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Transcription Factor CCG-8 as a New Regulator in the Adaptation to Antifungal Azole Stress

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Antifungal azoles are widely used for controlling fungal infections. Fungi are able to change the expression of many genes when they adapt to azole stress, and increased expression of some of these genes can elevate resistance to azoles. However, the regulatory mechanisms behind transcriptional adaption to azoles in filamentous fungi are poorly understood. In this study, we found that deletion of the transcription factor gene *ccg-8*, which is known to be a clock-controlled gene, made *Neurospora crassa* hypersensitive to azoles. A comparative genome-wide analysis of the responses to ketoconazole of the wild type and the *ccg-8* mutant revealed that the transcriptional responses to ketoconazole of 78 of the 488 transcriptionally ketoconazole-upregulated genes and the 427 transcriptionally ketoconazole-downregulated genes in the wild type were regulated by CCG-8. Ketoconazole sensitivity testing of all available knockout mutants for CCG-8-regulated genes revealed that CCG-8 contributed to azole adaption by regulating the ketoconazole responses of many genes, including the target gene (*erg11*), an azole transporter gene (*cdr4*), a hexose transporter gene (*hxt13*), a stress response gene (locus number NCU06317, named *kts-1*), two transcription factor genes (NCU01386 [named *kts-2*] and *fsd-1/ndt80*), four enzyme-encoding genes, and six unknown-function genes. CCG-8 also regulated phospholipid synthesis in *N. crassa* in a manner similar to that of its homolog in *Saccharomyces cerevisiae*, Opi1p. However, there was no cross talk between phospholipid synthesis and azole resistance in *N. crassa*. CCG-8 homologs are conserved and are common in filamentous fungi. Deletion of the CCG-8 homolog-encoding gene in *Fusarium verticillioides* (*Fvccg-8*) also made this fungus hypersensitive to antifungal azoles.

Antifungal azoles, which inhibit ergosterol biosynthesis by disrupting the essential P450 superfamily protein lanosterol 14α-demethylase CYP51 (also called ERG11), are the most widely used drugs for controlling fungal infections. However, when subjected to antifungal azole stress, fungi are able to transcriptionally change the expression of a number of genes (1–4). For example, *Candida albicans* can elevate the transcriptional levels of the azole efflux pump genes *CDR1* and *CDR2* and the azole drug target gene *ERG11* in response to ketoconazole treatment (5, 6). Similar responses were found in *Saccharomyces cerevisiae* (4), *Aspergillus fumigatus* (3), *Trichophyton rubrum* (1), and *Fusarium graminearum* (2, 7). Overexpression of these genes increases resistance to azoles in many fungal species (8–14). In addition to these genes, several other genes that are transcriptionally elevated by antifungal azoles have been found. For example, in *S. cerevisiae*, many genes downstream of *ERG11* in ergosterol biosynthesis and genes involved in lipid and cell wall biosynthesis are also transcriptionally elevated by ketoconazole treatment (4). However, the roles of the majority of azole-responsive genes in azole adaption are unknown.

Transcriptional responses to azole stress are known to be regulated by several transcription factors in *S. cerevisiae* and *C. albicans*. In *S. cerevisiae*, the transcription factors Pdr1p and Pdr3p regulate the transcription of multidrug efflux pump genes, such as *PDR5*, *SNQ2*, *YOR1*, and *FLR1* (15). Their *C. albicans* homologs, Fcr1p and Fcr3p, have similar roles (16). Our BLASTp search found that filamentous fungi do not contain proteins that are homologous to Pdr1p or Pdr3p. A zinc finger domain containing transcription factor Upc2p in *C. albicans* and its paralog in *S. cerevisiae*, Ecm22p, regulate ergosterol synthesis genes (17–20). Upc2p can directly bind to the promoters of ergosterol biosynthesis genes, such as *NCP1*, *ERG11*, and *ERG2*, and to multidrug efflux genes, including *CDR1*, *MDR1*, and *YOR1*, in *C. albicans* (21). Although Upc2p homologs are present in filamentous fungi, their functions in the azole response are unknown. To date, only a bzip-type transcription factor, AP-1, is known to be important for azole resistance in both yeasts and filamentous fungi (22–24). Thus, compared with those of *C. albicans* and *S. cerevisiae*, the regulatory mechanisms behind azole responses in filamentous fungi are poorly understood. The unknown transcription factors that regulate azole responses need to be identified in filamentous fungi.

Neurospora crassa has transcriptional responses to ketoconazole that are similar to those of pathogenic fungi for both ergosterol biosynthesis genes and the Pdr5p-like ABC transporter gene *cdr4* (25, 26), and about 70% of the genes in *N. crassa* have knockout mutants, which means that *N. crassa* may be an excellent model for identifying regulatory genes in drug resistance. Using this model, we have demonstrated that sterol C-22 desaturase ERG5 is required for wild-type azole resistance in *N. crassa* and *Fusarium verticillioides* (26). In this study, we found that the transcription factor CCG-8 positively regulates the ketoconazole re-

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sponses of many genes in *N. crassa* and is required for wild-type resistance to antifungal azoles in *N. crassa* and *F. verticillioides*.

MATERIALS AND METHODS

Strains and culture conditions. Most of the *N. crassa* strains, including FGSC 4200 (wild-type strain), FGSC 20378 (*ccg-8*; mating type *a*), and knockout mutants for genes regulated by *ccg-8* deletion, were obtained from the Fungal Genetics Stock Center (www.fgsc.net/; University of Kansas Medical Center). Vogel's minimum medium (27), supplemented with 2% (wt/vol) sucrose for slants or 2% glucose for plates and the liquid medium, was used for culturing the strains. All cultures were grown at 28°C. Antifungal compounds were added as needed.

F. verticillioides wild-type strain 7600 and its transformants were maintained on potato dextrose agar (PDA) (Difco, Detroit, MI).

