Coordinated Regulation of Membrane Homeostasis and Drug Accumulation by Novel Kinase STK-17 in Response to Antifungal Azole Treatment

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ABSTRACT The emergence of antifungal resistance, especially to the most widely used azole class of ergosterol biosynthesis inhibitors, makes fungal infections difficult to treat in clinics and agriculture. When exposed to azoles, fungi can make adaptive responses to alleviate azole toxicity and produce azole tolerance. However, except for azole efflux pumps and ergosterol biosynthesis genes, the role of most azole responsive genes in azole resistance is unknown. In this study, STK-17, whose transcription is upregulated by azoles, was characterized as a novel kinase that is required for azole resistance. Deletion or dysfunction of STK-17 led to azole hypersensitivity in Neurospora crassa and to other ergosterol biosynthesis inhibitors such as amorolfine, terbinafine, and amphotericin B, but not fatty acid and ceramide biosynthesis inhibitors. STK-17 was also required for oxidative stress resistance, but this was not connected to azole resistance. RNA-seq results showed that stk-17 deletion affected the basal expression and the response to ketoconazole of some membrane protein genes, indicating functional association of STK-17 with the membrane. Notably, deletion of stk-17 affected the normal response to azoles of erg genes, including the azole target-encoding gene erg11, and erg2, erg6, and erg24, and led to abnormal accumulation of sterols in the presence of azoles. HPLC-MS/MS analysis revealed increased intracellular azole accumulation in the stk-17 mutant, possibly due to enhanced azole influx and reduced azole efflux that was independent of the major efflux pump CDR4. Importantly, STK-17 was widely distributed and functionally conserved among fungi, thus providing a potential antifungal target.

IMPORTANCE Antifungal resistance is increasing worldwide, especially to the most widely used azole class of ergosterol biosynthesis inhibitors, making control of fungal infections more challenging. A lot of effort has been expended in elucidating the mechanism of azole resistance and revealing potential antifungal targets. In this study, by analyzing azole-responsive genes in Neurospora crassa, we discovered STK-17, a novel kinase, that is required for azole resistance in several types of fungi. It has a role in regulating membrane homeostasis, responses to azole by ergosterol biosynthesis genes and azole accumulation, thus, deepening our understanding on the mechanism of azole stress response. Additionally, STK-17 is conserved among fungi and plays important roles in fungal development and stress resistance. Kinase inhibitors are broadly used for treating diseases, and our study pinpoints a potential drug target for antifungal development.

KEYWORDS azole resistance, kinase, STK-17, Neurospora crassa, Fusarium verticilioides, sterol biosynthesis, azole accumulation

Fungal infections have large impacts on human health and agriculture (1). In spite of decades of research, only limited classes of antifungal drugs and fungicides are currently available (2, 3). Azoles, which inhibit ergosterol biosynthesis by directly binding to sterol 14α-demethylase, are the most widely used antifungals in clinics and agriculture. However,
numerous fungal pathogens have developed azole resistance and this is now a serious problem leading to therapy failure (2–4). Given the difficulties in developing antifungal drugs, increasing our understanding of how fungi neutralize antifungal compounds such as azoles would be helpful for maximizing their efficacy and to identify new antifungal targets.

When challenged by azole stress, fungi can make adaptive responses by changing expression of relevant genes across the genome, including those involved in ergosterol biosynthesis, efflux pumps, cell wall maintenance, lipid and amino acid metabolism, cell cycle regulation, and signal transduction (5–9). These changes allow fungi to quickly adjust their physiological state and maximize survival against azole exposure (10). Fungi lacking these responses are extremely sensitive to azoles (11). Deletions or mutation in transcription factors that regulate azole stress response, such as Pdr1/3p and Upc2p in yeast, Tac1p and Upc2p in Candida albicans, AtrR and SrbA in Aspergillus fumigatus, and CCG-8, ADS-4, CSP-1 in Neurospora crassa (5, 6, 12–18), cause alterations in azole sensitivity. Among these azole-responsive genes, the well-studied are those encoding the azole efflux pumps and sterol 14α-demethylase, as well as other ergosterol biosynthesis enzymes (2). Mutations leading to over-expression of azole efflux pumps, and those affecting sterol 14α-demethylase and enzymes involved in ergosterol biosynthesis, are frequently detected in azole-resistant isolates (2–4). Thus, genes required for reducing azole stress are often transcriptionally induced by azoles and this response can eventually result in resistance.

However, except for genes encoding azole efflux pumps and ergosterol biosynthesis enzymes, the roles of most of the azole-responsive genes in azole resistance have not been well characterized. One interesting and potentially important observation is that expression of some kinase genes is affected by azoles. Kinases, which regulate protein functions by phosphorylation, play important roles in adapting to stresses in fungi (19), and because they are more conserved than transcription factors, they may be useful as potential antifungal targets (19, 20). Some kinase genes were found to be co-regulated with azole efflux pumps and ergosterol biosynthesis genes, and were proposed to function in regulating plasma membrane function (21). Therefore, to gain a more comprehensive understanding of azole resistance and hopefully reveal more potential drug targets, this study focused on the functional characterization of the azole-responsive kinase genes.

We examined a previously reported azole-responsive kinase, STK-17, from N. crassa (16) and found that it could confer azole resistance in N. crassa and Fusarium verticillioides. STK-17 has a positive role in azole resistance through regulating membrane and ergosterol homeostasis and intracellular azole accumulation. Also, the azole resistance mediated by STK-17 is conserved among fungi and, thus, is a potential target for antifungal drug development.

RESULTS

Kinase STK-17 confers azole sensitivity in N. crassa. In our previous study we found that NCU04990, which encodes kinase STK-17, increased its transcript level under ketoconazole (KTC) stress (16). However, the function of STK-17 under azole stress is unknown. To address this question, the stk-17 deletion mutant (stk-17<sup>Δ</sup>) was first phenotypically characterized. As shown in Fig. 1A and B, although deletion of stk-17 in N. crassa resulted in reduced conidial production on slants as previously reported (22), the colony growth of stk-17<sup>Δ</sup> on agar plates was similar (95% ± 5%) to that of wild-type (WT) (Fig. 1B). However, on plates containing 2 μg/mL KTC, the colony size of stk-17<sup>Δ</sup> was only 30% ± 3% of WT after 52-h growth (Fig. 1B), indicating that the stk-17<sup>Δ</sup> strain was hypersensitive to KTC. The stk-17<sup>Δ</sup> strain was also hypersensitive to several other antifungal azoles, including voriconazole (Fig. 1B), fluconazole, and itraconazole (Fig. S1A). KTC was used as the primary azole drug in subsequent experiments with N. crassa.

