTTCGCTTCCCA
	Qncu05278-R	TCATCGACTCAAGCTGCTCCATGT
cdr4	Qncu05591-F	ACGCTTTGGAAATGGATGGTGACG
	Qncu05591-R	ATGAACAAGGCGACGGAAATGCAG
N*crta2*	Qncu05209-F	TGAGCAAGATCATTGTCCTAAT
	Qncu05209-R	AAATACCACAGCCATCTCAC
N*cabcB*	Qncu03776-F	CGGTGATGCAGGAAGTTATC
	Qncu03776-R	CTTCAACACCGCCACTAAA
N*cmnn4*	Qncu03213-F	GGTGGTGGAACAAGCAGAT
	Qncu03213-R	GGTCTCCGTTGGAGAAGTTAG

Genetics Stock Center (FGSC) (Kansas City, MO) and are listed in Table S1 in the supplemental material. Vogel's medium (26), supplemented with 2% (wt/vol) sucrose for slants or 2% glucose for liquid and plate media, were used to culture *N. crassa*. All of the *N. crassa* strains were cultured at 28°C.

Aspergillus fumigatus wild-type strain YJ407 and the CEA17 strain (Δ*pyrG89*) were grown in complete medium (CM). All *Aspergillus fumigatus* cultures were grown at 37°C.

Drug sensitivity tests. Ketoconazole, itraconazole, and fluconazole were dissolved in dimethyl sulfoxide (DMSO) and then aseptically added to autoclaved medium before it was poured into agar plates. The final DMSO concentration was below 0.25% (vol/vol). The plates (diameter, 9 cm) were inoculated with 2 μl of conidial suspension, with or without antifungal drugs, and incubated in the dark.

Complementation of *ads-4* deletion mutant. To complement the *ads-4*-knockout mutant, the *ads-4*-knockout mutant (FGSC 11386) was crossed with mutant FGSC 6103 (*his-3*; type A), which cannot synthesize histidine, to generate the *ads-4*-knockout strain named NCW 1, which has a *his-3* background. To create the complementary plasmid, the whole length of the *ads-4* coding sequence (1,260 bp), with a 1,948-bp upstream region and a 1,976-bp downstream region, was amplified using primers ads4-F and ads4-R (Table 1), to create a 5,184-bp complementation fragment. The PCR product was inserted into the pBM61 vector (27) at the SmaI site to form the complementary plasmid pBM61-ads4. The PBM61-ads4 construct was transformed into the *ads-4* deletion mutant with a *his-3* background (NCW 1) by using a previously reported method (28). Transformants were screened on Vogel's medium without histidine and were verified by PCR using primers ads4v-F and ads4v-R (Table 1).

Overexpression of *ads-4*. The *cfp* promoter was used to overexpress *ads-4* (29). The *cfp* promoter (888 bp) was amplified from the wild-type *N. crassa* genome by PCR using primers cfp-F and cfp-R (Table 1). The *ads-4* coding region, tagged with 5×cMyc-6×His, was amplified from the Qa5myc6his-ads4 vector (constructed by inserting the *ads-4* coding sequence into the Qa5myc6his plasmid) by PCR using the primers hismycads4-F and hismycads4-R (Table 1). The *trpc* terminator (997 bp) was amplified from the pCSN43 vector using primers trpc-F and trpc-R (Table 1). The three fragments were purified and fused together by fusion PCR. The fused fragment (3,423 bp) was ligated to the pCSN43 vector, which

produced the *ads-4* overexpression vector pCSN43-ads4^{OE}. The pCSN43-ads4^{OE} vector was transformed into the *N. crassa* wild-type strain (FGSC 4200) by protoplast transformation, as reported previously (20). The transformants were screened on Vogel's medium with hygromycin and were verified by PCR using primers cfp-F and trpc-R (Table 1).

RNA extraction and transcriptional analysis by qRT-PCR. RNA extraction and cDNA synthesis were performed according to previously described methods (24). Quantitative reverse transcription (qRT)-PCR was carried out using the iQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) with SYBR green detection (SYBR PrimeScript RT-PCR kit; TaKaRa Biotechnology Co., Ltd.), according to the manufacturer's instructions. Each cDNA sample was analyzed in triplicate, and the average threshold cycle was calculated. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (30). The results were normalized to the β-tubulin expression level. Gene-specific primers are shown in Table 1.

Transcript profile analysis. Briefly, conidia from the wild-type strain and the *ads-4* deletion mutant were added to 20 ml Vogel's liquid medium in a plate (diameter, 9 cm) and incubated at 28°C in the dark for 24 h until a mycelial mat formed on the surface of the liquid medium. The mycelial mat was then cut into small pieces (diameter, 10 mm) and transferred to Vogel's liquid medium (two pieces in 100 ml) in 150-ml flasks. The cultures were incubated at 28°C for 12 h with shaking. KTC, at a final concentration of 2.5 μg/ml, was then added to the medium. After 24 h of incubation, total RNA was extracted and subjected to transcriptome sequencing (RNA-seq) analysis. Genes with transcriptional ratios of more than 2.0 or less than 0.5 in two samples were considered to be differentially expressed.

