INTRODUCTION

To date, modern triazole antifungals, such as voriconazole (VRC), itraconazole (ITC) and posaconazole (POC), are still the most commonly used drugs for A. fumigatus infections (Fisher et al., 2018; Groll et al., 2017; Traummueller et al., 2011). However, the emergence of azole-resistant isolates resulting from continued azole stress or natural mutation leads to therapeutic failures and an increase in mortality (Chowdhary et al., 2017; Denning & Bromley, 2015; Snelders et al., 2008). Thus, understanding potential azole resistance mechanisms is key for developing new drugs and managing aspergillosis.

Previous studies have provided convincing evidence that azoles work as antifungals by targeting the key ergosterol biosynthesis enzymes lanosterol 14α-demethylase Erg11 (also known as Cyp51) encoded by two homologous genes, erg11A (cyp51A) and erg11B (cyp51B), resulting in an accumulation of 14α-methyl sterols (Becher & Wirsel, 2012; Georgopapadakou & Walsh, 1996; Joseph-Horne et al., 1996). Excess 14α-methyl sterols impair fungal growth by altering cell membrane permeability and stability (Cowen & Steinbach, 2012).
Recent studies have proposed that azole antifungals also exert their fungicidal activity by triggering the synthesis of cell wall carbohydrate patches that penetrate the plasma membrane, thereby killing the fungus (Geissel et al., 2018). Investigation of azole-resistant A. fumigatus suggests that the primary genetic alteration responsible for azole resistance is found within the erg11A locus (Burks et al., 2021). Among these azole-resistant isolates, the substitution of leucine 98 for histidine (L98H) in the erg11A gene along with two copies of a specific 34-bp tandem repeat (TR34) in the erg11A promoter (TR34/L98H) resulted in the overexpression of erg11A, which was found to be the predominant resistance mechanism (Chowdhary et al., 2017; Mellado et al., 2007; Snelders et al., 2008; Zhang et al., 2019). Understanding the regulatory mechanisms of erg11A expression can provide important insight into azole resistance mechanisms in fungal pathogens.

The expression of erg11A has been reported to be regulated by several transcription factors, including SrbA, AtrR, CBC (CCAAT-binding complex), and NCT complex (negative cofactor two A and B), by directly binding to the promoter region of erg11A and consequently regulating azole susceptibility (Furukawa, Scheven, et al., 2020; Zhang et al., 2019). SrbA, a transcriptional regulator that belongs to the sterol regulatory element-binding protein (SREBP) family, directly binds to the 34-mer of erg11A promoter and positively regulate erg11A expression to resist azoles (Bat-Ochir et al., 2016; Blosser & Cramer, 2012; Willger et al., 2008, 2012). SrbA also binds to its own promoter to autoregulate its expression, as well as the promoters of sterol biosynthesis-related genes in response to hypoxia (Blatzer et al., 2011; Chung et al., 2014). During hypoxia, endoplasmic reticulum (ER)-associated full-length SrbA undergoes protein cleavage involving the rhomboid protease RdbB, Dsc ubiquitin E3 ligase complex (Dsc-A-E), and signal peptide peptidase SppA, resulting in the release of the N-terminal helix-loop-helix (HLH) transcription factor domain into the nucleus to function as a transcription factor (Bat-Ochir et al., 2016; Dhingra et al., 2016; Vaknin et al., 2016; Willger et al., 2012). The nuclear translocation of the N-terminal SrbA also occurs upon azole stress in A. fumigatus (Song et al., 2017). Consistent with the defective phenotypes observed in the srbA deletion mutant under hypoxia and azole conditions, dscA-E null mutants show increased susceptibility to hypoxia and azole drugs (Dhingra et al., 2016; Willger et al., 2012). The Zn₁-Cys₂ transcription factor AtrR has also been shown to be a positive regulator of erg11A in A. fumigatus. Similar to SrbA, the binding site of AtrR also falls in the 34-mer region of the erg11A promoter (Hagiwara et al., 2017; Paul et al., 2019). CBC, a heterotrimer composed of HapB, HapC, and HapE, is a negative regulatory complex of erg11A in A. fumigatus. CBC directly binds to the CCAAT motif within the 34-mer of the erg11A promoter, and CBC dysfunction increases A. fumigatus resistance to azoles (Gsaller et al., 2016). Notably, a clinically relevant HapE Prm mutation in A. fumigatus is reported to significantly perturb the binding affinity of CBC to the erg11A promoter, resulting in an azole-resistant phenotype (Camps et al., 2012; Hortschansky et al., 2017). Recently, the subunits of A. fumigatus NCT complex (negative cofactor two A and B), NctA and NctB, have been identified as a key regulator of azole resistance by directly binding to the TATA box-like AT-rich motifs within promoter regions of erg11A, hapC, srbA and atrR and regulating their expressions (Furukawa, van Rhijn, et al., 2020). Several studies have implicated that the molecular mechanism of azole resistance in A. fumigatus is highly complex and tightly regulated by a network of transcriptional activators and repressors. In this study, we analyzed an isolate 415-2 selected from our previously reported azole-resistant A. fumigatus library (Wei et al., 2017). Through next-generation sequencing (NGS), we successfully identified a nonsense mutation in hapB conferring 415-2 azole resistance. Based on a previous study showing that azole tolerance is governed by the opposing actions of SrbA and CBC on erg11A expression, we found that the increased expression of Erg11A and azole resistance induced by loss of HapB is dependent on SrbA. Additionally, we found that the lack of HapB not only increases SrbA expression but also promotes SrbA cleavage and nuclear translocation, which is probably due to the increased expression of RdbB, the Dsc complex, and SppA. These findings broaden our understanding of how SrbA and CBC coordinately regulate Erg11A expression and azole resistance and may provide a potential avenue for overcoming the resistance to azole drugs.