To examine if the azole hypersensitivity phenotype of this mutant was concentration dependent, the growth of stk-17<sup>Δ</sup> at various concentrations (0 to 2 μg/mL) of KTC was compared with that of WT. As shown in Fig. 1C, stk-17<sup>Δ</sup> was more inhibited than WT at each concentration. The growth of stk-17<sup>Δ</sup> was completely inhibited by 1.5 μg/mL KTC, while complete inhibition of WT required 2 μg/mL KTC. This result was further verified by determination of MIC of KTC, which turned out to be 1.5 μg/mL for stk-17<sup>Δ</sup> and 2.0 μg/mL for WT.
To confirm that the azole hypersensitivity in the mutant was caused by stk-17 deletion, the stk-17MO strain was complemented with its own coding sequence flanked by the native promoter and terminator. The complemented strain (stk-17com) grew normally on slants and produced abundant conidia similar to WT (Fig. 1A) and regained WT sensitivity to azoles (Fig. 1B), confirming the role of STK-17 in conferring basal azole resistance and conidiation.

To determine if the kinase activity of STK-17 was responsible for its function, a sequence expressing a 5x-myc-6x-his-tagged kinase-dead (D183A) version of the kinase (stk-17D183A) under the control of its own promoter and terminator was introduced into the stk-17KO strain, and a control strain expressing a 5x-myc-6x-his-tagged non-mutated kinase (stk-17Tcom) was also generated. The re-introduction of stk-17 in the obtained strains was verified by qRT-PCR (Fig. S2A). And we noticed that its expression is 19-fold higher in the stk-17D183A than that in the WT and stk-17Tcom strain (Fig. S2A). The stk-17Tcom strain displayed KTC susceptibility and conidial production similar to WT and stk-17com, while the stk-17D183A strain was similar to stk-17KO (Fig. 1A and B). Thus, the STK-17 kinase activity is required for basal resistance to azole antifungals, as well as normal conidial development. Surprisingly, the protein of STK-17 could not be detected in above-mentioned strains (Fig. S2B), indicating that STK-17 may be a low abundant protein.

STK-17 is required for basal resistance to ergosterol biosynthesis inhibitors and oxidative stress. In addition to azoles, the stk-17MO strain and the stk-17D183A strain displayed greater sensitivity to terbinafine and amorolfine (Fig. 1B), while the stk-17D183A strain and the stk-17Tcom strain exhibited sensitivity to these two drugs similar to WT. Terbinafine targets squalene epoxidase ERG1, an enzyme upstream of ERG11, while amorolfine targets sterol C8-C7 isomerase and sterol reductase (ERG2 and ERG24, respectively), two enzymes downstream of ERG11 in the ergosterol biosynthetic pathway. Deletion of stk-17 also resulted in hypersensitive to amphotericin B, which directly binds to ergosterol in the membrane (Fig. S1B). Thus, deletion of stk-17 or functional loss of STK-17 kinase activity generally impaired the basal resistance to ergosterol biosynthesis inhibitors with different antifungal targets.

Because sterols are important membrane lipids, we then tested whether STK-17 function was also associated with other membrane lipids by examining the drug sensitivity of the stk-17MO strain to two other inhibitors of membrane lipid biosynthesis, cerulenin and myriocin. These compounds were previously used in N. crassa to inhibit fatty acid and ceramide biosynthesis, respectively (23, 24). Under treatment...
with each antifungal, the growth of both WT and the stk-17KO strain was inhibited by at least 50% compared with control. However, no difference in susceptibility to each of these antifungals was found between the two strains (Fig. S1C).

We also tested the sensitivity of the stk-17KO strain to other stresses, including osmotic stress induced by NaCl, cell wall stress induced by caspofungin, oxidative stress induced by menadione, and cell membrane stress induced by SDS. The stk-17KO strain and the stk-17D183A strain, but not the stk-17com strain and the stk-17Tcom strain, displayed greater sensitivity to menadione (Fig. 1B), indicating a role of STK-17 in the oxidative stress response. However, deletion of stk-17 did not affect the sensitivity to 1 M NaCl, 1 μg/mL caspofungin, or 0.005% SDS (Fig. S1DEF). This suggests that STK-17 is required for basal resistance to ergosterol biosynthesis inhibitors, as well as oxidative stress.

Oxidative stress is not a mediator of increased azole sensitivity in the stk-17KO strain. Deletion of stk-17 increased sensitivity of N. crassa to both azole stress and oxidative stress (Fig. 1B). Because azoles like miconazole exert toxicity by inducing oxidative stress (25, 26), it is possible that the azole hypersensitivity of the stk-17KO strain resulted from the oxidative stress induced by azoles. If that is the case, genes involved in scavenging ROS would be induced by azoles and their deletion mutants would be hypersensitive to azoles. In our previous results, we found that only cat-2, a catalase gene, was dramatically induced by ketoconazole (5, 6). Thus, we examined the sensitivity of cat-2KO to azoles, as well as mutants of other two catalase genes cat-1 and cat-3. However, deletion of cat-1 or cat-3 did not result in hypersensitive to azoles (Fig. 2A). Interestingly, deletion of cat-2 led to resistance to ketoconazole, although this mutant was hypersensitive to H2O2 (Fig. 2B). To further explore the putative relationship between azole hypersensitivity and sensitivity to oxidative stress in the stk-17KO strain, the antioxidant N-acetyl cysteine (NAC) was added to the medium for the azole sensitivity test. At a concentration of 5 mM that did not affect the growth of either WT or the stk-17KO strain, NAC had no effect on azole sensitivity of WT or the stk-17KO strain (Fig. 2C). Above results mean that ketoconazole toxicity may be independent of oxidative stress in N. crassa. And the hypersensitivity to oxidative stress and to azoles in the stk-17KO strain may be independent and caused by different mechanisms.

Genome-wide transcriptional responses to stk-17 deletion. To gain insights into the molecular basis by which STK-17 regulates basal azole resistance, we determined STK-17-dependent gene expression using RNA-sequencing in the presence or absence of azoles. Transcripts of a total of 9,990 genes were detected and 6,713 genes were used for subsequent identification of differentially expressed genes after removing genes with FPKM <5 in all samples.