Knockout of *ads-4*-homologous gene in *Aspergillus fumigatus*. A deletion cassette containing the *pyrG* gene as the selectable marker was constructed to knock out Afu1g16460 in *Aspergillus fumigatus*. PCR primers Afu1g16460U-XhoI-F and Afu1g16460U-ClaI-SmaI-R (Table 1) were designed to amplify the upstream sequence (1,462 bp) of Afu1g16460 before the ATG start codon, and Afu1g16460D-ClaI-F and Afu1g16460D-BamHI-R (Table 1) were used to amplify the downstream flanking sequence (821 bp) of Afu1g16460 after the stop codon. The upstream and downstream noncoding fragments were digested with XhoI/ClaI and ClaI/BamHI, respectively, and then cloned into the pBlueScript (pSK) vector to form the pSK-UD vector. The *pyrG* gene selectable marker, which was released from pCDA14 (31) by HpaI digestion, was inserted into pSK-UD at the SmaI site between the upstream and downstream Afu1g16460 sequences. The deletion vectors were linearized by digestion with NotI. They were then transformed into *A. fumigatus* CEA17 protoplasts and plated under uridine and uracil autotrophy selection. The deletion mutants were confirmed using PCR analysis with primers Afu1g16460V-F and Afu1g16460V-R (Table 1), to amplify the coding sequence for Afu1g16460 (32).

RESULTS

ADS-4 responds transcriptionally to ketoconazole in *Neurospora crassa*. NCU08744 is a transcription factor composed of 430 amino acids with a bZIP DNA-binding domain. NCU08744 was named ADS-4 (*a*ntifungal *d*rug *s*ensitive-4) according to the azole-hypersensitive phenotype of its deletion mutant. RNA-seq analysis showed that KTC (2.5 μg/ml) treatment (24 h) resulted in a 6.7-fold increase in *ads-4* transcript levels (see Data Set S1 in the supplemental material). The *ads-4* transcriptional increase after treatment with KTC was confirmed by a time course experiment in which *ads-4* transcript levels were measured after 2 h, 6 h, 12 h, 18 h, 24 h, and 30 h of KTC treatment. Figure 1A shows that the *ads-4* transcript levels increased 0.68-fold after 2 h of KTC treatment and continued to increase with time. Thus, ADS-4 is a transcriptional factor that responds to KTC stress.

Deletion of *ads-4* causes hypersensitivity to azoles in *N. crassa*. To test whether ADS-4 makes a potential contribution to azole resistance, the sensitivities of an *ads-4* deletion mutant (FGSC 11386) and the WT strain (FGSC 4200) to two azole drugs were analyzed comparatively. When grown on medium without drugs, the *ads-4* mutant had a growth rate similar to that of the WT strain. When grown on medium containing KTC or fluconazole (FLC), however, the *ads-4* mutant grew significantly more slowly than the WT strain (Fig. 2). The inhibition rates for the WT strain treated with KTC and FLC were 72.29% and 61.25%, respectively, while the inhibition rates for the mutant strain were 90.33% and 69.96%, respectively. Statistical analysis indicated that *ads-4* deletion significantly increased the sensitivity to KTC and FLC (Table 2).

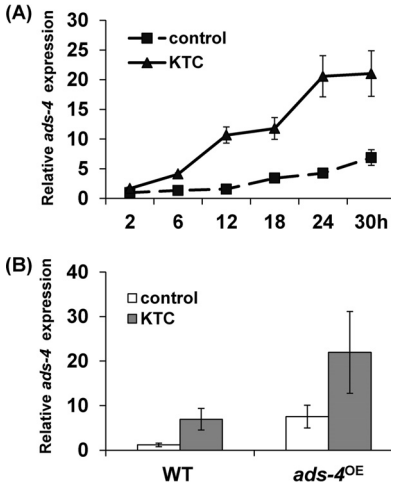

FIG 1 Effects of ketoconazole treatment and *ads-4* overexpression on the transcription of *ads-4*. (A) *ads-4* transcriptional responses to ketoconazole (KTC) in *N. crassa* WT cells. The WT cells were inoculated and cultured for 12 h at 28°C with shaking (200 rpm), and then KTC was added to reach a final concentration of 2.5 μg/ml. The control medium did not contain KTC. Mycelia were harvested at 2 h, 6 h, 12 h, 18 h, 24 h, and 30 h, and the transcription of *ads-4* was detected by qRT-PCR. The *ads-4* transcript levels are shown in terms of relative quantity (the *ads-4* transcript level at 2 h in WT cells without KTC treatment was defined as 1). The *ads-4* transcript levels in WT cells with or without KTC are shown. (B) Detection of *ads-4* transcripts in the strain with *ads-4* overexpression (*ads-4*^{OE}) and the WT strain. Expression of *ads-4* was detected by qRT-PCR 24 h after treatment with 2.5 μg/ml KTC. The control medium did not contain KTC.

To confirm the role of *ads-4* in azole resistance, the NCW 2 strain (Δ*ads-4*::*ads-4*), with complementation of the *ads-4* deletion, was created. As shown in Fig. 2 and Table 2, the complemented strain exhibited wild-type sensitivities to KTC and FLC, which indicated that ADS-4 is required if normal resistance to azoles is to be maintained.