2 MATERIALS AND METHODS

2.1 Strains, media, and culture conditions

All A. fumigatus strains used in this study are listed in Table A1. In general, these strains were grown on solid minimal media (MM), which contained 0.02 g/ml agar, 0.01 g/ml glucose, 1 ml/L trace elements, and 50 ml/L 20x salt solution (Zhang & Lu, 2017). The liquid MM recipe does not contain agar. Uridine (5 mM) and uracil (10 mM) are required for uracil and uridine auxotrophic strains. To test the sensitivity of A. fumigatus to azole drugs, ITC and VRC were supplemented in MM or MM plus uridine and uracil (MMU). For the plate assay, a 2 µl slurry containing 2 x 10⁸ spores was spotted onto solid MM at 37°C for 2 or 2.5 days. Longer culture time (4 days) was required for the observation of the growth phenotypes on MM with ITC or VRC.

2.2 Next-generation sequencing analysis sequencing and single-nucleotide polymorphism analysis

The fresh conidial spores of isolate 415-2 were inoculated into liquid MM and shaken for 24 h at 37°C at 200 rpm, and the resulting mycelial pellets were dried and extracted to obtain genome DNA (gDNA). The next-generation sequencing (NGS) experiment was performed at Shanghai OE Biotechnology Co., Ltd., as a commercial service. gDNA of 415-2 was sequenced by using the Illumina HiSeq 2000 platform with 100-bp paired-end reads in a high-output mode. An average depth of each nucleotide was gained.
Sequence assembly and mapping were referred to the A. fumigatus A1163 genome (http://www.ncbi.nlm.nih.gov/assembly/GCA_000150145.1). Analysis of mapping quality and SNPs was performed by using a next-generation sequencing data analysis suite, SHORE software.

2.3 Constructs for deletion, truncation, and GFP labeling strains

Strains (Δerg11A, hapB165, Erg11A-GFP, SppA-GFP, RbdB-GFP, and DscA/B/C/D/E-GFP) were constructed at their native locus by our MMEJ-CRISPR system as described previously (Zhang & Lu, 2017; Zhang et al., 2016). The sgRNA targeting the related gene was synthesized using the MEGAscript T7 Kit (Invitrogen). The corresponding repair templates with microhomology arms were obtained by PCR. Then, the repair template and sgRNA were transformed into a Cas9-expressing A. fumigatus strain (ZC03/WT). WtGFP-srbA, GFP-SrbAΔ, SrbAΔ, and SrbAΔ strains were constructed by transformation with plasmid prg3-AMAI-NotI (Aleksenko & Clutterbuck, 1997; Aleksenko et al., 1996). For the recycling usage of the selectable marker pyr4, 1 mg/ml 5-FOA was used to screen recipient strains. For deleting srbA, the traditional homologous recombination strategy was employed. All primers are listed in Table A2. A. fumigatus transformation was carried out as previously described (Zhang & Lu, 2017; Zhang et al., 2016).