Drug susceptibility test. Ketoconazole, fluconazole, and itraconazole were dissolved in dimethyl sulfoxide (DMSO) and then aseptically added to the autoclaved medium before the agar plates were made. The final concentrations of ketoconazole, fluconazole, and itraconazole in the agar plates were 2, 15, and 6 μg/ml, respectively. The final DMSO concentration was below 0.25% (vol/vol). Two microliters of conidial suspension was inoculated onto individual plates (diameter [Φ] = 9 cm) with or without antifungal drugs and incubated at 28°C.

Complementation of the *N. crassa ccg-8* mutant. To complement the *ccg-8* knockout mutant, a complementary plasmid, pCB1532-ccg8, was created by inserting a 4,301-bp DNA fragment containing the *ccg-8* gene (2,015 bp) flanked by a 1,383-bp upstream regulatory region and a 903-bp downstream region into the pCB1532 plasmid, which also contained a sulfonylurea resistance allele of the *Magnaporthe grisea ILV1* gene as a selective marker (28). The insert was amplified from the wild-type strain, FGSC 4200, using primers ccg8(p)F-SmaI (TCCCCCGGGCTTCTTACA TAGGTAGTCGGATTGG) and ccg8(3)R-EcoRI (CGGAATTCGCTCCA AGTTGTTTGCCAT), digested by SmaI and EcoRI, and then ligated into the pCB1532 plasmid. The pCB1532-ccg8 construct was transformed into the *ccg-8* deletion mutant, FGSC 20378 (*ccg-8*; *a*), using the previously reported protoplast transformation method (29). Chlorimuron ethyl (15 μg/ml; Sigma) was added to the top of the agar to inhibit the growth of nontransformed protoplasts.

RNA extraction and transcriptional analysis by reverse transcription-quantitative PCR (qRT-PCR). Mycelia were harvested, immediately frozen, and then ground into a fine powder in liquid nitrogen. Total RNA was extracted and treated with DNase I to remove genomic DNA according to the standard TRIzol protocol (Invitrogen, Carlsbad, CA, USA). The cDNAs were synthesized from total cellular RNA using a cDNA synthesis kit (Fermentas, Burlington, Canada) according to the manufacturer's protocol.

Gene-specific primers (Table 1) were designed using online tools from PrimerQuest. The 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) according to the manufacturer's instructions. Each cDNA sample was analyzed in triplicate, and the average threshold cycle (C_T) was calculated. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (30). The results were normalized to the expression level of β-tubulin.

Transcriptomic profiling analysis. Genome-wide transcriptional profiles for the wild type and the *ccg-8* deletion mutant in Vogel's medium with or without ketoconazole treatment (2.5 μg/ml for 24 h) were obtained by digital gene expression (DGE) profiling (31). Briefly, conidia from the wild-type strain and the *ccg-8* deletion mutant were added to 20 ml liquid medium in a plate (Φ = 9 cm) and incubated for 24 h at 28°C in the dark until they formed a mycelial mat on the surface of the liquid medium. The mycelial mat was then cut into small pieces (Φ = 10 mm) and transferred to Vogel's liquid medium (two pieces/100 ml) in 250-ml flasks. The cultures were shaken at 180 rpm and incubated at 28°C for 12 h. Ketoconazole was then added to the medium to reach a final concentration of 2.5 μg/ml. After 24 h of incubation, mycelia were harvested and total RNA was extracted and subjected to DGE analysis as described by Sun et al. (32). In this study, the transcriptional ratios between two samples of more than 2 and less than 0.5 were used as the thresholds for defining differentially expressed genes.

Deletion of the *ccg-8* homolog gene in *F. verticillioides*. The vector for deleting the *ccg-8*-homologous gene in *F. verticillioides*, *Fvccg8* (FVEG_06643), was constructed as follows. The 5′-end-flanking sequences (1,351 bp) of FVEG_06643 were amplified using primers

TABLE 1 Gene-specific primer pairs used for reverse transcription-quantitative PCR assay

Gene	Primer name	Nucleotide sequence (5′→3′)
NCU09686 (*ccg-8*)	QN9686F	AAGGTGGCTCTCTCCTTTA
	QN9686R	GGTCATTTGGTTCATCTTCTTG
NCU02624 (*erg11*)	QN2624F	CCGCCATTGTCAAGGAAA
	QN2624R	ACGTGATCGGTCGGAATA
NCU05591 (*cdr4*)	QN5591F	ACGCTTTGGAAATGGATGGTGACG
	QN5591R	ATGAACAAGGCGACGGAAATGCAG
NCU06666 (*ino1*)	QN6666F	CACACCGTTGTGATCAAGTA
	QN6666R	CACGTTGAAGAGCGAGATG
NCU06317 (*kts-1*)	QN6317F	CACTACCACCACCAACAAG
	QN6317R	GGACCAGCATGTGCAATA
NCU01386 (*kts-2*)	QN1386F	ATGGCACCCTTTGTGATG
	QN1386R	CAGTCCATCTCTCTTGGAAAC
NCU09915 (*fsd-1/ndt80*)	QN9915F	ATCCGAGAGGTGGCTATG
	QN9915R	GTAGATCGTTGCAGGGAAAT
NCU09691 (*kts-3*)	QN9691F	GATTGCGACCCGAAGAAG
	QN9691R	GGATCATCCACACAAGTCAG
NCU02084 (*arginase*)	QN2084F	GATAGTCACATCGACACATGG
	QN2084R	ATGGAGGTGTTGTGGATTAAG
NCU09635 (*kts-4*)	QN9635F	CATCCCTCCTAACAACAAGAC
	QN9635R	CCGTTGGCATAATTGACATAAG
NCU01315 (*gt41-1*)	QN1315F	CTGATTGCTGGCGATGATTA
	QN1315R	CACCACCAGCACCATTATAC
NCU08957 (*kts-5*)	QN8957F	CTCTGACATCTCGGACTTTG
	QN8957R	TTGGAGGAGGAGGAAGATT
NCU01633 (*hxt13*)	QN1633F	GACTCCATTGGCTACTTCTATG
	QN1633R	TACATGGTCTCGATCTCCTC
NCU00247 (*kts-6*)	QN0247F	CGCCTTCTTCAGCTTCTTT
	QN0247R	GATGATGGTGGGATGAATGAG
NCU01302 (*hydrolase*)	QN1302F	CATTCCCTACCAAGAGATCAAG
	QN1302R	GTTGCAGATAGGACGAGTATG
NCU00611 (*kts-7*)	QN0611F	CATATCTCCATGCCACTCTTG
	QN0611R	CAAGGAGTTCAGCTGGTAAG
NCU00248 (*kts-8*)	QN0248F	GAGGTAGGAGGAACGACAT
	QN0248R	GGGCATCCCTCTGATAAGTA
NCU04334 (*cpn10*)	QN4334F	CCCGAGTCCTCCGTTAAG
	QN4334R	GCCGTACTGAGGGATAAGAA
NCU04923 (*gcy-1*)	QN4923F	GAGAGCAACTTCCAGATTCC
	QN4923R	CCAGACATCGTAGCCAAAG
β-*tub*	QbtubF	CCCAAGAACATGATGGCTGCTTCT
	QbtubR	TTGTTCTGAACGTTGCGCATCTGG