Overexpression of *ads-4* increases azole resistance in *N. crassa*. To identify the role of *ads-4* during azole resistance, an *ads-4* overexpression strain, *ads-4*^{OE}, was generated, in which ADS-4 was tagged with 5×cMyc-6×His at its N terminus and was driven by a *cfp* promoter (29). The *ads-4* transcriptional levels in the *ads-4*^{OE} strain were 5.25-fold higher than those in the WT strain in liquid medium without azoles (Fig. 1B). When cells were treated with KTC (2.5 μg/ml) for 24 h, the *ads-4* transcriptional levels in the *ads-4*^{OE} strain were 17.26-fold higher than those in the WT strain (Fig. 1B). On plates without azoles, the *ads-4*^{OE} strain grew at a rate similar to that of the WT strain. On plates with KTC or FLC, the *ads-4*^{OE} strain grew faster than the WT strain (Fig. 2).

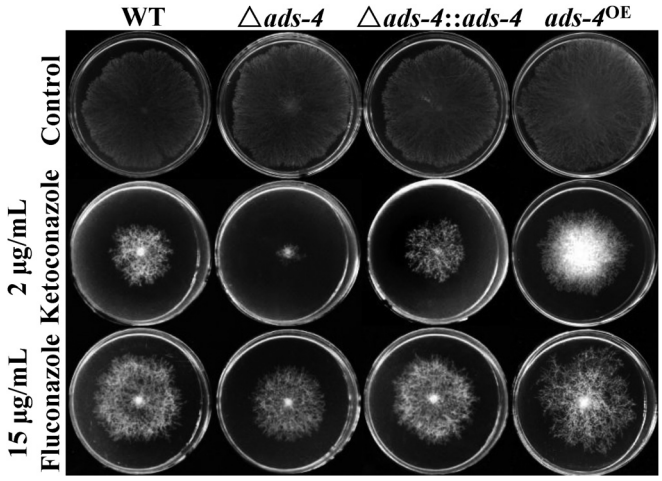

FIG 2 Role of ADS-4 under azole stress. Two-microliter aliquots of conidial suspensions (1×10^6 conidia/ml) of the *N. crassa* WT strain, the *ads-4*-knockout mutant (Δ*ads-4*), the *ads-4*-complemented strain (Δ*ads-4*::*ads-4*), and the *ads-4* overexpression strain (*ads-4*^{OE}) were spotted onto the center of Vogel's plates (diameter, 9 cm) with or without antifungal drugs. The final concentrations of ketoconazole and fluconazole were 2 μg/ml and 15 μg/ml, respectively. Plates were incubated at 28°C in the dark. Photographs were captured at 24 h for the control plates and at 48 h for the ketoconazole- and fluconazole-treated plates.

The inhibition rates for the WT strain treated with KTC and FLC were 72.29% and 61.25%, respectively, while the inhibition rates for the *ads-4*^{oe} strain were 67.93%, and 58.51%, respectively. The Waller-Duncan *t* test showed that the *ads-4*^{oe} inhibition rates with these azoles were significantly lower than the WT rates (Table 2). These results indicate that the *ads-4* transcriptional increase during azole stress improves azole resistance.

Deletion of ADS-4 affects genome-wide transcriptional responses to ketoconazole in *N. crassa*. The *ads-4* deletion strain and the WT genome-wide transcriptional responses to KTC were comparatively analyzed by RNA-seq in order to ascertain whether ADS-4 mediates transcriptional responses to azoles. The RNA-seq data showed that, with KTC treatment, 488 genes were upregulated in the WT strain, while only 398 genes were upregulated in the *ads-4* deletion mutant. KTC treatment also caused downregulation of 427 genes in the WT strain, whereas only 342 genes were downregulated by KTC treatment in the *ads-4* deletion mutant (see Data Set S1 in the supplemental material).

ADS-4 regulates transcriptional responses by the sterol C-22 desaturase ERG5 to ketoconazole. ERG5 is a sterol C-22 desaturase that plays a role in ergosterol biosynthesis. Deletion of *erg5* in *N. crassa* and *Fusarium verticillioides* caused hypersensitivity to azoles (25). The RNA-seq data showed that the *erg5* transcript level in KTC-treated WT cells was 2.1-fold higher than that in non-KTC-treated WT cells. In the *ads-4*-knockout mutant, KTC treatment did not significantly change the *erg5* transcript level (see Data Set S1 in the supplemental material). Consistent with the RNA-seq data, qRT-PCR analysis showed that the expression of *erg5* in KTC-treated WT cells was 3.76-fold greater than that in non-KTC-treated WT cells. In contrast, no significant *erg5* transcriptional increase was detected in the *ads-4*-knockout mutant after KTC treatment (Fig. 3A).

In the strain with *ads-4* overexpression, *ads-4*^{OE}, the *erg5* transcript level was 2.06-fold higher than that the WT strain in liquid medium without KTC. With KTC treatment, the *erg5* transcript level in the *ads-4*^{OE} strain was also significantly higher than that in the WT strain (Fig. 3A).

These results show that ADS-4 is a transcription factor that regulates the expression of *erg5* and is essential for the *erg5* transcriptional response to KTC stress. The *ads-4* transcriptional increase during KTC stress should promote the expression of *erg5*. Promotion of *erg5* expression is probably a mechanism by which ADS-4 regulates the adaptation to azole stress.