2.4 Molecular cloning

The plasmid p-Ama1-PsrbA-srbA for labeling SrbA with GFP was constructed as follows: using primers Ama1-srbA-F and Ama1-srbA-R, the PsrbA-gfp-srbA fragment containing the srbA promoter, GFP and srbA ORF was amplified from the gDNA of an N-tagged GFP-SrbA strain and then subcloned into the BamHI site of the plasmid prg3-AMAI-NotI, yielding the plasmid p-Ama1-PsrbA-gfp-srbA.

For colocalization analysis of GFP-SrbA with the nucleus, the plasmid p-Ama1-PsrbA-gfp-srbA-PgpdA-rfp-H2A was generated as follows: the fragment Ppord-gfp-srbA was amplified from p-Ama1-PsrbA-gfp-srbA with primers Ama1-srbA-F and gpd-srbA-R. The fragment PgpdA-rfp-H2A was amplified from pBARGPE-PgpdA-RFP-H2A using primers gpd-F and Ama1-trpC-R. Then, the two fragments were cloned into the BamHI site of the plasmid prg3-AMAI-NotI, yielding p-Ama1-PsrbA-gfp-srbA-PgpdA-rfp-H2A.

To analyze the localization of truncated SrbA (SrbAΔ), it contains residues 1 to 380 of the N-terminus of SrbA), the plasmid p-Ama1-PsrbA-gfp-srbA-PgpdA-rfp-H2A was generated as follows: The fragment PsrbA-gfp-srbA was amplified from p-Ama1-PsrbA-gfp-srbA with primers Ama1-srbA-F and Ama1-srbA-R. The fragment PgpdA-rfp-H2A was amplified from pBARGPE-PgpdA-RFP-H2A using primers gpd-srbA-R and Ama1-trpC-R. Then, the two fragments were cloned into the BamHI site of the plasmid prg3-AMAI-NotI, yielding p-Ama1-PsrbA-gfp-srbA-PgpdA-rfp-H2A.

The plasmid p-Ama1-PsrbA-srbAΔ/srbAΔ was generated as follows: the fragment Ppord-srbAΔ/Ppord-srbAΔ was amplified from the gDNA of A. fumigatus with primers Ama1-srbA-F and Ama1-srbA-R/Ama1-srbA-R. Then, the fragment was subcloned into the BamHI site of the plasmid prg3-AMAI-NotI, yielding p-Ama1-PsrbA-srbAΔ/srbAΔ.

The above plasmids were transformed into different background strains, which are listed in data Table A1.

2.5 Fluorescence microscopy

Fresh spores in 0.5 ml of liquid MM were grown under different treatment conditions (see legends) on sterile glass at 37°C after a set time. The coverslips with hyphae were gently washed with PBS buffer three times and then fixed with 4% paraformaldehyde. Then, hyphae were washed again with PBS. To detect nuclei, the hyphae were stained with Hoechst 33528 at a final concentration of 0.1 mg/ml for 30 min. The fluorescent images of the cells were directly captured with a Zeiss Axio Imager A1 microscope (Zeiss).