Genome-wide transcriptional change was induced by deletion of stk-17. In the absence of KTC treatment, expression of 181 genes (139 upregulated and 42 downregulated) were concomitantly affected by the stk-17 deletion compared with WT and the stk-17com strain.
genes were KTC downregulated but less repressed in the GO enrichment analysis (10 genes, Fig. S3F, Fig. 3) and only the term biosynthesis genes was enriched in the KEGG enrichment analysis (two genes, Fig. S3G). The enriched steroid metabolism in response to azole treatment. Thus, the upregulation of these genes in the STK-17 deleted strain by Venn analysis. The expression of 282 KTC-upregulated genes was higher in the STK-17 deletion strain compared with the 776 genes affected by KTC treatment of the wild-type (WT) strain. Interestingly, many of these genes were KTC-responsive in our previous study in N. crassa, in which KTC concentration was higher and the treatment time was longer. Thus, like ADS-4, these genes may also need to be upregulated to higher levels to overcome azole stress. Expression of 343 genes (221 upregulated and 122 downregulated) were only changed under KTC due to STK-17 deletion (Fig. S3E, Data set S1 Sheet 2). Several of them were affected under the non-KTC condition, but the majority of them showed responses to KTC in the STK-17 deletion strain (Data set S1 Sheet 2). Interestingly, many of these genes were KTC-responsive in our previous study in N. crassa, in which KTC concentration was higher and the treatment time was longer. Thus, like ADS-4, these genes may also need to be upregulated to higher levels to overcome azole stress. Only 43 genes showed reduced response to KTC in the STK-17 deletion strain as revealed by Venn analysis (Fig. S3E, Data set S1 Sheet 2), among which 24 genes were KTC-upregulated but less induced in the STK-17 deletion strain by KTC and 19 genes were KTC downregulated but less repressed in the STK-17 deletion strain by KTC. Thus, deletion of STK-17 affected transcriptional responses of certain genes to azole stress.

**STK-17 regulates expression of genes encoding transmembrane proteins.** To further explore the biological processes affected by STK-17 deletion, the 181 genes affected by STK-17 deletion under non-KTC condition and 43 KTC responsive genes affected by STK-17 deletion under KTC condition were subjected to enrichment analysis. Functional categories enrichment analysis of the 181 genes revealed that 54 of them were enriched in the category of transmembrane and transmembrane helix (Fig. S2B, Data set S1 Sheet 1), and most of them (41 out of 54) were upregulated (Fig. 3, Data set S1 Sheet 1), indicating that deletion of STK-17 affected the expression of membrane proteins. Moreover, only three GO terms, integral component of membrane (47 genes), transmembrane transport (7 genes) and transporter activity (4 genes), were enriched with P values < 0.05 (Fig. S3C, Fig. 3). Among these genes, transcription of the genes encoding INSIG domain-containing protein (NCU07869), fatty acid elongase (NCU08976), and neutral ceramidase (NCU04721) were increased 2.4-, 1.7-, and 1.7-fold, respectively (Fig. 3, Data set S1 Sheet 1). The INSIG domain is involved in sterol homeostasis, while fatty acid elongase and neutral ceramidase are required for membrane lipid biosynthesis and metabolism (17, 27, 28). In addition, the majority of the upregulated transmembrane genes, such as NCU07869, NCU08976, NCU04721, NCU08052, NCU05639, and NCU05567, were KTC-responsive and their responses to KTC were enhanced in the STK-17 deletion strain (Fig. 3, Data set S1 Sheet 1), indicating a role of these genes in membrane homeostasis in response to azole treatment. Thus, the upregulation of these genes in the STK-17 deletion strain suggests that normal membrane function may be impaired in the STK-17 deletion strain.

For the 43 genes, only the term “integral component of membrane” was enriched in GO enrichment analysis (10 genes, Fig. S3F, Fig. 3) and only the term “steroid biosynthesis” was enriched in the KEGG enrichment analysis (two genes, Fig. S3G). The enriched steroid biosynthesis genes were erg11 and erg6, whose expression in STK-17 deletion under KTC was only 66% and 64% that of wild-type level, respectively (Data set S1 Sheet 2); therefore, STK-17 may be involved in normal membrane function and responses to azole by ergosterol biosynthesis genes.
FIG 3 STK-17 regulates the expression of membrane genes. Heatmap showing expression of genes in WT, stk-17Δ strain, and stk-17Δcom strain under ketoconazole treated (+K) and non-treated condition. Genes selected were enriched membrane genes whose basal expression or responses to azoles were affected by stk-17 deletion. The average FPKM values of each gene in each sample were used for heatmap construction after log transformation and scaling. The heatmap was constructed, visualized, and edited with TBtools. The thick lines in the middle of the figure indicate the GO terms of each gene.
STK-17 is required for ergosterol biosynthesis genes to azoles. The RNA-seq analysis revealed that deletion of stk-17 affected the responses of erg11 and erg6 to azoles. We checked the expression of other erg genes and found that their responses to KTC were also affected by stk-17 deletion, including erg2 (NCU04156), erg3 (NCU06207, NCU04983), erg24 (NCU08762), and erg25 (NCU06402) (Fig. 4A), although the fold change was <1.5. Thus, STK-17 globally regulates ergosterol biosynthesis in response to azole exposure. To confirm the above results, we measured the expression of erg11 in stk-17KO strain, as well as WT, treated with or without azoles, by qRT-PCR. In WT, the transcript level of erg11...
TABLE 1 Sterol contents in N. crassa WT and stk-17KO strain treated with or without ketoconazole

| Sample          | Normalized relative amount |     |
|-----------------|----------------------------|-----|
|                 | Ergosterol (RT = 7.43 m/z = 379) | Lanosterol (RT = 8.8 m/z = 409) | Eburicol (RT = 9.7 m/z = 423) | 14α-methyl-3,6-diol (RT = 4.88 m/z = 411) | Unknown (RT = 4.135 m/z = 377) |
| First time      |                            |     |     |     |     |
| WT              | 1                          | 1   | 1   | NA  | 1   |
| stk-17KO        | 0.936963293                | 1.4558808 | 0.5395822 | NA  | 0.4183652 |
| WT-K            | 0.399318266                | 5.7413887 | 34.803441 | 1   | 0.5321747 |
| stk-17KO-K      | 0.261997774                | 12.311462 | 131.52387 | 0.99419263 | 8.4854378 |
| Second time     |                            |     |     |     |     |
| WT              | 1                          | 1   | 1   | NA  | 1   |
| stk-17KO        | 0.92234653                 | 4.6967224 | 0.4599823 | NA  | 0.0911043 |
| WT-K            | 0.549896763                | 20.015507 | 17.158954 | 1   | 0.6625718 |
| stk-17KO-K      | 0.406244225                | 22.815779 | 28.971442 | 1.09345252 | 3.426908 |

*WT, wild type; WT-K or stk-17KO, wild type or stk-17KO mutant treated with ketoconazole, respectively; RT, retention time; m/z, mass-to-charge ratio.

increased 9-fold after incubation with 1 μg/mL KTC for 12 h (Fig. 4B). The basal expression of erg11 was not affected by stk-17 deletion, but it increased only 6-fold in response to KTC (Fig. 4B). The response of erg11 to KTC in stk-17KO strain was similar to WT, confirming the requirement of STK-17 in maintaining the proper transcriptional response of erg11 to azoles. The response of erg11 to treatment with different concentrations of KTC or for a longer period of 24 h was consistently affected by stk-17 deletion (Fig. 5A and B). We also measured the ERG11 protein level in WT and the stk-17KO strain using an ERG11-specific antibody. As expected, the ERG11 protein level increased with KTC but was attenuated by stk-17 deletion (Fig. 4C). Thus, STK-17 regulates the response of erg11 to azole at both the transcription and protein level. The expression of other erg genes was also determined by qRT-PCR. In N. crassa, erg2 (NCU04156), erg5 (NCU05278), erg6 (NCU03006), and erg24 (NCU08762) all showed a transcriptional response to azoles and were required for azole resistance (29); therefore, we only tested these genes. Similar to erg11, the responses of erg2, erg6 and erg24 (but not erg5) to azoles were attenuated by stk-17 deletion (Fig. 4B, Fig. 5A), in agreement with the RNA-seq results. Thus, STK-17 is required for the responses of other erg genes to azoles.