ADS-4 activates transcriptional responses by the azole efflux pump CDR4 to ketoconazole. CDR4, the ortholog of Cdr1p in *C. albicans*, is the only Pdr5p-like ABC transporter that has a detectable role in azole resistance in *N. crassa* (24). Deletion of *cdr4* significantly increases the sensitivity to azoles (24) (Fig. 4). The RNA-seq data showed that the *cdr4* transcript level increased 68.5-fold in the WT strain after 24 h of KTC treatment, whereas the increase was only 16.1-fold in the *ads-4*-knockout mutant (see Data Set S1 in the supplemental material). The qRT-PCR analysis showed that deletion of *ads-4* did not significantly affect the expression of *cdr4* in liquid medium without KTC. KTC treatment resulted in an 18.0-fold *cdr4* transcriptional increase. KTC treatment also increased the *cdr4* transcriptional level in the *ads-4*-knockout mutant but only by 2.7-fold (Fig. 3B).

The expression of *cdr4* was not significantly higher in the strain with *ads-4* overexpression, *ads-4*^{OE}, compared to the WT strain, under both KTC-treated and non-KTC-treated conditions. Thus, ADS-4 is important in the transcriptional responses of *cdr4* to azoles, but upregulation of *ads-4* may not promote the expression of *cdr4*.

ADS-4 regulates transcriptional responses by the ABC multidrug transporter NcAbcB to ketoconazole. The RNA-seq data showed that 24 h of KTC treatment resulted in a 18.1-fold increase in NCU03776 transcription. However, only a 4.3-fold increase in NCU03776 transcription was seen in the *ads-4*-knockout mutant (see Data Set S1 in the supplemental material). Phylogenic analysis of NCU03776 with its homologs in *S. cerevisiae* and *Aspergillus fumigates* showed that NCU03776 is the ortholog of Ycf1p in *S. cerevisiae* and AbcB (Afu1g10390) in *A. fumigates* (see Fig. S2 in the supplemental material). *S. cerevisiae* Ycf1p is an ABC transporter that transports a broad range of toxins into vacuoles (33, 34). Transcription of *A. fumigates abcB* can be induced by voriconazole, and the *abcB*-knockout mutant is hypersensitive to

TABLE 2 Inhibition rates with azoles for *Neurospora crassa* WT, Δ*ads-4*, Δ*ads-4*::*ads-4*, and *ads-4*^{OE} strains

Drug	Inhibition rate (%)[a]			
	WT	Δ*ads-4*	Δ*ads-4*::*ads-4*	*ads-4*^{OE}
KTC	72.29 ± 0.02	90.33 ± 0.02[b]	72.92 ± 0.02	67.93 ± 0.02[c]
FLC	61.25 ± 0.01	69.96 ± 0.01[b]	62.63 ± 0.01	58.51 ± 0.01[c]

[a] Tested strains are the wild-type (WT) strain, the *ads-4*-knockout strain (Δ*ads-4*), the *ads-4*-complemented strain (Δ*ads-4*::*ads-4*), and the *ads-4* overexpression strain (*ads-4*^{OE}). Radii of colonies were measured after 24 h of inoculation for nontreated plates and 48 h for drug-treated plates, and growth rates of strains were calculated by the equation colony radius (mm)/incubation time (h). The means of the relative inhibition rates for each fungicide were calculated with the following equation: (growth rate on plates without fungicide − growth rate on plates with fungicide)/growth rate on plates without fungicide. Differences between the mutants and the WT strain were statistically analyzed with the Waller-Duncan test.

[b] Significantly different from the WT strain, $P < 0.01$.

[c] Significantly different from the WT strain, $P < 0.05$.