2.6 Quantitative real-time PCR analysis

Fresh A. fumigatus conidia were grown in MM in a rotary shaker at 220 rpm for 37°C for 48 h. For measuring the relative mRNA expression levels of target genes, total RNA of related strains was extracted using the UNIQ-10 Column TRizol Total RNA Isolation Kit (Sangon Biotech, B511361-0020), following the manufacturer’s introduction. Then, cDNA synthesis was performed with the HiScript II Q RT SuperMix for qPCR Kit (Vazyme, R223-01). For detecting the relative srbA gene copy number, the gDNA of A. fumigatus was extracted using Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, B518259-0050). At least three biological replicates had been performed for each independent assay. The relative transcript levels of target genes and srbA gene copy number were calculated by the comparative threshold cycle (ΔΔCT) and normalized against the expression of tubA mRNA level and tubA gene copy number, respectively. The difference of the relative mRNA expression and srbA gene copy number was determined as 2^{-ΔΔCT}. All the RT-qPCR or qPCR primers and annotations are listed in data Table A2.

2.7 Western blotting

To extract GFP fusion proteins from A. fumigatus mycelia, 10^8 conidia were inoculated into 100 ml of liquid MM under different treatment conditions (see legends) for a set time. Mycelia were collected, frozen in liquid nitrogen, and ground with a mortar and pestle. In general, protein extraction was performed using a previously described alkaline lysis strategy (Nandakumar et al., 2003). For extracting the nucleoprotein, the commercial nucleoprotein extraction kit (Beyotime, P0027) was used according to the manufacturer’s instructions. The GFP fusion protein was detected by using...
4 of 21

ZHANG et al.

4 of 21

ZHANG et al.

an anti-GFP mouse monoclonal antibody (Roche) at a 1:3000 dilution. Actin mouse monoclonal antibody (Proteintech, 66009-1) at 1:5000 dilution against actin was used as an internal loading control. Detailed procedures of protein extraction and western blotting were described previously (Nandakumar et al., 2003; Zhang et al., 2018; Zhang et al., 2016).

2.8 | Recombinant CBC protein purification and electrophoretic mobility shift assay

To express His-labeled CBC subunits in vitro, the exons of hapB, hapC, and hapE were amplified with three pairs of primers EmsA-hapB-F/EmsA-hapB-R, EmsA-hapC-F/EmsA-hapC-R, and EmsA-hapE-F/EmsA-hapE-R, respectively, and then ligated into the pET30a vector, subsequently transformed into BL21(DE3) Competent Cells were grown in LB medium at 37°C to an OD600 between 0.6 and 0.8, followed by addition of 0.1 mM isopropyl β-D-thiogalactoside. Protein purification was performed as previously described using a rapid Ni-nitrilotriacetic acid (NTA) agarose minicolumn (Huang et al., 2015). EMSA was carried out according to previously described with minor modifications (Huang et al., 2015; Long et al., 2018). In briefly, each reaction contains consisted of 6 µl of 5x EMSA binding buffer, 1.5 µl of 1 mg/ml salmon sperm DNA (nonspecific competitor), 60 ng Cy5-labeled probe (double-stranded DNA), 0.5 µg HapB, 0.8 µg HapC, and 0.6 µg HapE. For competitive testing, a 30-fold nonlabeled DNA probe (1.8 µg) as a competitive cold probe was added to the reaction. To confirm the specific binding of CBC to the binding motif CCAAT, the CCAAT motif within the promoter of the target gene was randomly mutated into a non-CCAAT sequence. The reaction mixtures were incubated at 37°C for 0.5 h and then separated on a 5% polyacrylamide gel in 0.5× Tris-borate EDTA buffer. Subsequently, the Cy5-labeled probes were detected with an Odyssey machine (LI-COR).

3 | RESULTS

3.1 | The azole resistance of isolate 415-2 is due to the hapB mutation

Our previous study has obtained a library of azole-resistant strains through a long-term induction of azole treatment, however, the resistance mechanisms for a majority of these isolates have not been experimentally investigated (Wei et al., 2017). From this library, we found isolate 415-2 without the mutations in erg11A showed high resistance to azoles. In comparison to the wild-type strain, isolate 415-2 displayed partial growth defects and high resistance to ITC and VRC (Figure1-a). To identify the potential mutated genes leading to azole resistance, next-generation sequencing (NGS) was implemented on isolate 415-2. After BLAST