Next, we compared the sterol profiles of WT and the stk-17KO strain treated with or without KTC. Under non-KTC conditions, the WT sterol profile and that of the stk-17KO strain were similar. As previously reported for N. crassa (16, 30), KTC treatment led to reduction in ergosterol level and accumulation of sterol intermediates such as lanosterol, eburicol, and a toxic sterol, 14α-methyl-3,6-diol (Table 1), as revealed by two independent analyses. However, deletion of stk-17 altered the sterol profiles under KTC treatment. In WT, KTC treatment reduced ergosterol levels to 55% and 40%, respectively, in two independent analysis, while the decrease was 15% more in the stk-17KO strain than in WT (Table 1). In addition, the concentration of lanosterol and eburicol under KTC treatment in the stk-17KO strain was higher than that in wild type (Table 1). We did not find a higher level of 14α-methyl-3,6-diol in the stk-17KO strain (Table 1), although it is a by-product from lanosterol and eburicol through an alternative biosynthetic pathway, indicating its conversion pathway was affected. Unexpectedly, an unknown sterol with a UV absorbance peak at 280 nm and m/z of 377 and 393 was found to be 4-fold higher in the stk-17KO strain (Table 1 and Fig. S5). Thus, STK-17 is required for sterol homeostasis under azole stress.

Deletion of stk-17 affects intracellular KTC accumulation independent of the azole efflux pump, CDR4. Intracellular accumulation of KTC can be detected along with sterol profile analysis. After a 24-h treatment, the intracellular KTC in the stk-17KO strain was 7-fold higher than that in WT (Fig. 5A and B). Similar results were obtained for KTC accumulation after a 12-h treatment (Fig. 5A and B).

Azole accumulation is largely affected by efflux pump activity (2), and CDR4 is the primary azole efflux pump in N. crassa (30–32). To determine if CDR4-mediated azole efflux affected the accumulation of KTC in the stk-17KO strain, we compared the expression of cdr4 in WT and the stk-17KO strain. Deletion of stk-17 did not affect the basal expression of cdr4 under non-treatment conditions (Fig. 5C). KTC treatment significantly upregulated cdr4 expression 4-fold in WT and similar results were found in the stk-17KO strain (Fig. 5C), indicating that STK-17 do
not regulates CDR4 transcriptionally. We also compared intracellular KTC in a \( \text{cdr4}\text{KO} \) strain with the \( \text{stk-17}\text{KO} \) strain. As reported before (30), after a 24-h KTC treatment, deletion of \( \text{cdr4} \) resulted in 2-fold higher accumulation of intracellular KTC compared to WT. However, \( \text{stk-17} \) deletion led to much higher KTC accumulation than either the WT or the \( \text{cdr4}\text{KO} \) strain (Fig. 5B). Above all, CDR4 is unlikely to contribute to azole accumulation in the \( \text{stk-17}\text{KO} \) strain. We further verified this conclusion by determining the azole sensitivity of the double-deletion strain lacking both \( \text{stk-17} \) and \( \text{cdr4} \). The double mutant was much more sensitive to KTC than either single-deletion mutant (Fig. 5D), proving that CDR4 has little effect on KTC hypersensitivity in the \( \text{stk-17}\text{KO} \) strain.

In fungi, rhodamine 6G (R6G) influx/efflux is used to mimic azole influx and efflux (33). To explore how STK-17 regulates intracellular azole accumulation, we compared R6G influx/efflux in WT with that in the \( \text{stk-17}\text{KO} \) strain. The influx of R6G was detected under glucose-starved condition. After incubating the starved cells with R6G for 1 h, the R6G fluorescence signal was detected in the geminated conidia of each strain (Fig. 5E). The percentage of conidia with high fluorescence intensity was higher in the \( \text{stk-17}\text{KO} \) strain compared to WT and \( \text{stk-17}\text{com} \) strain. The mean fluorescence intensity was 1.8-fold higher in the \( \text{stk-17}\text{KO} \) strain than...
that in WT and the stk-17coom strain (Fig. 5E). Therefore, the stk-17 deletion enhanced drug uptake. To determine R6G efflux, R6G incubated germinated conidia mentioned above were then incubated in PBS with glucose for 40 min. This treatment led to the loss of the high fluorescence intensity peak (Fig. 5E and F), indicating R6G efflux; however, there was a peak shift in the stk-17coom strain relative to WT and the stk-17com strain (Fig. 5F). The mean fluorescence intensity was 3.8-fold higher in the stk-17coom strain (Fig. 5F), indicating that drug efflux may also be mediated by STK-17. In summary, STK-17 may regulate azole accumulation by modulating both azole influx and efflux independent of the major azole efflux pump, CDR4.

**STK-17 has no interaction with known transcription factors that regulate azole stress response.** Because STK-17 affects sterol homeostasis, we examined if it acted through regulation of some known transcription factors. The function of sterol regulatory element binding proteins (SREBPs), the major regulator of ergosterol homeostasis and azole resistance in fungi (30), was first examined. However, deletion of SREBP homolog encoding gene sah-2, as well as the gene encoding rhomboid protease RBD-2 that is required for the activation of SREBP (30) in *N. crassa* did not result in azole hypersensitivity (Fig. S6AB). AttrR is another important transcription factor regulating ergosterol biosynthesis in *Aspergillus fumigatus* (15). Function of this transcription factor was also examined. However, deletion of atrR (NCU01478) in *N. crassa* did not change the sensitivity to ketoconazole (Fig. S6C). These results may indicate that STK-17 regulates ergosterol biosynthesis through some other genes.

Because deletion of ccg-8 in *N. crassa* also resulted in azole accumulation and attenuated responses of erg genes to azoles similar to what was seen with the stk-17coom strain (6, 31), we examined the relationship between STK-17 and the transcription factor CCG-8. However, the double-deletion mutant of stk-17 and ccg-8 was more sensitive than either of the single-deletion mutants (Fig. S6D), indicating a weak connection between STK-17 and CCG-8.