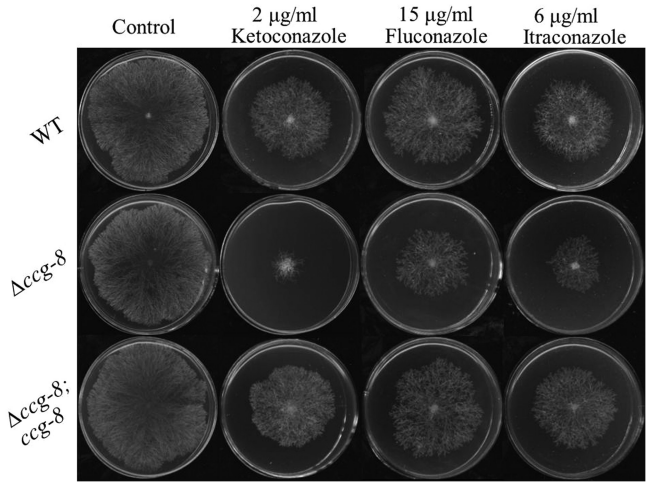

FIG 1 Drug susceptibility analysis of the *N. crassa* wild type (WT), the *ccg-8* knockout mutant (Δ*ccg-8*), and the *ccg-8*-complemented strain (Δ*ccg-8*; *ccg-8*). Two microliters of conidial suspension (1×10^4 conidia/ml) was inoculated onto the center of each plate ($\Phi = 9$ cm) with or without antifungal drugs; the final concentrations of ketoconazole, fluconazole, and itraconazole were 2 μg/ml, 15 μg/ml, and 6 μg/ml, respectively. The plates were incubated at 28°C in the dark for about 66 h. Each test had three replicates, and the experiment was independently repeated twice.

Fv06643(p)F-KpnI (TGGGGTACCCTTCTCCTCAACGCACCCT) and Fv06643(p)R-XhoI (CCGCTCGAGCGCAAGACGATAGCCCAC), and the 3′-end-flanking sequences (1,597 bp) of FVEG_06643 were amplified using primers Fv06643(3)F-SmaI (TCCCCCGGGCGTTTGGTGGGCTTTGTA) and Fv06643(3)R-NotI (ATAAGAATGCGGCCGCGAATGACGGGAGGCGATGA). The resulting PCR products were cloned into the pCSN44 plasmid (33) to create the knockout construct pCSN44-ΔFv06643, in which the PCR product was ligated with the hygromycin phosphotransferase (*hph*) gene. Then, the deletion cassette was transformed into *F. verticillioides* 7600 to create the deletion mutants. Fungal transformation followed the protocol reported by Miller et al. (34), with minor modifications, as described by Li et al. (35).

RESULTS

***N. crassa ccg-8* deletion elevates azole sensitivity.** By screening the knockout mutants of the transcription factor-encoding genes, we successfully isolated a mutant, FGSC 20378, that displayed hypersensitivity to multiple antifungal azoles. FGSC 20378 lacks the *ccg-8* (locus number NCU09686) gene, which has previously been identified as a clock-controlled gene (36). Without antifungal drugs, the differences in the growth rates (Fig. 1) and conidial yields (data not shown) between the *ccg-8* deletion mutant and the wild-type strain were not significant. However, when the strains were grown on agar plates with ketoconazole, fluconazole, or itraconazole, the growth of the *ccg-8* deletion mutant was more inhibited by these azoles than was that of the wild-type strain (Fig. 1). The rates of inhibition of the *ccg-8* deletion mutant by ketoconazole (2 μg/ml), fluconazole (15 μg/ml), and itraconazole (6 μg/ml) were 80% ± 3%, 44% ± 2%, and 58% ± 2%, respectively. In contrast, the rates of inhibition of the wild type by these drugs were only 30% ± 2%, 22% ± 2%, and 34% ± 3%, respectively.

***N. crassa ccg-8* deletion does not affect sensitivities to other stresses.** In addition to testing sensitivities to antifungal drugs, we tested the sensitivity of the *ccg-8* mutant to other stresses, including high temperature (40°C), osmotic stress (1 M NaCl), and oxidative stress (18 μg/ml menadione). The *ccg-8* mutant displayed wild-type levels of sensitivity to these stresses (data not shown), which suggested that CCG-8 is not involved in adaptation to these stresses.

Complementation of the *ccg-8* deletion mutant. To complement the *ccg-8* deletion mutant, we transformed the complementary pCB1532-ccg8 plasmid into the *ccg-8* deletion mutant. Ten transformants were obtained, and six transformants displayed wild-type azole sensitivity (the phenotypic characteristics of a representative transformant are shown in Fig. 1), which confirmed that the hypersensitivity to azoles in the *ccg-8* mutant was caused by *ccg-8* deletion.