![Image](image-url)
analysis of the whole genomic sequence assembly based on the 
A. fumigatus A1163 genome, hundreds of single nucleotide polymorphisms (SNPs) were detected in the 415-2 genome (Figure 1-b). Most of these SNPs were located in untranslated regions (UTRs), and 38 SNPs existed within the open reading frame (ORF), which contained 23 missense mutations, 2 nonsense mutations, 11 silent mutations, and 2 mutations in splice regions. Notably, one of the two nonsense mutations created a premature stop codon with the hapB gene at amino acid position 165 that led to the premature transcription termination of hapB. HapB is a subunit of CBC, which is a multimeric transcription factor complex comprising three subunits (HapB/HapC/HapE) and can bind to CC(G)AAT motif (Gsaller et al., 2016). Inactivation of the CBC by the absence of any of its subunits increases tolerance to different classes of drugs targeting ergosterol biosynthesis, including azoles, allylamines (terbinafine), and statins (simvastatin) (Gsaller et al., 2016). To test whether the azole resistance of isolate 415-2 results from dysfunction of HapB/CBC, we transformed a wild-type hapB gene into isolate 415-2, generating 415-2hapB strain. As shown in Figures 1-a, 415-2hapB exhibited similar colony growth and azole susceptibility to the wild-type strain, demonstrating that the loss of HapB contributes to the azole resistance of 415-2 isolate. Moreover, hapB truncation mutant that mimics the mutation in 415-2 isolate (hapBΔ165) and the CBC deletion mutant (ΔhapB, ΔhapC, and ΔhapE) phenocopied the 415-2 mutant with or without treatment of azole (Figure 1-a), further demonstrating that the azole resistance of isolate 415-2 is due to dysfunction of the HapB/CBC complex.

3.2 | Increased expression of Erg11A and azole resistance induced by loss of HapB is dependent on SrbA

A previous study showed that CBC represses the mRNA expression of erg11A by binding the azole resistance-associated 34 mer in the erg11A promoter (Gsaller et al., 2016). To further explore whether CBC and SrbA coordinately control Erg11A expression at the protein level, we labeled GFP at the C-terminus of Erg11A in the wild-type, ΔhapB, ΔsrbA, and ΔhapBΔsrbA background strains. Immunoblotting analysis and fluorescence microscopic observation were performed to determine Erg11A-GFP fusion protein expression in related strains in the presence or absence of ITC. Consistent with the increase in erg11A mRNA expression after treatment withazole drugs ITC and VRC (Blosser & Cramer, 2012; Du et al., 2021), the protein expression of Erg11A-GFP also displayed a significant elevation after exposure to ITC for 2h (Figure 2-a). Especially, enhanced Erg11A-GFP protein expression was much greater in ΔhapB than that in wild-type under the ITC treatment condition. Interestingly, the protein expression of Erg11A-GFP was almost completely suppressed in ΔsrbA and ΔhapBΔsrbA compared to that of wild-type strain irrespective of ITC treatment, confirming that SrbA is required for Erg11A protein expression. In line with immunoblotting results, fluorescence observation also showed that Erg11A-GFP exhibited stronger GFP signals with the endoplasmic reticulum (ER)-localized pattern in the ΔhapB strain than that in the wild-type strain, and the GFP signals were barely observed in the ΔsrbA and ΔhapBΔsrbA strains (Figure 2-b). Moreover, the phenotypic analysis showed that ΔhapBΔsrbA presented a sensitive phenotype on ITC/ VRC-amended medium, which is similar to the ΔsrbA strain (Figure 2-c), suggesting that the increased expression of Erg11A and azole resistance induced by loss of HapB is dependent on SrbA.

3.3 | The protein expression and localization of SrbA and HapB under azole treatment conditions