**STK-17 genetically interacts with ERG11 under specific conditions.** Given that we found that STK-17 regulated the positive response of erg11 to azoles, we further examined the relationship between STK-17 and ERG11 by constitutively overexpressing ERG11 in WT and the stk-17coom strain. Western blotting showed that ERG11 was successfully overexpressed in WT and the stk-17coom strain (Fig. 6A). Drug sensitivity test showed that overexpression of erg11 alleviated the azole sensitivity of both WT and the stk-17coom strain, indicating that overexpression of erg11 can contribute to azole sensitivity (Fig. 6B). Overexpression of erg11 also restored the menadione sensitivity of the stk-17coom strain to WT level, but its overexpression in WT had little effect on menadione sensitivity (Fig. 6B). This indicates a genetic interaction between STK-17 and ERG11. However, overexpression of erg11 had little effect on conidiation and sensitivity to amorolfine and terbinafine in both WT and the stk-17coom strain (Fig. 5E and F), meaning that the genetic interaction between STK-17 and ERG11 may be condition-specific.

**STK-17-mediated azole and oxidative stress sensitivity is functionally conserved among fungi.** The role of STK-17 in azole resistance in *N. crassa* is important for broadening our understanding of azole resistance in other fungi, especially pathogenic fungi. Protein sequence analysis and phylogenetic analysis indicate that STK-17 is widely distributed and conserved among fungi (Fig. S7A and B). To determine if STK-17 is functionally conserved in other fungi, we complemented the *N. crassa* stk-17coom strain with the STK-17 homologs, Vhs1p and Sks1p from *Saccharomyces cerevisiae*, Ran1 from *A. fumigatus*, and Ran1 from *F. verticillioides*, three phylogenetically divergent species. All these genes were controlled by the promoter of stk-17. Although *S. cerevisiae* Sks1p and Vhs1p only attenuated the defects in stress sensitivity but not conidiation, *A. fumigatus* Ran1 and *F. verticillioides* Ran1 restored all the phenotypes of the stk-17coom strain in drug sensitivity, oxidative stress, and conidiation (Fig. 7A and B, Fig. 5C and D), indicating a conserved function of STK-17 for stress resistance among fungi, especially filamentous fungi. We then deleted ran1 in *F. verticillioides*, a plant pathogenic fungus. No defects in growth and conidiation were found in the deletion mutants; but, similar to the *N. crassa* stk-17coom strain, deletion of *ran1* in *F. verticillioides* resulted in hypersensitivity to posaconazole, vireoconazole, and itraconazole, as well as to the oxidative stress inducer H$_2$O$_2$ (Fig. 7C). Thus, STK-17 protein is functionally conserved in regulating azole and oxidative stress resistance among fungi.
Azole resistance is a serious problem for clinicians in controlling fungal infections but also in agriculture. Fungi can make adaptive responses to azole stress by inducing expression of genes needed for azole resistance. However, the function of most azole responsive genes is poorly understood. In this study, a previously discovered azole-responsive kinase gene, \textit{stk-17}, was functionally characterized and its deletion resulted in hypersensitive to azoles in \textit{N. crassa} and \textit{F. verticilioides}. Except for signal pathways, this is the first identified kinase that is required for azole resistance. Importantly, this kinase is conserved from yeast to filamentous fungi and has important roles in development and stress responses in \textit{N. crassa} and other fungi (22, 34, 35), suggesting that it

**FIG 6** STK-17 has a genetic interaction with ERG11. (A) Western blotting detection of 5\(_x\)myc-6\(_x\)his tagged ERG11 in \textit{erg11} overexpression strains using an anti-myc antiserum. The tagged ERG11 had a mass of 78 kDa (arrow). (B) Sensitivity test of WT, \textit{stk-17}\(_{\text{KO}}\), and \textit{erg11} overexpression strains to ketoconazole (KTC) and menadione (MEN). Conidia suspensions (2 \(\mu\)L of 2 \(\times\) 10\(^6\)/mL) for each strain were inoculated on Vogel's plates with or without indicated chemicals. The plates were incubated at 28°C and the colonies were documented at the indicated time points.

**FIG 7** STK-17 protein is conserved among fungi. (A) and (B) The drug hypersensitive phenotypes of the \textit{stk-17}\(_{\text{KO}}\) strain can be restored by STK-17 homologs from \textit{Saccharomyces cerevisiae}, \textit{Fusarium verticilioides}, and \textit{Aspergillus fumigatus}. \textit{Fvrant} (\textit{stk-17} homolog of \textit{F. verticilioides}); \textit{ScVHS1}, and \textit{ScSKS1} (\textit{stk-17} homologs of \textit{S. cerevisiae}); \textit{Afrant} (\textit{stk-17} homolog of \textit{A. fumigatus}). (C) Deletion of STK-17 homolog \textit{Ran1} resulted in hypersensitive to azole and oxidative stress. Conidia of WT and \textit{ran1}\(_{\text{KO}}\) strain were serially diluted to 10\(^7\), 10\(^6\), 10\(^5\), and 10\(^4\) conidia/mL. The conidia suspensions (2 \(\mu\)L) were inoculated on plates with or without indicated chemicals. The plates were incubated at 28°C and the colonies were documented after 40 h.
may be a promising drug target. Moreover, deletion of stk-17 had broad effects on azole resistance, including ergosterol biosynthesis, azole uptake, and azole efflux, which are critical aspects of azole resistance, thus deepening our understanding on the regulation of the azole stress response.

STK-17 is an azole responsive kinase in *N. crassa* (6, 29). Moreover, STK-17 homologs in several other fungi have transcriptional responses to azoles and hypoxia (7, 8, 11, 36, 37). Hypoxia can inhibit ergosterol biosynthesis through oxygen-requiring steps (17). So, stk-17 and its homologous genes may respond to perturbations in ergosterol biosynthesis. Our results clearly showed that stk-17 deletion resulted in hypersensitive to azoles, chemicals that inhibit ergosterol biosynthesis in *N. crassa* and *F. verticillioides*. Similarly, chemical genomic analysis of *S. cerevisiae* revealed that mutants of the STK-17 homologs, Vhs1p and Sks1p, are hypersensitive to several azoles (38). Thus, the response of STK-17 to azole stress may contribute to azole resistance, and this could be directly associated with two mechanisms described below.

In several fungal species under azole stress, STK-17 and its homologs have been shown to be co-regulated with ergosterol biosynthesis. In *C. albicans*, Sha3p, a homolog of STK-17, was directly regulated by Upc2p, a major sterol biosynthesis regulator (39). In *N. crassa*, STK-17 was shown to be directly controlled under azole stress by CSP-1, a global transcriptional repressor, while CSP-1 was a negative regulator of ergosterol biosynthesis genes (16). This co-regulation of STK-17 and its homologs with ergosterol biosynthesis genes may imply a functional connection between each other. This hypothesis is further supported by our data showing that STK-17 genetically interacted with ERG11, an important enzyme in the ergosterol biosynthesis pathway. Our results also showed that deletion of stk-17 altered the responses of ergosterol biosynthesis genes to azoles. This would partially explain the involvement of STK-17 in azole resistance. Azole inhibition resulted in ergosterol depletion and the accumulation of sterol intermediates (30). In response to changes in sterol profiles under azole stress, fungi could maintain sterol homeostasis by transcriptionally upregulating ergosterol biosynthesis genes, such as erg2, erg6, erg5, erg24, and erg11. To date, erg11 and erg5 were found to be beneficial to azole resistance (2, 29). Therefore, deletion of stk-17 affected the response of ergosterol biosynthesis genes to azoles, resulting in azole-induced abnormal sterol profiles and hypersensitivity.