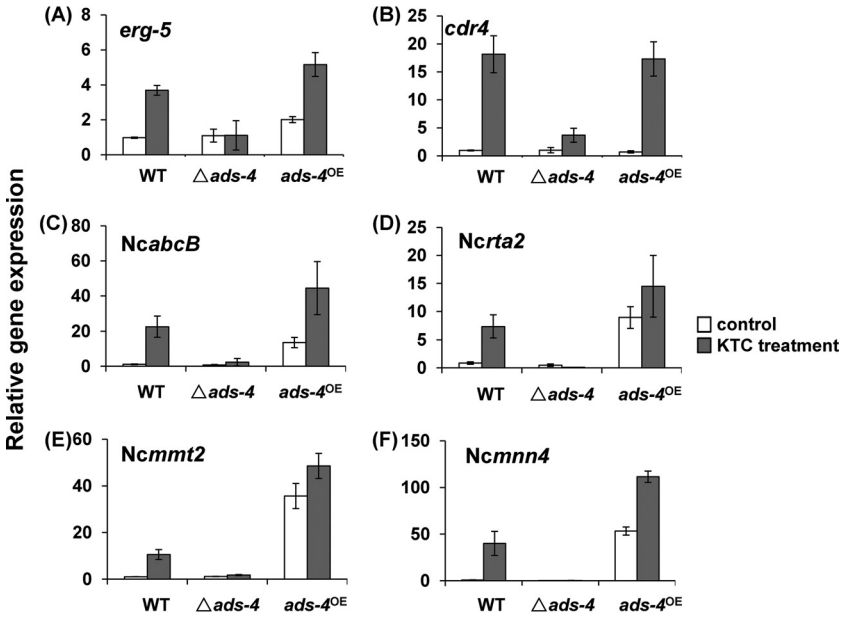

FIG 3 Differential expression of genes in the WT strain, the *ads-4* deletion mutant (Δ*ads-4*), and the *ads-4* overexpression strain (*ads-4*^{OE}). Strains were grown in Vogel's liquid medium at 28°C, with shaking at 180 rpm, for 12 h before treatment. Ketoconazole (KTC) was then added to the medium to reach a final concentration of 2.5 μg/ml. After incubation for 24 h, transcripts of *erg5*, *cdr4*, N*cabcB*, N*crta2*, N*cmmt2*, and N*cmnn4* were analyzed by qRT-PCR. Values shown are the means of three independent replicates. Standard deviations are indicated by error bars.

azoles (35, 36). NCU03776 was named N*cabcB* in this study. The qRT-PCR analysis showed that deletion of *ads-4* did not significantly affect the transcription of N*cabcB* in medium without KTC. KTC treatment caused an 18.85-fold increase in the N*cabcB* transcript level in the WT strain. However, the N*cabcB* transcript level increased only 2.28-fold in the *ads-4*-knockout mutant after KTC treatment (Fig. 3C).

N*cabcB* expression increased 10.96-fold in the *ads-4* overexpression strain *ads-4*^{OE}, compared with the WT strain, with no KTC treatment. With KTC treatment, the transcriptional level of N*cabcB* in the *ads-4*^{OE} strain was significantly higher than that in the WT strain (Fig. 3C). Thus, the *ads-4* transcriptional increase with exposure to KTC probably promotes N*cabcB* expression. Although the N*cabcB* homolog *abcB* is known to contribute to azole resistance in *A. fumigates* (35, 36), the deletion of N*cabcB* does not affect the sensitivity to KTC in *N. crassa* (Fig. 4), which indicates that N*cabcB* is not a major contributor to azole resistance in *N. crassa*.

ADS-4 regulates transcriptional responses by NcRTA2 to azole stress. *C. albicans* Rta2p was thought to be a lipid transporter and has predicted transmembrane domains. Rta2p is also involved in calcineurin-mediated azole resistance (37, 38). Our RNA-seq data showed that transcriptional responses by the RTA2 homolog NCU05209 (named N*crta2* in this study) were affected by *ads-4* deletion. The N*crta2* transcription level increased 5.17-fold in the WT strain during KTC treatment, but there was not a significant N*crta2* transcriptional increase in the *ads-4*-knockout strain (see Data Set S1 in the supplemental material). This result was confirmed by qRT-PCR, which showed that there was a 7.48-fold increase in N*crta2* transcription in the WT strain after KTC treatment but no significant N*crta2* transcriptional increase in the *ads-4*-knockout strain (Fig. 3D). This indicated that ADS-4 is required for the N*crta2* transcriptional response to KTC.

Without KTC treatment, the N*crta2* transcript level in the strain with *ads-4* overexpression, *ads-4*^{OE}, was 10.29-fold higher than that in the WT strain; with KTC treatment, the N*crta2* transcript level was 16.63-fold higher than that in the non-KTC-

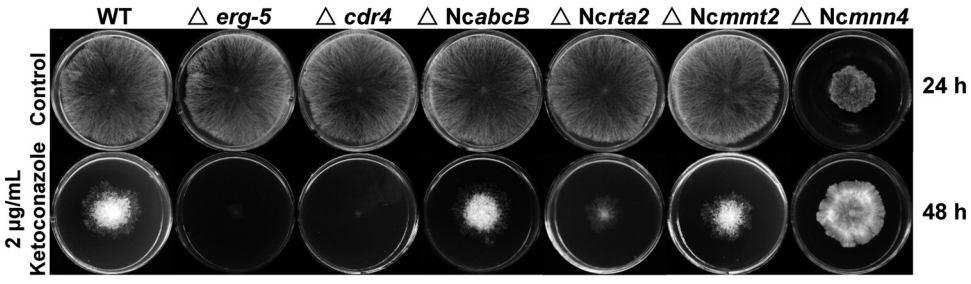

FIG 4 Ketoconazole susceptibility analyses of the *N. crassa* knockout mutant for genes regulated by ADS-4. Two-microliter aliquots of conidial suspension (1×10^6 conidia/ml) were inoculated onto the center of Vogel's plates (diameter, 9 cm) with or without ketoconazole (2 μg/ml). The plates were incubated at 28°C in the dark. Photographs were taken at 24 h for the control plates and at 48 h for the ketoconazole-treated plates (at 24 h, no colony for any tested strain was formed on the plates with ketoconazole).

treated WT cells, which was significantly higher than that in the KTC-treated WT cells. These results indicate that the Nc*rta2* transcriptional response is regulated by ADS-4.

The Nc*rta2*-knockout mutant was hypersensitive to KTC (Fig. 4), which was consistent with observations for the *C. albicans RTA2*-knockout mutant (37, 38). Thus, the Nc*rta2* transcriptional increase should improve resistance to azoles.