Transcription of *ccg-8* is induced by ketoconazole. Genome-wide transcriptional responses to ketoconazole treatment were analyzed by the transcriptome sequencing (RNA-seq) method, and the transcriptional level of *ccg-8* increased by at least three times after ketoconazole (2.5 μg/ml) treatment for 24 h (Table 2). The transcriptional levels of *ccg-8* in the wild type treated with or without ketoconazole were further analyzed by qRT-PCR. The *ccg-8* transcriptional level in the wild type increased 3.6 times after 24 h of ketoconazole treatment (2.5 μg/ml) relative to the levels without treatment (Fig. 2).

CCG-8 regulates transcriptional responses to ketoconazole stress. We analyzed genome-wide transcriptional profiles in the wild type and the *ccg-8* deletion mutant in Vogel's medium with or without ketoconazole (2.5 μg/ml for 24 h) using the DGE method in order to understand how CCG-8 influences azole sensitivity.

After ketoconazole treatment, 488 genes were upregulated and 427 genes were downregulated in the wild-type strain (see Table S1 in the supplemental material). The transcriptional levels for 70 of the 488 genes upregulated by ketoconazole stress in the wild type were significantly lower in the *ccg-8* mutant than in the wild type (Tables S1 and S2 in the supplemental material), and transcriptional levels for 8 (NCU07332, NCU00999, NCU07454, NCU02579, NCU01219, NCU04334, NCU04720, and NCU04923) of the 427 genes downregulated by ketoconazole stress in the wild type were significantly higher in the *ccg-8* mutant than in the wild-type strain (Tables S1 and S2). These results indicated that *ccg-8* deletion can affect the normal transcriptional responses to ketoconazole.

The DEG data also showed that without ketoconazole treatment, *ccg-8* deletion upregulated 570 genes and downregulated 386 genes relative to transcriptional levels in the wild type (see Table S3 in the supplemental material). There were no obvious defects in growth and development under the tested culture conditions without azole, so the transcriptional changes caused by *ccg-8* deletion should not affect growth and development.

Roles of genes regulated by CCG-8 in azole resistance. Seventy-eight genes are differentially expressed between the *ccg-8* mutant and the wild type during ketoconazole stress, and overexpression of either of two of these genes, *cdr4* (the *PDR5* homolog) and *erg11*, has previously been shown to confer azole resistance in many fungi (8–14). The sensitivity to ketoconazole of their knockout mutants was tested in order to understand the roles of the remaining CCG-8-regulated genes in azole resistance. For 70 genes positively regulated by CCG-8, 56 single-gene-knockout mutants are available from the Fungal Genetics Stock Center. Our test showed that 14 mutants with single-gene deletions were hypersensitive to ketoconazole compared to the wild type (Fig. 3; Tables 2 and 3). Among the knocked-out genes were two transcription factor-encoding genes, *fsd-1* or *ndt80* (NCU09915) and

TABLE 2 Comparison of the transcriptional responses by the wild type and the *ccg-8* mutant to ketoconazole stress by gene set[a]

Locus and/or category[b]	Gene	Function	TPM in WT	TPM in WT(k)	Fold change [WT(k)/WT]	*P* value	TPM in Δ*ccg-8*	TPM in Δ*ccg-8*(k)	Fold change [Δ*ccg-8*(k)/ Δ*ccg-8*]	*P* value	Fold change [Δ*ccg-8*(k)/ WT(k)]	Sensitivity to ketoconazole compared to WT's
Phospholipid synthesis pathways												
NCU06666	*ino1*	Inositol-3-phosphate synthase	38.86	21.55	0.55	2.65E−05	180.9	17.25	0.09	4.7E−117	0.80	Similar
NCU08045	*cho2*	Phosphatidylethanolamine *N*-methyltransferase	11.22	6.16	0.55	0.022589	110	27.06	0.25	3.34E−41	4.39	NA
NCU03695	*psd1*	Phosphatidylserine decarboxylase proenzyme 1	28.78	51.28	1.78	1.87E−14	69.7	115.08	1.65	7.63E−10	2.26	Similar
NCU02381	*cho1*	Phosphatidylserine synthase	18.13	8.12	0.45	0.000217	29.96	13.08	0.44	1.91E−06	1.61	NA
NCU09643	*cds1*	Phosphatidate cytidylyltransferase	17.56	13.43	0.76	0.165267	24.02	18.73	0.78	0.138875	1.39	NA
Kinases participating in Opi1p (CCG-8 homolog in *S. cerevisiae*) phosphorylation												
NCU06240	*pkac-1*	Catalytic subunit protein kinase A	26.48	24.63	0.93	0.6266	40.04	9.81	0.25	5.40E−16	0.40	Similar
NCU06544	*pkc*	Protein kinase C	24.75	67.16	2.71	0	52.79	24.98	0.47	5.28E−09	0.37	Similar
NCU03124	*ck2*	Catalytic subunit of protein kinase CK2	68.21	155.87	2.29	1.61E−13	52.2	60.07	1.15	0.173376	0.39	Similar
Target enzyme of azoles and multidrug transporters												
NCU02624	*erg11*	Cytochrome P450 lanosterol 14a-demethylase	69.08	676.66	9.80	0	113.9	254.84	2.24	4.01E−13	0.38	NA
NCU05591	*cdr4*	ABC transporter CDR4	3.74	259.97	69.51	2.17E−07	4.75	54.42	11.46	4.10E−08	0.21	Hypersensitive
NCU04161		Multidrug resistance-associated protein 5	2.59	20.99	8.10	2.57E−06	4.75	6.84	1.44	0.264586	0.33	Similar
Stress responses												
NCU01499		Related to hsp70	16.12	39.74	2.47	1.97E−09	35.29	147.49	4.18	0	3.71	Similar
NCU06317	*kts-1*	Stress response RCI peptide	123.19	1142.04	9.27	1.56E−13	323.3	412.45	1.28	1.56E−09	0.36	Hypersensitive
NCU09873	*pck-1/acu-6*	Phosphoenolpyruvate carboxykinase	4.89	31.06	6.35	1.68E−08	300.1	275.96	0.92	0.064493	8.88	Similar
NCU00355	*cat-3*	Catalase-3	135.85	252.42	1.86	0	54.28	24.98	0.46	1.08E−09	0.10	Similar
Transcription factors												
NCU09686	*ccg-8*	Clock-controlled protein 8	33.97	127.89	3.76	3.94E−13	0	0				Hypersensitive
NCU01386	*kts-2*	Zn(II)2Cys6 domain-containing protein	0.58	26.86	46.31	0.000112	1.78	5.95	3.34	0.00582	0.22	Hypersensitive
NCU01629		C2H2 finger domain-containing protein	4.03	53.45	13.26	1.15E−07	28.77	25.57	0.89	0.427838	0.48	Similar
NCU05536		Zn(II)2Cys6 domain-containing protein	6.04	48.97	8.11	1.25E−09	6.23	8.33	1.34	0.317684	0.17	Similar
NCU09496		C2H2 transcription factor	10.07	97.94	9.73	2.66E−13	37.37	47.58	1.27	0.042249	0.49	NA
NCU04022		C2H2 finger domain-containing protein	12.66	120.89	9.55	6.62E−14	33.22	29.44	0.89	0.38236	0.24	NA
NCU09915	*fsd-1/ndt80*	Female sexual development-1 protein	7.77	46.17	5.94	2.51E−11	46.56	21.41	0.46	1.60E−08	0.46	Hypersensitive