STK-17 also negatively regulates azole accumulation. To effectively inhibit ergosterol biosynthesis, azoles need to accumulate in the cell to a certain concentration, which is affected by both azole influx and efflux (2). Higher levels of azole accumulation resulted in azole hypersensitivity while lower accumulations led to resistance (30). Deleting stk-17 affected both azole influx and efflux allowing azoles to accumulate in *N. crassa* and produce hypersensitivity. Azole import is mediated by unknown proteins (2). CDR4 is the major ABC azole transporter in *N. crassa*, but CDR4 is not regulated by STK-17 (32), suggesting the existence of other mechanisms of azole accumulation that are associated with STK-17. As described above, STK-17 coordinately regulates ergosterol biosynthesis and azole accumulation under azole stress.

How STK-17 exerts such a broad effect on the azole resistance is unclear. One explanation is that STK-17 regulates membrane function and homeostasis. A group of membrane genes, including those encoding INSIG domain-containing protein (NCU07869), fatty acid elongase (NCU08976), and neutral ceramidase (NCU04721), were transcriptionally altered by the deletion of stk-17, mostly by upregulation of expression, and some of them could be induced by azoles, which impair cell membranes. Thus, it is possible that stk-17 deletion prevents proper functioning of the cell membrane. In *S. cerevisiae*, the homolog of STK-17, Vhs1p, physically interacts with diacylglycerol and phosphatidylethanolamine (40), suggesting a potential interaction between STK-17 and the cell membrane. Ergosterol homeostasis is an important aspect of membrane homeostasis. Azole accumulation is also affected by the function and integrity of the cell membrane. Membrane lipid raft disruption was previously shown to be associated with intracellular miconazole accumulation (41). Deletion of the ergosterol biosynthesis gene, ERG6, in yeast affects the rate of passive drug diffusion across the membrane,
without affecting Pdr5p-mediated drug export (42). In Candida glabrata, reduced activity of Erg1p, another enzyme in ergosterol biosynthesis, increases fluconazole import (43). In spite of these studies, the effects of stk-17 deletion on cell membrane integrity is largely unknown. It seems to be specifically associated with sterol metabolism, since the deletion only resulted in hypersensitive to ergosterol biosynthesis inhibitors but not to inhibitors of fatty acid and sphingolipid biosynthesis. This may mean that STK-17 functionally interacts with sterol-enriched membrane regions (44), but we did not find any differences between WT and stk-17KO strain by using filipin III to stain membrane sterols (unpublished data). Further investigations are needed.

Deletion of stk-17 had pleiotropic effects, primarily related to azole sensitivity and oxidative stress response. It may be that the hypersensitive to azoles of the stk-17KO strain is a result of oxidative stress because some azoles do induce oxidative stress (25, 26). However, only fungicidal azoles such as miconazole can induce oxidative stress (26), while most other azoles like ketoconazole and fluconazole used are fungistatic (2). In addition, the genes required for coping with oxidative stress are not necessarily required for azole resistance even the one (cat-2) whose expression is induced by ketoconazole. As well as in fission yeast, deletion of sty1, the homologous gene of yeast HOG1, resulted in increased sensitivity to oxidative stress but resistance to azoles (45, 46). Moreover, we found that antioxidant N-Acetylcysteine (NAC) could not make N. crassa resistant to azoles, which is different from the results in Aspergillus fumigatus (47). This would be due to the differences between fungi. But we noticed that NAC addition without pH adjustment resulted in azole resistance (our unpublished data). Further experiments involving comparison between different fungi may give a comprehensive explanation. Above all, STK-17’s regulation of resistance to azoles may be independent of its effects on oxidative stress. Although, the sensitivity of stk-17KO strain to amphotericin B is possibly associated with oxidative stress. Amphotericin B directly extract ergosterol in the membrane and induces iron leakage and oxidative stress subsequently (48). As ergosterol content was not changed in stk-17KO strain under normal condition, its higher sensitivity to amphotericin B may not be ergosterol dependent and more likely to be associated with oxidative stress.

The hypersensitive phenotype of the stk-17KO strain to oxidative stress may be linked to STK-17-regulated membrane function. We speculate that the connection between membrane homeostasis and the response of stk-17KO to oxidative stress may be through erg11 (lanosterol 14α-demethylase) because a deficiency in lanosterol 14α-demethylase in a C. glabrata strain resulted in enhanced oxidative killing (49). A possible explanation for this would be as a result of the ergosterol located the in mitochondrial outer membrane (50). However, we found that overexpression of erg11 in WT had no impact on oxidative stress resistance, indicating that it is specific to stk-17 deletion. Because erg11 is mainly involved in the biosynthesis of ergosterol, a major component of the membrane, it is possible that STK-17-regulated oxidative stress is associated with membrane function.

Unfortunately, proteins directly downstream of kinase STK-17 have not been identified. A limitation for this identification is the low abundance of STK-17 as the protein cannot be detected even in the overexpression strain. Its targets may also be low abundant and not easily detected in large scale identification through proteomics. We examined the relationship between STK-17 and some transcription factors as an alternative. However, no candidate was obtained. It’s interesting that SREBP and AtrR, two regulators for ergosterol biosynthesis in A. fumigatus (15, 17), were not responsible forazole resistance in N. crassa. It’s also reported in F. graminearum that deletion of SREBP did not lead toazole hypersensitivity (51). So, this may be another example for the regulatory evolution of ergosterol biosynthesis as that found in Saccharomycotina (18). Further investigation may show us some interesting observations.

STK-17-mediated stress resistance is functionally conserved among fungi because homologs of STK-17 in different fungi from yeast to filamentous fungi can restore drug sensitive phenotypes of stk-17KO strain. However, our results suggest the possibility of functional divergence on the part of STK-17 as well. STK-17 homologs in yeast cannot restore the conidiation phenotype to the stk-17KO strain. In addition, deletion of genes...
encoding the STK-17 homologs in *F. verticillioides* showed no defects in conidiation. In *A. nidulans*, no defects were found in STK-17 mutants, including conidiation (52). Moreover, several phenotypes of yeast Vhs1p, such as sensitive to hyperosmotic stress, were not evident in *N. crassa* (53). Thus, the functions of STK-17 in fungi seem to be divergent throughout evolution. Supported by our results of heterologous complementation experiment, this divergence could be a consequence of changes in kinase substrate specificity and alteration of phosphorylation events in fungi (54). Further investigation should provide more information on the modification of regulatory networks in fungi. Still, it is clear that STK-17 and its homologs play important roles in fungal physiology, such as pseudo hyphal growth in *C. albicans* and yeast (34), glucose starvation in yeast (34), meiosis in fission yeast (35), and conidiation and stress response in *N. crassa*, thus making it a promising antifungal target.