ADS-4 regulates transcriptional responses by the mitochondrial metal transporter NcMMT2 to ketoconazole. From the remainder of the differentially expressed genes, whose roles in azole resistance were not previously known, we chose two genes for which transcriptional responses to KTC were completely abolished by *ads-4* deletion for further analysis by qRT-PCR. The first gene chosen was NCU07879. The homolog of NCU07879 in *S. cerevisiae* is *MMT2* (mitochondrial metal transporter), a gene responsible for iron homeostasis (39, 40). NCU07879 was named Nc*mmt2* in this study. The RNA-seq data showed that Nc*mmt2* transcription rose 24.5-fold in the WT strain after KTC treatment, whereas no significant transcriptional increase was seen in the *ads-4*-knockout mutant (see Data Set S1 in the supplemental material). The qRT-PCR analysis showed that Nc*mmt2* transcription was 10.3-fold higher in KTC-treated WT cells than in non-KTC-treated WT cells, whereas Nc*mmt2* transcription was only 1.48-fold higher in KTC-treated *ads-4*-knockout mutant cells than in non-KTC-treated WT cells (Fig. 3E). Overexpression of *ads-4* resulted in a 33.68-fold increase in Nc*mmt2* transcription (Fig. 3E), which indicated that Nc*mmt2* expression was controlled by ADS-4. The Nc*mmt2*-knockout mutant had no obvious growth defects and showed almost wild-type sensitivity to KTC (Fig. 4).

ADS-4 controls transcriptional responses by the mannosylphosphorylation protein NcMNN4 to azole stress. The other gene analyzed by qRT-PCR was NCU03213. NCU03213 shares significant homology with *S. cerevisiae* Mnn4p. Mnn4p is mannosyl phosphate transferase, which is essential for the transfer of mannosyl phosphate to *N*- and *O*-oligosaccharides in *S. cerevisiae* cell walls (41, 42). NCU03213 was named Nc*mnn4* in this study. RNA-seq data showed that Nc*mnn4* had a strong response to azole treatment; Nc*mnn4* transcription increased 23.78-fold after KTC treatment in the WT strain, whereas there was no transcriptional increase in the *ads-4*-knockout strain (see Data Set S1 in the supplemental material). This trend was verified by qRT-PCR, which showed that Nc*mnn4* transcription increased 53.18-fold after KTC treatment in the WT strain. KTC treatment did not cause significant Nc*mnn4* transcriptional changes in the *ads-4*-knockout strain (Fig. 3F).

Without KTC treatment, the Nc*mnn4* transcript level in the *ads-4*^{OE} strain was 71.11-fold higher than that in non-KTC-treated WT cells. After 24 h of KTC treatment, the Nc*mnn4* transcript level in the *ads-4*^{OE} strain was 150.01-fold higher than that in non-KTC-treated WT cells, which was also significantly higher than that in KTC-treated WT cells (Fig. 3F).

The Nc*mnn4*-knockout mutant grew more slowly than the WT strain. On KTC-treated medium, however, the mutant was less sensitive to KTC than was the WT strain (Fig. 4).

ADS-4 is critical for azole resistance in the pathogenic fungus *A. fumigatus*. A BLASTp search revealed that a large number of ADS-4 homologs are present in filamentous ascomycetes but not in yeasts. Alignment analysis showed that ADS-4 homologs are highly conserved (see Fig. S1 in the supplemental material). Among fungal bZIP transcription factors, the functions of CYS-3, CPC-1, and AP-1 are relatively clear. Phylogenic analysis of fungal bZIP transcription factors showed that ADS-4, CYS-3, CPC-1, and AP-1 were distributed in four distinct clades (Fig. 5).

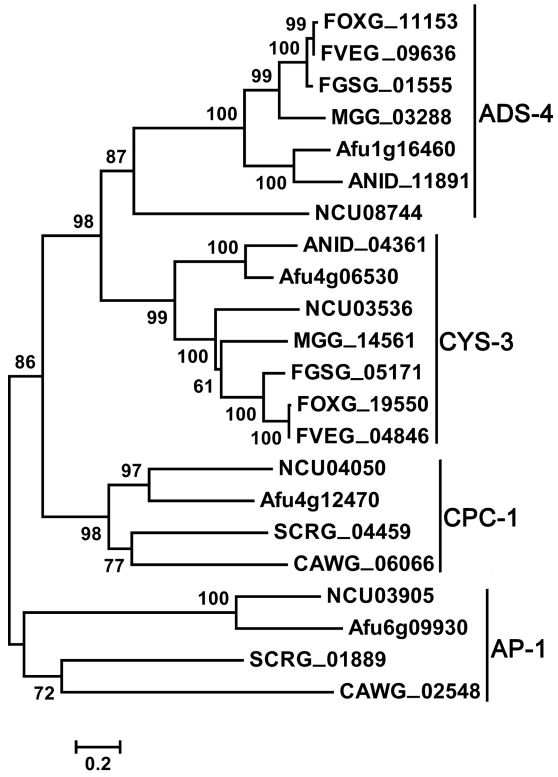

FIG 5 Phylogenic analysis of *N. crassa* bZIP transcription factors and their homologs. Protein sequences were aligned and the neighbor-joining tree was constructed using MEGA version 6 (61), with cutoff bootstrap values of 50% obtained from 1,000 replicates. NCU, *Neurospora crassa*; FOXG, *Fusarium oxysporum*; FVEG, *Fusarium verticillioides*; FGSG, *Fusarium graminearum*; MGG, *Magnaporthe oryzae*; Afu, *Aspergillus fumigatus*; ANID, *Aspergillus nidulans*; SCRG, *Saccharomyces cerevisiae*; CAWG, *Candida albicans*.