[a] WT, wild type; WT(k) or Δ*ccg-8*(k), wild type or Δ*ccg-8* mutant treated with ketoconazole, respectively; TPM, number of locus tags per million; NA, the mutant was not available.

[b] Locus numbers and function were annotated according to the *N. crassa* genome assembly (http://www.broadinstitute.org/annotation/genome/neurospora/).

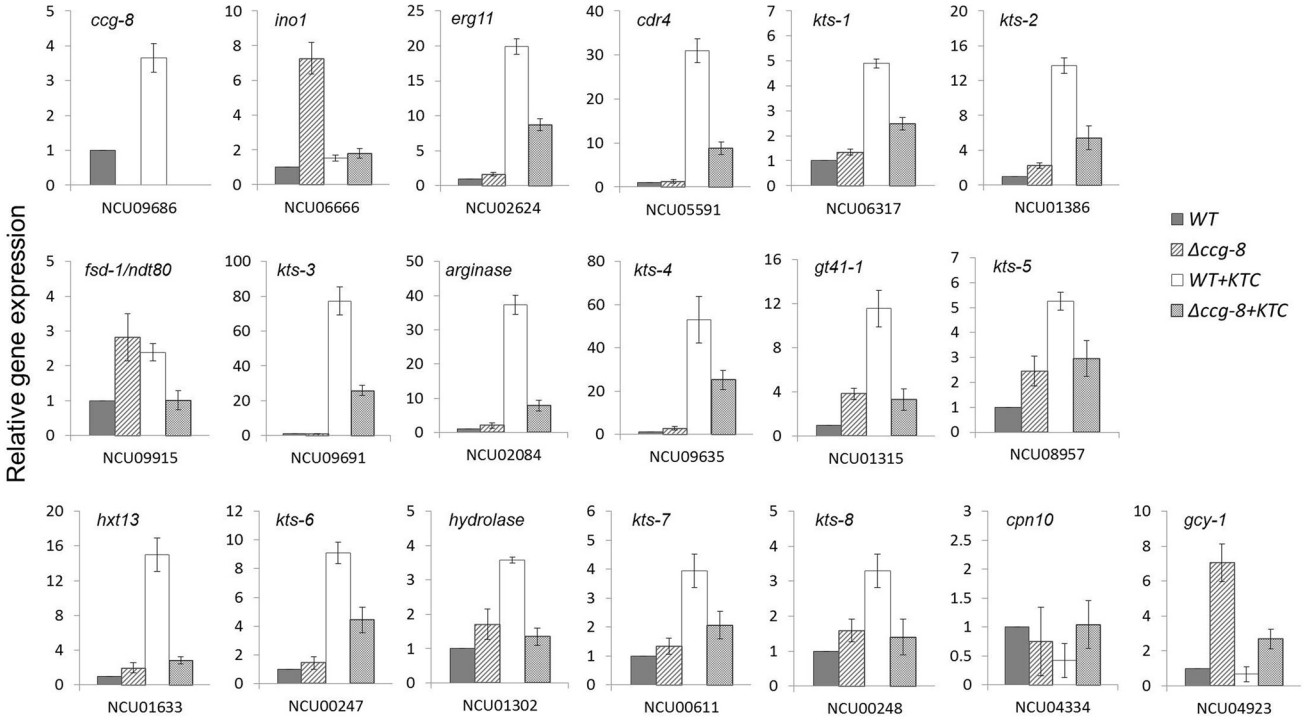

FIG 2 Differential expression of genes in the *ccg-8* deletion mutant relative to that in the wild-type strain in response to azole stress as determined by qRT-PCR. Values shown are means of results from three independent replicates. Standard deviations are indicated with error bars. The wild-type strain and the *ccg-8* deletion mutant cultures were inoculated into Vogel's liquid medium and incubated at 28°C with shaking at 180 rpm for 12 h. Ketoconazole (KTC) was then added into the medium to reach a final concentration of 2.5 μg/ml. After incubation for 24 h, mycelia were harvested and total RNA was extracted and subjected to qRT-PCR analysis.