Previous studies indicated that ER membrane-bound SrbA can be cleaved at its C-terminus and delivered into the nucleus to function as a transcription factor after hypoxia induction (Bat-Ochir et al., 2016; Vaknin et al., 2016; Willger et al., 2012). The azole drug has a similar mechanism of action that triggers nuclear transport of SrbA to the nucleus (Song et al., 2017; Vaknin et al., 2016). To explore whether azole-induced SrbA translocation was accompanied by the cleavage of SrbA, we generated the WTGFP−SrbA strain by expressing the GFP-SrbA fusion protein in the wild-type background. The resulting strain exhibited similar growth phenotypes compared to the wild-type strain under conditions with or without ITC treatment (Figure A 1), indicating that the N terminal GFP tag did not affect the function of SrbA. GFP signals were detected in the peripheral areas of the nucleus in WTGFP−SrbA strain without ITC treatment, as indicated by the nuclear dye Hoechst (Figure 3-a), indicating its localization in the ER. After ITC treatment for 2h, GFP-SrbA signals overlapped with the Hoechst signals, indicating the nuclear localization of SrbA. We confirmed that treatment with the DMSO control did not change the ER localization of GFP-SrbA. To determine if SrbA cleavage was associated with its nuclear localization, total protein was extracted from the WTGFP−SrbA strain using alkaline lysis followed by immunoblotting using a GFP antibody. Immunoblotting showed specific bands at approximately 150 kDa with or without ITC treatment, corresponding to the full-length SrbA-GFP fusion protein (hereafter named GFP-SrbA-F; GFP: ~27 kDa, SrbA: ~120 kDa), however, no cleaved SrbA bands were observed (Figure 3-b). In contrast, the nuclear forms of SrbA (the cleaved N-terminus, hereafter named GFP-SrbA-N) were clearly visible by using a specific nucleoprotein extraction kit (Figure 3-b), suggesting that ITC induces SrbA protein cleavage and nuclear translocation. To explore the effects of ITC on HapB protein, the HapB was labeled with an N-terminus GFP tag at its native locus. The GFP-HapB fusion protein did not cause any morphological phenotypes compared to the parental strain (Figure A1), indicating that it is fully functional. As shown in Figure 3-c, the GFP-HapB fusion protein was constantly located in the nucleus, irrespective of ITC treatment, indicating that the localization of HapB was not affected by ITC. Notably, the immunoblotting analysis showed that ITC treatment could induce upregulation of GFP-HapB expression (Figure 3-d), suggesting that the nuclear-localized HapB could respond to azole treatment.
3.4 The nuclear form of SrbA confers azole resistance and increases the expression of Erg11A in *A. fumigatus*

Considering that ITC induces the SrbA cleavage and nuclear translocation, we wondered whether the nuclear form of SrbA was associated with azole resistance. To test this, we constructed a truncated SrbA strain (GFP-SrbA<sup>T</sup>) that expresses a putative nuclear form of SrbA (SrbA<sup>T</sup>, which contains the first 380 aa of SrbA) fused with GFP at its N-terminus under the control of the *srbA* native promoter in the wild-type background (Figure 4-a). To examine whether the GFP-SrbA<sup>T</sup> fusion protein localizes to the nucleus, we coexpressed RFP-tagged histone H2A (RFP-H2A) as a nuclear marker. As shown in Figure 4-b, GFP signals of full-length GFP-SrbA<sup>T</sup> were detected in the peripheral areas of RFP-H2A, whereas GFP-SrbA<sup>T</sup> was colocalized with RFP-H2A in minimal medium, indicating that the mutant with a nuclear form of SrbA was successfully constructed.

To exclude the interference of the GFP tag with the function of SrbA<sup>T</sup>, we generated a SrbA<sup>T</sup> strain that expresses SrbA<sup>T</sup> without a GFP tag in the wild-type background using the AMA1 vector. A SrbA<sup>F</sup> strain expressing the full-length of *srbA* was also constructed similarly as a control. To exclude the possibility that the AMA1 plasmids may introduce different *srbA* gene copies between SrbA<sup>T</sup> and SrbA<sup>F</sup> strains, we compared the relative gene copy number of *srbA* by quantitative real-time PCR (qRT-PCR) analysis. The result showed no significant difference in *srbA* copy number between SrbA<sup>T</sup> and SrbA<sup>F</sup> strains (Figure A2-a). The colony phenotype of SrbA<sup>T</sup> strain was indistinguishable from SrbA<sup>F</sup> strain on minimal medium but displayed increased resistance to ITC (Figure 4-c), indicating the contribution of the nuclear form of SrbA to azole resistance. To verify...
whether expression of Erg11A could be changed in the SrbA\textsuperscript{T} strain, we expressed SrbA\textsuperscript{T} and SrbA\textsuperscript{F} in the Erg11A-GFP labeled strain, respectively. As shown in Figure 4-d, Erg11A-GFP expression in the SrbA\textsuperscript{T} strain was significantly higher than that in the SrbA\textsuperscript{F} strain in minimal media. Fluorescence microscopy further confirmed that Erg11A-GFP in the SrbA\textsuperscript{T} strain exhibited a strong ER-localization signal, whereas in the SrbA\textsuperscript{F} strain, GFP fluorescence was barely observed (Figure 4-e), suggesting that nuclear form of SrbA contributes to the increased expression of Erg11A, and the amount of nuclear form of SrbA in SrbA\textsuperscript{T} strain is greater than that in the SrbA\textsuperscript{F} strain. Taken together, these data suggest that the constitutive nucleolus-localized N-terminus of SrbA renders A. fumigatus resistant to azole by upregulating Erg11A.