The protein sharing the greatest similarity with ADS-4 in *A. fumigatus* is Afu1g16460 (33% sequence identical [45/135] and 49% sequence positive [67/135]), which was named AfADS-4 in this study. Afu1g16460 is the ortholog of ADS-4 (Fig. 5). Af*ads-4* was deleted in *A. fumigatus* in order to test the functional conservation of ADS-4 among fungi. Figure 6 shows that the Af*ads-4*-knockout strain was hypersensitive to itraconazole and KTC, compared to the WT strain, which indicated that ADS-4 orthologs are functionally conserved with regard to adaptation and resistance to antifungal azoles.

DISCUSSION

To survive under antifungal azole stress, fungi adjust the transcriptional levels of a number of genes (20, 36, 43–45). Many of these azole-responsive genes are known to contribute to azole adaptation and resistance (17, 20, 23, 46–49). In filamentous fungi, only two transcription factors, AP-1 and CCG-8, are known to be involved in the regulation of azole responses and adaptation (20, 23). This study identified the third transcription factor controlling azole adaptation. The only reported homolog of ADS-4 is ZipA (ANID_11891) in *Aspergillus nidulans* (Fig. 5). ZipA negatively regulates resistance to oxidative stress (50). However, its role in

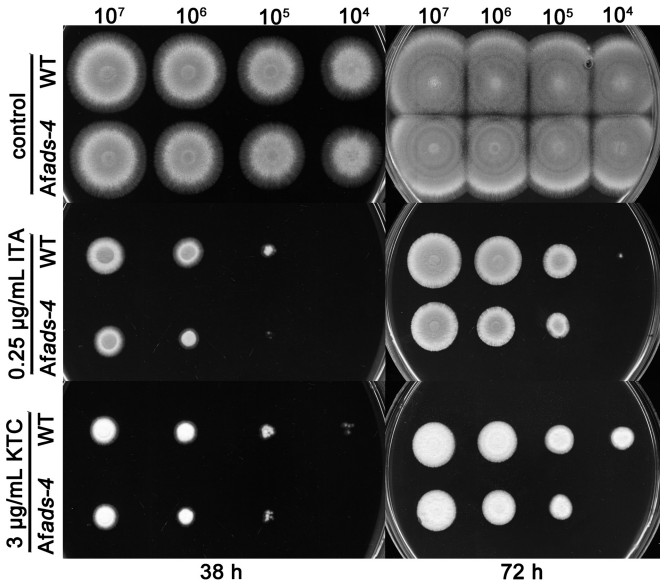

FIG 6 Hypersensitivity of *Aspergillus fumigatus* ADS-4 homolog Afu1g16460 (Af*ads-4*)-knockout mutant to azoles. A series of conidial suspensions (1×10^7, 1×10^6, 1×10^5, and 1×10^4 conidia/ml) of the *A. fumigatus* WT strain was prepared, and 2 μl of each conidial suspension was inoculated onto CM agar plates (diameter, 15 cm) with or without 3 μg/ml ketoconazole or 0.25 μg/ml itraconazole (ITA). Images of the plates were captured after 38 h and 72 h of cultivation at 37°C. The experiment was independently repeated twice.

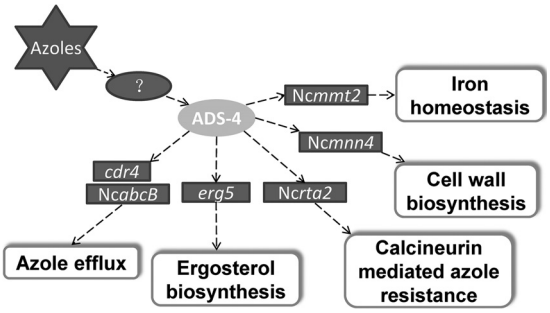

FIG 7 Proposed mechanisms of ADS-4 in azole responses and resistance. Azole stress activates *ads-4* transcription by an unknown signaling pathway, and then ADS-4 activates transcription of downstream genes (indicted in rectangles) involved in biological processes important to azole adaptation and resistance.

antifungal drug resistance has not been studied. The functional conservation of ADS-4 in *N. crassa* and *A. fumigatus* suggests that it is possible to use ADS-4 as a new target for antifungal drug design. Our study also identified some new azole-responsive genes, such as Nc*mmt2* and *Ncmnn4*.

Since overexpression of *ads-4* increased azole resistance, spontaneous mutations that cause overexpression of *ads-4* might increase azole resistance in pathogenic fungi. Gain-of-function mutations that cause overexpression of the transcription factor Mrr1p in *C. albicans* increase resistance to azoles (51). Similarly, gain-of-function mutations that improve Pdr1p transcriptional activity in *S. cerevisiae* increase resistance to azoles (52). Therefore, azole resistance caused by *ads-4* overexpression might exist in nature.