NCU01386. Transcription factor FSD-1 is essential for female fertility in *N. crassa* (37). An FSD-1 homolog in *S. cerevisiae*, Ndt80, controls the expression of genes that regulate meiotic progression and spore formation (38, 39). NCU01386 encodes a Zn(II)Cys6-type transcription factor, and its highly conserved homologs are present in filamentous fungi, including the human pathogen *A. fumigatus*, but not in yeasts. To date, none of its homologs has previously been characterized. Mutants for the hexose transporter

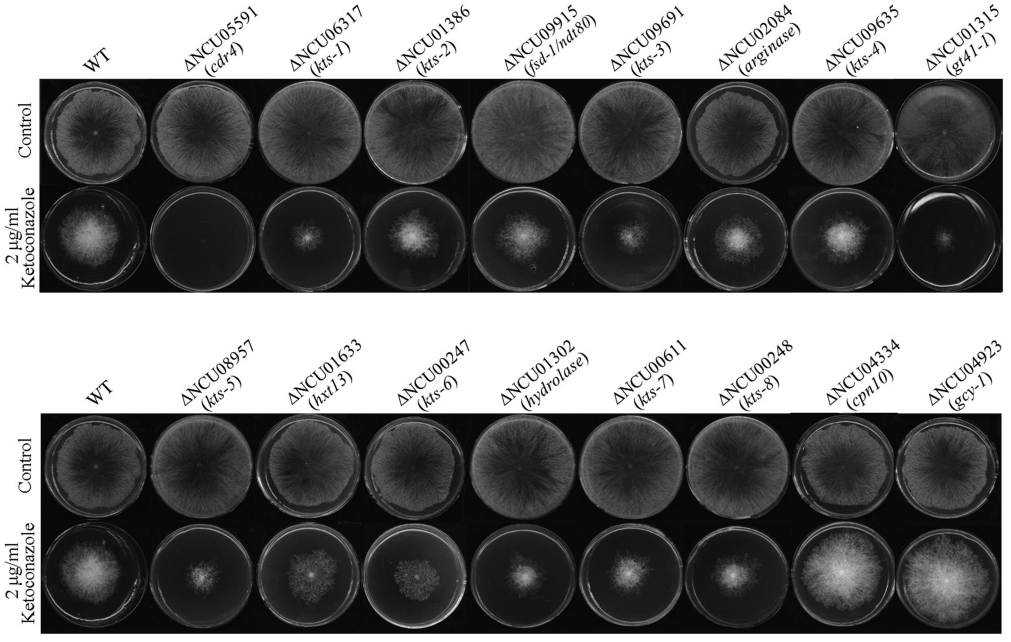

FIG 3 Ketoconazole susceptibility analyses of *N. crassa* knockout mutants for genes regulated by CCG-8. Two microliters of conidial suspension (1×10^4 conidia/ml) was inoculated onto the center of each plate ($\Phi = 9$ cm) with or without ketoconazole (2 μg/ml), and the plates were incubated at 28°C in the dark for about 66 h.

HXT13 gene *hxt13* (NCU01633), the azole transporter gene *cdr4* (NCU05591), a stress-responsive gene (NCU06317), a putative UDP-*N*-acetylglucosaminyltransferase gene, *gt41-1* (NCU01315), an arginase-encoding gene (NCU02084), and a hydrolase-encoding gene (NCU01302) were also hypersensitive to ketoconazole (Fig. 3). In addition, 6 mutants with deletions of genes encoding hypothetical proteins with unknown functions, NCU09691, NCU09635, NCU08957, NCU00247, NCU00611, and NCU00248, were also hypersensitive to ketoconazole (Fig. 3). We named all unannotated genes as shown in Tables 2 and 3, based on the ketoconazole-hypersensitive phenotypes of the knockout mutants. For example, NCU06317 was named the ketoconazole-sensitive 1 (*kts-1*) gene, and NCU01386 was named the ketoconazole-sensitive 2 (*kts-2*) gene.

The positive regulation of these genes by CCG-8 under ketoconazole stress was confirmed by qRT-PCR (Fig. 3). In response to ketoconazole, the expressions of these genes in both the wild type and the *ccg-8* mutant were induced. However, their expression levels were lower in the *ccg-8* mutant than in the wild type under the ketoconazole treatment condition. Since deletion of these genes results in increased sensitivity to ketoconazole, lower levels of expression of these genes may be the reason behind the hypersensitivity to antifungal azoles in the *ccg-8* mutant.

Of the eight genes negatively regulated by CCG-8 (see Table S2 in the supplemental material), six currently have knockout mutants available. Two mutants with single genes (NCU04334 and NCU04923) knocked out were more resistant to ketoconazole than the wild type (Fig. 3). NCU04334 is a chaperonin-encoding gene, *cpn10* (http://www.broadinstitute.org/annotation/genome/neurospora/GeneDetails.html?sp=S7000007580545759), and NCU04923 is a glycerol dehydrogenase-encoding gene, *gcy-1* (http://www.broadinstitute.org/annotation/genome/neurospora/GeneDetails.html?sp=S7000007580553568). The negative regulation of NCU04923 (*gcy-1*) by CCG-8 under ketoconazole stress was confirmed by qRT-PCR (Fig. 2). Since their deletion is able to elevate resistance to ketoconazole, negative regulation of their expression by CCG-8 should make *N. crassa* resistant to azoles.

CCG-8 regulates phospholipid synthesis that is not involved in azole resistance. The homolog of CCG-8 in *S. cerevisiae* is Opi1p (40), which plays a negative regulatory role in the expression of inositol-3-phosphate synthase upstream activation sequence (UAS_{INO})-containing genes involved in phospholipid synthesis (41). In *N. crassa*, CCG-8 also regulates phospholipid synthesis because most UAS_{INO}-containing genes were upregulated in response to *ccg-8* deletion. For example, in response to *ccg-8* deletion, the inositol-3-phosphate synthase gene *ino1* (NCU06666), phosphatidylserine decarboxylase proenzyme gene *psd1* (NCU03695), and phosphatidylethanolamine *N*-methyltransferase gene *cho2* (NCU08045) were transcriptionally increased by 4.6, 2.4, and 9.8 times, respectively (Table 2). However, the UAS_{INO}-containing genes showed no transcriptional response to ketoconazole stress in the wild type (Table 2). Furthermore, all available mutants for UAS_{INO}-containing genes displayed wild-type ketoconazole susceptibility (Table 2). These results indicated that phospholipid synthesis was not involved in azole adaption. The regulation of phospholipid synthesis and the regulation of azole responses are two independent functions of CCG-8.