**MATERIALS AND METHODS**

**Strains and culture conditions.** *N. crassa* strains used in this study are listed in Table S1A. The WT strain FGSC#4200 and the stk-17 deletion mutant FGSC#18049 (stk-17<sup>ΔΔ</sup>) were obtained from the Fungal Genetics Stock Center (www.fgsc.net; University of Kansas Medical Center). The double deletion mutants were created by crossing the corresponding single deletion mutants. All the strains were cultured on Vogel’s medium (Vogel’s minimum medium, supplemented with 2% sucrose for slants or glucose for plates, and 1.5% agar) (55). Regeneration medium was used for protoplast transformation and consisted of Vogel’s minimum medium plus 1 M sorbitol, 0.5 g/L fructose, 0.5 g/L glucose, 20 g/L sorbose, and 1.5% agar. Sorbose medium was used for electroporation and consisted of Vogel’s minimum medium with 20 g/L sorbose, 0.5 g/L fructose, 0.5 g/L glucose, and for solid medium, 1.5% agar (56). All cultures were grown at 28°C. Hygromycin B (150 mg/L; Amresco) and chlorimuron ethyl (15 mg/L; Sigma) were added as needed.

YPG liquid medium (0.3% yeast extract, 1% peptone, and 2% glucose) and PDA medium (20% potato, 2% glucose, and 1.5% agar) were used for culturing the *F. verticillioides* strains listed in Table S1A. Protoplasts of *F. verticillioides* were recovered on regeneration medium (yeast extract 0.1%, casein hydrolysate 0.1%, sucrose 0.8%, and 1.5% agar) after transformation. All *F. verticillioides* cultures were grown at 28°C. Hygromycin B at a final concentration of 150 mg/L was added as needed.

**Drug susceptibility test.** *N. crassa* strains were cultured for 5 to 7 days and conidia were then washed with sterile water to generate conidial suspension (2 × 10<sup>5</sup> per mL). Two microliter of conidial suspension were inoculated onto the center of Vogel’s plates (diameter Φ = 9 cm) with or without antifungal drugs. All the plates were then incubated at 28°C. Antifungal azoles were dissolved in dimethyl sulfoxide (DMSO) and added to the Vogel’s medium before plating. The antifungals were tested at final concentrations of 2.0 μg/mL for ketoconazole (KTC, Sigma), 0.5 μg/mL for voriconazole (VOR, Sigma), 25 μg/mL for fluconazole (Sigma), and 10 μg/mL for itraconazole (Sigma). For other drugs or stresses, the final concentrations were 5 μg/mL for menadione (Sigma), 0.25 μg/mL for amorolfine (J&K), 2 μg/mL for terbinafine (J&K), 0.025 μg/mL for amphotericin B (Sigma), 0 μg/mL for cerulenin (Enzo Life Sciences), 20 ng/mL for myriocin (Enzo Life Sciences), 8% for NaCl, 1 μg/mL for caspofungin (Apexbio), and 0.005% for SDS (Sangon Biotech). Colonies of the strains were sampled and documented at various time points during growth.

*F. verticillioides* conidia from 7-day-old cultures were suspended in sterile water and the concentration was adjusted to 10<sup>3</sup> per mL, and diluted to 10<sup>4</sup>, 10<sup>5</sup>, and 10<sup>6</sup> conidia per mL. Two microliter of each conidial suspension were inoculated onto PDA plates (diameter Φ = 15 cm) with or without the antifungal drugs, posaconazole (Apexbio), VOR, and itraconazole, all at a concentration of 1 μg/mL. The plates were then incubated at 28°C for 72 h and photographed.

**Plasmid construction and transformation.** Plasmid pCB1532 containing the chlorimuron ethyl resistance gene was used to construct the plasmid pCOMstk-17 for stk-17<sup>ΔΔ</sup> strain complementation. Briefly, a 4,608-bp fragment containing the stk-17 coding sequence (1,616 bp) flanked by a 1,430-bp upstream regulatory region and a 1,561-bp downstream region was amplified using primers Pstk-17F and Tstk-17R (Table S1B) and inserted into pCB1532 digested with *Xba*I and *Hind*III.

The plasmid expressing 5×myc-6-his-tagged STK-17 was also constructed to complement the stalk-17<sup>ΔΔ</sup> strain. The upstream and downstream regions of stk-17<sup>ΔΔ</sup> were first amplified using primer pairs, Pstk-17F/Pstk-17R and Tstk-17F/Tstk-17R, respectively (Table S1B) and inserted into pCB1532 digested with *Xba*I and *Hind*III, while the upstream and downstream sequences were double digested with enzyme pairs *Xba*I/EcoRI and *Eco*RI/*Hind*III, respectively. This resulted in the plasmid pCB1532-up-down. The 5×myc-6-his was tagged to the N-terminus of STK-17 by simply inserting the coding sequence of stk-17 into the Qa plasmid (57) using primers, stk-17F and stk-17R (Table S1B). The tagged sequence was then amplified using primers, Tagstk-17F and Tagstk-17R (Table S1B), digested with EcoRI and inserted into EcoRI digested pCB1532-up-down, resulting in the complementary plasmid pTCOMstk-17.

The kinase-dead (KD) plasmid was constructed from pTCOMstk-17. The primers stk-17KD-F and stk-17KD-R (Table S1B) containing the KD point mutation (D183A) were designed, and the first half and second half of 5×myc-6-his tagged STK-17 flanked with the native promoter and terminator of stk-17 were respectively amplified with the primer pairs, Pstk-17F/stk-17KD-R and stk-17KD-F/Tstk-17R (Table S1B) from pTCOMstk-17. The two sequences were then fused by overlap-extension PCR because primers stk-17KD-F and stk-17KD-R were complementary. The final plasmid pKDstk-17 was constructed by inserting an *Xba*I- and *Hind*III-digested fusion fragment into pCB1532.

An stk-17 overexpression plasmid was constructed by a method similar to what had been reported previously using the cfp promoter (5, 16, 58). The expression of *cfp* gene is not changed by azoles and oxidative...
stress (6, 59), making it a promoter suitable for gene overexpression in our study. Briefly, the minimal cdp promoter (58) and trpc terminator were amplified using primer pairs PcfpF/PcfpR and TrpcCF/TrpcCR, respectively (Table S1B), and inserted into Xbal/HindIII-digested pCB1532, while the upstream and downstream fragments were digested by Xbal/EcoRI and EcoRI/HindIII, respectively, resulting in the plasmid, pOE-UD. The 5'-myc-6×his tagged stk-17 was then amplified using primers Tagstk-17F and Tagstk-17R (Table S1B), and digested by EcoRI and inserted into the EcoRI-digested pOE-UD, resulting in the plasmid pOEstk-17.