ADS-4 mediates transcriptional responses to ketoconazole for a number of genes, among which at least six genes, including *cdr4*, *erg5*, Nc*abcB*, Nc*mmt2*, Nc*RTA2*, and Nc*mnn4*, might be related to azole adaptation and resistance. On the basis of the functions of these genes, we propose that ADS-4 regulates azole adaptation and resistance by the following possible mechanisms (Fig. 7).

First, ADS-4 should positively contribute to azole efflux. CDR4 is the ortholog of the azole efflux pump Cdr1p in *C. albicans* (24). The roles of *cdr4* and its orthologs in azole resistance in many fungi have been demonstrated previously (24, 25, 53). The ortholog of Nc*abcB* in *A. fumigates* is the ABC multidrug transporter AbcB, which positively contributes to azole resistance (35, 36). Increases in *cdr4* and Nc*abcB* transcript levels should reduce the accumulation of azoles in cells. Thus, upregulation of *cdr4* and Nc*abcB* by ADS-4 should contribute positively to azole efflux.

Second, ADS-4 is linked to calcineurin-mediated azole resistance. Azole stress could activate the calcineurin signaling cascade, which leads to dephosphorylation of the transcription factor Crz1p in *C. albicans* (54). Dephosphorylated Crz1p activates the expression of Rta2p, which transports sphingolipid long-chain bases from the inner leaflet to the outer leaflet of the plasma membrane. The disruption of *RTA2* affects raft formation, making *C. albicans* more susceptible to azoles (37, 38, 55). The Rta2p homolog Rsb1p in *S. cerevisiae* has the same function (56). Thus, ADS-4 might be linked to calcineurin-mediated azole resistance by activating Ncrta2 transcription under azole stress.

Third, ADS-4 might mediate ergosterol biosynthesis under azole stress. Azole treatment affects the composition of sterols in fungal membranes (9, 25). The sterol C-22 desaturase ERG5 is an essential enzyme in ergosterol biosynthesis. Deletion of *erg5* in *N. crassa* completely blocked ergosterol biosynthesis and increased azole susceptibilities (25). Thus, the promotion of *erg5* expression by ADS-4 might be beneficial for maintaining the proper sterol composition in membranes under azole stress.

Fourth, ADS-4 might mediate mitochondrial iron transport during azole stress. The mitochondrial iron transporter Nc*MMT-2* had a strong response to KTC, and its response was completely controlled by ADS-4. Although its role in azole resistance has not been reported previously, a linkage between iron and azole susceptibility has been reported. In *S. cerevisiae*, mitochondria serve as an iron reservoir, and mitochondrial iron transporters redistribute iron from intracellular compartments into the mitochondria (39). Iron deprivation can enhance membrane fluidity and increase the passive diffusion of drugs, which leads to increased drug susceptibility in *C. albicans* (57). In addition, ERG-25, an enzyme involved in ergosterol biosynthesis, needs iron to fulfill its function (58). Thus, iron plays an important role in azole resistance. The increase in *Ncmmt-2* expression produced by ADS-4 might enhance iron transport into mitochondria and counteract membrane iron leakage.

Fifth, ADS-4 might mediate the adaptive alterations in cell wall composition during azole stress. Mnn4p transfers mannosyl phosphate to *N*- and *O*-oligosaccharides in *S. cerevisiae* cell walls (41, 42, 59, 60). The *Ncmnn4* transcriptional increase during ketoconazole treatment suggests that the composition of cell walls might have adaptive alterations under azole stress. In *Candida glabrata*, structural changes in cell walls could lead to the strain hypersensitive to itraconazole (60). Thus, ADS-4 might mediate cell wall biosynthesis through upregulation of *Ncmnn4* under azole stress.

In addition, this study partially revealed the relationship be-

tween ADS-4 and CCG-8, two transcription factors that positively regulate responses to azoles. The RNA-seq data showed that ADS-4 and CCG-8 did not regulate each other. A comparison of the genes regulated by ADS-4 and the genes regulated by CCG-8 showed that only *cdr4* was regulated by both ADS-4 and CCG-8. Deletion of either *ads-4* or *ccg-8* did not completely block the *cdr4* transcriptional response to ketoconazole (20). Therefore, the *cdr4* transcriptional responses to azole stresses are probably regulated by a number of transcription factors. In addition to *ads-4* and *ccg-8*, genes encoding other transcription factors were also found in the ketoconazole-responsive gene group. Thus, it should be possible to find new transcription factors that regulate the mechanisms controlling azole responses and resistance.

In summary, this study identified a new regulator of azole responses and partially revealed its mechanism. However, its detailed regulatory mechanisms, such as the consensus DNA motifs that ADS-4 might recognize and bind to and the signaling pathway that activates *ads-4* transcription during azole stress, remain to be further clarified.

ACKNOWLEDGMENTS

This project was supported by grants from the National Natural Science Foundation of China (grants 31461143002 and 31371986) and the China Ocean Mineral Resources R & D Association (grant DY125-15-T-07).

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