Deletion of the *ccg-8* homolog gene increases azole susceptibility in *F. verticillioides*. CCG-8 homologs are widely distributed in filamentous fungi (40). We chose the plant-pathogenic fungus *F. verticillioides* as a test species to investigate whether CCG-8 in

TABLE 3 Significantly differently expressed genes between the wild type and the *ccg-8* mutant in response to ketoconazole stress[a]

Category and locus[b]	Gene	Function	TPM in WT	TPM in WT(k)	Fold change [WT(k)/WT]	*P* value	TPM in Δ*ccg-8*	TPM in Δ*ccg-8*(k)	Fold change [Δ*ccg-8*(k)/Δ*ccg-8*]	*P* value	Fold change [Δ*ccg-8*(k)/WT(k)]	Sensitivity to ketoconazole compared to WT's
Upregulated genes												
NCU09691	*kts-3*	Hypothetical protein	0.01	14.83	1483	8.88E−16	0.01	3.87	387	0.00012	0.26	Hypersensitive
NCU02084		Arginase	13.82	1007.43	72.90	9.08E−14	67.92	304.5	4.48	2.78E−13	0.30	Hypersensitive
NCU09635	*kts-4*	Hypothetical protein	3.17	216.04	68.15	7.57E−07	8.3	69.88	8.42	2.00E−11	0.32	Hypersensitive
NCU01315	*gt41-1*	UDP-*N*-acetylglucosaminyltransferase	2.59	38.9	15.02	2.57E−06	20.46	11.3	0.55	0.002807	0.29	Hypersensitive
NCU08957	*kts-5*	Hypothetical protein	5.47	69.4	12.69	4.61E−09	16.31	27.06	1.66	0.002712	0.39	Hypersensitive
NCU01633	*hxt13*	Hexose transporter HXT13	377.04	4679.24	12.41	1.73E−12	622.5	1352.73	2.17	0	0.29	Hypersensitive
NCU00247	*kts-6*	Hypothetical protein	6.62	64.36	9.72	3.39E−10	7.71	13.98	1.81	0.013643	0.22	Hypersensitive
NCU01302		Hydrolase	8.63	41.98	4.86	3.78E−12	17.2	9.81	0.57	0.009111	0.23	Hypersensitive
NCU00611	*kts-7*	Hypothetical protein	8.06	30.78	3.82	1.54E−11	6.52	14.57	2.23	0.001244	0.47	Hypersensitive
NCU00248	*kts-8*	Hypothetical protein	59	216.04	3.66	3.92E−13	92.24	85.05	0.92	0.322136	0.39	Hypersensitive
Downregulated genes												
NCU04334	*cpn10*	Chaperonin	45.76	14.55	0.32	1.18E−14	18.09	31.22	1.73	0.000581	2.15	Resistant
NCU04923	*gcy-1*	Glycerol dehydrogenase	83.76	33.86	0.40	1.77E−18	462.4	163.85	0.35	1.7E−110	4.84	Resistant

[a] WT, wild type; WT(k) or Δ*ccg-8*(k), wild type or Δ*ccg-8* mutant treated with ketoconazole, respectively; TPM, number of locus tags per million; NA, the mutant was not available.
[b] Locus numbers and function were annotated according to the *N. crassa* genome assembly (http://www.broadinstitute.org/annotation/genome/neurospora/).

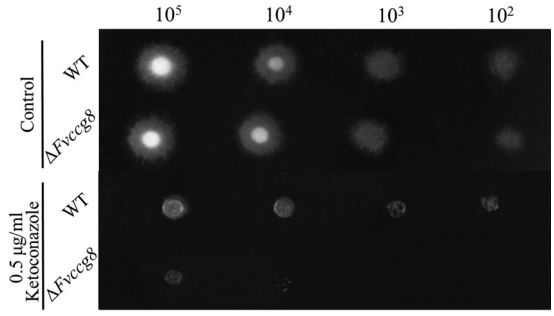

FIG 4 Ketoconazole susceptibility analysis of the *Fvccg*-8 (FVEG_06643) knockout mutant. Two-microliter specimens of conidial suspensions with different concentrations (1×10^5, 1×10^4, 1×10^3, or 1×10^2 conidia/ml) were inoculated onto the plates ($\Phi = 9$ cm), with or without ketoconazole, and incubated at 28°C for 72 h. Each test had three replicates, and the experiment was independently repeated twice.

other filamentous fungi also mediates azole sensitivity. *F. verticillioides* not only is a primary pathogen of maize but also can cause diseases in other crops. Moreover, ingestion of food contaminated by this fungus can cause esophageal cancer and neural tube defects in animal and human diseases, likely due to toxic metabolites produced by *F. verticillioides*, such as fumonisins, fusarins, and bikaverin (42). The *ccg-8*-homologous gene FVEG_06643 (*Fvccg-8* in this study) was deleted in *F. verticillioides*. On the medium without the drug, the growth rate of the *Fvccg-8* deletion mutant was similar to that of the wild-type strain (Fig. 4) and no conidiation defects were observed in the mutant (data not shown). When inoculated onto the medium with 0.5 μg/ml ketoconazole, the *Fvccg-8* deletion mutant showed greater growth inhibition than the wild-type strain, which indicated that the CCG-8 homologs have similar azole resistance roles in different fungal species.

DISCUSSION

Fungi are able to adjust the transcriptional levels of many genes when they adapt to azole stresses. In filamentous fungi, only transcription factor AP-1 was previously known as a regulator in azole adaption (24). In this study, we have shown that a second transcription factor, CCG-8, was also involved in the regulatory mechanism behind azole adaption in filamentous fungi by demonstrating that CCG-8 regulates transcriptional responses to ketoconazole in *N. crassa* and that CCG-8 is required for wild-type azole resistance in both *N. crassa* and *F. verticillioides*. Although CCG-8 in *N. crassa* has been identified as a clock-controlled gene (36) and its homolog Opi1p has previously been shown to be a negative regulator of UAS_{INO}-containing genes, which are involved in phospholipid synthesis (40, 41), the role of CCG-8 or its homologs in antifungal drug resistance has not previously been identified. In addition, this study has provided the first report of transcriptomic responses to antifungal azoles in the model fungus *N. crassa* and tested the ketoconazole sensitivities of 62 ketoconazole-responsive genes, which resulted in the identification of 15 new genes involved in azole adaption.