The erg11 overexpression plasmid was generated from pOEstk-17 by replacing stk-17 with erg11. Briefly, the coding sequence of erg11 and a new trpc terminator were first amplified with primer pairs erg11F/erg11R and TrpcCF/TrpcCR, respectively, and then inserted into Ascl/HindIII-digested pOEstk-17 after digesting with Ascl/Xmal and Xmal/HindIII, respectively, resulting in plasmid pOEerg11.

The pCOMstk-17, pTCOMstk-17, pOEstk-17, and pOEerg11 were then transformed into the stk-17Δ strain and/or the WT strain using protoplast transformation or electroporation transformation as previously described (6). The chlorimuron ethyl-resistance gene was used as the selective marker. The transformants were verified by PCR and the primers are listed in Table S1B.

**RNA extraction and gene expression analysis using qRT-PCR.** Samples for RNA extraction were prepared as previously described (32). Briefly, 15 small pieces of mycelial mats (1 to 2 mm²) were grown in liquid Vogel’s medium for 13 h and then treated with or without ketoconazole for 12 h or 24 h. Harvested samples were immediately frozen, and then ground into fine powder in liquid nitrogen. Total RNA extraction was performed according to the standard TRIzol protocol (Invitrogen, Carlsbad, CA, USA). The cDNAs were then synthesized from 2 to 3 μg total RNA using the FastQuant RT kit with qDNase (Tiangen, China) according to the manufacturer’s protocol, and diluted to a final volume of 50 μL. Gene-specific primers for qRT-PCR analysis were designed using online tools PrimerQuest or Primer 6 and listed in Table S1C. The qRT-PCR was performed on a Bio-Rad, Hercules, CA (Bio-Rad) machine (ProteinSimple, USA), according to the manufacturer’s instructions. At least three independent experiments were carried out and each cDNA sample was analyzed in duplicate. The average threshold cycle (CT) values were used to calculate relative expression levels normalized to expression of β-tubulin using the 2^(-DDct) method (60).

**RNA-seq and data analysis.** The transcriptomic profiles of the WT strain, the stk-17Δ strain, and the stk-17Δmog strain were analyzed by RNA-sequencing (RNA-seq) and compared. The mycelia of those strains treated with or without a sub-inhibitory dosage (1 μg/mL) of KTC for 12 h were harvested and RNA was extracted as described above. The total RNA samples were then sent to the Beijing Genomics Institute (Wuhan, China) for RNA quality control, sequencing library construction, single-end RNA-sequencing (BGseq-500S platform), and data processing as described before (61). Three biological replicates for each sample were analyzed. The transcript level of a gene was determined by calculating the fragments per kilobase of transcript per million mapped reads (FPKM) and represented as average FPKM from three biological replicates for each sample. Genes with FPKM value <5 in all samples were considered as low-abundance genes and excluded from subsequent analysis. Genes with log2(fold change) levels ≥ 0.67 and FDR (false discovery rate) values <0.001 in comparisons between two samples were considered to be differentially expressed. Because stk-17Δmog was supposed to be identical to WT, the genes concomitantly affected in WT and stk-17Δmog versus stk-17Δ were included in the total of STK-17 dependent expression. Venn diagramming and heatmap construction were performed with TBtools (62). GO, KEGG, and functional categories enrichment analysis were performed on DAVID (63).

**HPLC-MS analysis of sterol contents and intercellular ketoconazole.** The samples for sterol extraction were prepared by the same method as for RNA extraction. In order to obtain more stable results of sterol profiles, samples with 24-h KTC treatment were used for the subsequent analysis. The collected mycelia of different strains were heat dried at 80°C and then ground into fine powder for sterol extraction and HPLC-MS analysis as reported previously (29). Ergosterol (J&K Scientific Ltd., China) and KTC standards were run for comparison.

**Detection of ERG11 expression by Western blotting.** The samples for protein extraction were prepared using the same method as for RNA extraction. The collected mycelia were immediately frozen and ground into powder for protein extraction. Proteins were extracted as previously reported (57) and quantified with the 2-D Quant Kit (GE, USA). Western blotting was performed with Wes, a simple western machine (ProteinSimple, USA), according to the manufacturer’s instructions. ERG11 protein was detected using an anti-ERG11 antibody generated by our laboratory. This antibody was tested for specificity and sensitivity and proven before use. β-tubulin antibody was used as a loading control. Standard Western blotting was also used to detect 5′-myc-6×his tagged ERG11 with an anti-myc antibody (Abbkine, USA).

**Complementation of N. crassa stk-17Δ strain with STK-17 homologs from S. cerevisiae, A. fumigatus, and F. verticillioides.** In order to complement the N. crassa stk-17Δ strain, the STK-17 coding sequence in the plasmid pTCOMstk-17 was replaced with coding sequences of A. fumigatus Ran1 (Afu3g10530), F. verticillioides Ran1 (FVEG10227), S. cerevisiae VHS1, and S. cerevisiae SKS1 to generate the complement plasmids. For A. fumigatus ran1, the coding region of ran1 and terminator of stk-17 were amplified with Afran1comF/R and Tstk-17F/R, respectively (Table S1B). The amplicons were digested with Ascl/KpnI and KpnI/EcoRI, respectively, and inserted into Ascl/EcoRI-digested pTCOMstk-17, resulting in pTCOMAfra1. For F. verticillioides ran1, the coding region of ran1 and the terminator of stk-17 were first amplified by PCR using primer pairs Fvrn1comF/R and Tstk-17F/R, respectively (Table S1B). Then the obtained fragments were inserted into Ascl/HindIII-digested pTCOMstk-17 by homologous recombination using the ClonExpress Multis one-step cloning kit (Vazyme, Nanjing, China), resulting in pTCOMFvra1. For S. cerevisiae VHS1, the coding region of VHS1 and the terminator of stk-17 were first amplified by PCR using primer pairs SVHS1comF/R and Tstk-17F/R, respectively (Table S1B). Then the amplicons were inserted into Ascl/HindIII-digested pTCOMstk-17 by homologous recombination, resulting in pTCOMSVHS1. For S. cerevisiae SKS1, the coding region of SKS1 and the terminator of stk-17 were first amplified by PCR using primer pairs ScSKS1comF/R and Tstk-17F/R, respectively.
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SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.7 MB.

SUPPLEMENTAL FILE 3, PDF file, 3.1 MB.

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C.H., S.L., and X.S. designed the study and wrote the manuscript. C.H., M.Z., X.C., W.X., and Z.Z. performed the experiments. C.H., X.S., and S.L. contributed to the data analysis and the data interpretation.

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