CCG-8 activates the transcriptional responses of the azole target gene *erg11* and the azole pump gene *cdr4* to ketoconazole stress in *N. crassa*. In *C. albicans*, the responses of the azole target gene *ERG11* and the azole pump gene *CDR1* (the *cdr4* homolog) to azoles are regulated by transcription factor Upc2p, which can directly bind to the promoters of *ERG11* and the azole pump gene *CDR1* (21). Deletion of Upc2p resulted in hypersensitivity to antifungal azoles in *C. albicans* (18, 19). Although it is unknown whether a Upc2p homolog binds to the promoters of the azole target gene *erg11* and azole pump gene *cdr4*, by screening knockout mutants, we found that a mutant with a deletion of the Upc2p-homologous gene *tah-3* (NCU03686), FGSC 11076, was not hypersensitive to ketoconazole (data not shown). This suggests that the role of the Upc2p homolog in *N. crassa* is not as important as that of Upc2p in *C. albicans*. In addition, as mentioned in the introduction, filamentous fungi do not have homologs for the transcription factors Pdr1p and Pdr3p, which regulate the transcription of the azole pump gene in *S. cerevisiae*, *PDR5* (15). These results suggested that filamentous fungi and yeasts may have different sets of transcription factors that regulate the responses to azole stress. However, since it is already known that transcription factor AP-1 is important for azole resistance in both yeasts and filamentous fungi (22–24), filamentous fungi and yeasts might share some common transcription factors in regulating azole adaptation. Homologs of CCG-8 are also found in *C. albicans* and other human-pathogenic *Candida* spp. (40). It is necessary to test the roles of CCG-8 homologs of *Candida* spp. in azole response and adaptation. CCG-8 activates the transcriptional responses of the azole target gene *erg11* and the azole pump gene *cdr4*. Overexpression of either the *cdr4* or *erg11* homolog is able to confer azole resistance in many fungi. Thus, CCG-8 probably positively regulates resistance to azoles by transcriptional activation of *cdr4* and *erg11* during azole stress. This study has shown that 13 of the 70 genes positively regulated by CCG-8, in addition to *erg11* and *cdr4*, made a significant contribution to azole resistance. Thus, CCG-8 may also positively regulate resistance to azoles by transcriptional activation of these genes during azole stress. Moreover, the reduced expression of two genes among the eight negatively regulated by CCG-8 was able to increase the organism's resistance to ketoconazole. Therefore, CCG-8 may elevate resistance to azoles by transcriptionally repressing these genes during azole stress. In addition to the genes that made significant contributions to azole resistance, many other CCG-8-regulated genes may contribute to azole resistance. For example, a multidrug resistance-associated protein 5-encoding gene, NCU04161 (http://www.broadinstitute.org/annotation/genome/neurospora/GeneDetails.html?sp=S7000007580550095), and three stress-responsive genes, NCU01499, *pck-1* (NCU09873), and *catalase-3* (NCU00355), were transcriptionally responsive to ketoconazole stress and were regulated by CCG-8 (Table 2). Increased expression of these genes may improve fungal survival under azole stress, although their roles may be detected by single-gene deletion. Not all genes differentially expressed between the *ccg-8* mutant and the wild type were directly regulated by CCG-8. We showed that transcription factors FSD-1 and KTS-2 (NCU01386) were regulated by CCG-8 and were involved in azole resistance. In addition to these two transcription factors, four other transcription factors (NCU01629, NCU05536, NCU09496, and NCU04022) responded to ketoconazole stress, and their transcriptional responses to ketoconazole were regulated by CCG-8 (Table 2). Thus, many of the genes differentially expressed between the *ccg-8* mutant and the wild type may be regulated by these transcription factors.

With the exceptions of those of *erg11* and *cdr4*, links to azole resistance for the CCG-8-regulated genes identified in this study have not been previously reported. Among them, stress-responsive gene NCU06317 (*kts-1*), UDP-*N*-acetylglucosaminyltrans-

ferase-encoding gene *gt41-1* (NCU01315), arginase-encoding gene NCU02084, and hydrolase-encoding gene NCU01302 may be generally required for adaptation to stresses because their transcriptional levels were also induced by oxidative stress (43). The ketoconazole-hypersensitive phenotype of the mutant with a deletion of the hexose transporter HXT13 gene *hxt13* (NCU01633) suggested that this transporter may be involved in azole transportation or that the transportation of hexose might contribute to azole resistance under azole stress. Deletion of the glycerol dehydrogenase-encoding gene *gcy-1* caused an increase in ketoconazole resistance. This suggested that glycerol metabolism may be involved in azole adaption. It has been reported that treatment by the fungicide fludioxonil, a derivative of the phenylpyrroles, which are structurally different from azoles, caused an accumulation of glycerol in *N. crassa* (44). Deletion of *gcy-1* theoretically reduces the conversion from glycerol to glycerone, which probably results in the accumulation of glycerol in cells. However, the role of glycerol in azole resistance still needs to be investigated. For the rest of the genes identified in this study, more functional information is required to explain how they contribute to azole sensitivity. The discovery of their roles in azole resistance is important when it comes to identifying the genetic causes of drug resistance in clinical or agricultural isolates of fungal pathogens.

In the future, the consensus DNA motifs bound by CCG-8 need to be identified, as does whether CCG-8 homologs in yeasts also regulate transcriptional responses to azoles. In addition, since CCG-8 is a clock-controlled protein (36), the relationship between the circadian clock and azole resistance needs to be investigated.

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