3.5 Lack of HapB increases SrbA expression and prolongs its nuclear retention

Based on the above findings, SrbA positively regulates Erg11A expression by its nuclear form, and overexpression of Erg11A is the main cause for azole resistance of the hapB deletion mutant. We next expressed the GFP-SrbA by AMA1 vector in the ΔhapB and wild-type background to examine whether a lack of HapB would affect the levels of the nuclear form of SrbA. The qRT-PCR analysis showed no significant difference in srbA copy number between WT\textsuperscript{GFP-SrbA} and ΔhapB\textsuperscript{GFP-SrbA} mutants (Figure A2-b). Using fluorescence microscopy, we examined the localization of GFP-SrbA in the ΔhapB\textsuperscript{GFP-SrbA} and WT\textsuperscript{GFP-SrbA}. As shown in Figure 5-a, GFP-SrbA showed ER-localized patterns in the ΔhapB and wild-type strains when grown in minimal medium without ITC treatment and moved into the nucleus after ITC treatment for 2 and 4 h. Notably, with the prolongation of ITC treatment to 6 h, the nucleus-localized fluorescence of GFP-SrbA in the wild-type strain showed the punctate GFP signals throughout the hyphae. In contrast, GFP-SrbA in ΔhapB hyphae still exhibited a distinct nuclear localization pattern at the same time-point, which suggests that the lack of HapB prolongs the retention time of GFP-SrbA in the nucleus. Using immunoblotting with a GFP antibody, we examined the cleavage levels of SrbA in the ΔhapB and its parental wild-type strains after ITC or DMSO treatment for 4 h. As shown in Figure 5-b, the ΔhapB\textsuperscript{GFP-SrbA} strain showed more accumulation of the nuclear forms of SrbA than the control strain WT\textsuperscript{GFP-SrbA} even under non-induced conditions with DMSO treatment. In addition, we found that the ΔhapB mutant showed an increased protein level of SrbA compared to the wild-type strain without ITC treatment, suggesting that HapB could be a negative regulator of SrbA. To confirm this, we conducted the qRT-PCR analysis to compare the srbA transcription level in the wild-type and ΔhapB mutant. As shown in Figure 5-c, the mRNA level of srbA increased in the ΔhapB mutant. Previous studies have shown that CBC regulates the expression of downstream targets through binding to the 5′-CCAAT-3′ within their promoter regions (Gsaller et al., 2016; Hortschansky et al., 2017; Steidl et al., 2001). Sequence analysis revealed that two CCAAT motifs are located at position -849 and 195 base pairs (-849 and -195) upstream of the srbA translation start site. To further investigate whether CBC can directly bind to these two CCAAT motifs, we expressed and purified the HapB/C/E proteins in Escherichia coli, respectively, and then mixed them to form CBC complex for electrophoretic mobility shift assays (EMSA). As shown in Figure 5-d, the srbA probe 1 (position: -849) mixed with CBC displayed a slow shift compared to the free probe without CBC treatment. Excess of unlabeled probe (cold probe) significantly reduced the binding activity of CBC with the Cy5-labeled probe. Moreover, the mutated probe without the CCAAT motif showed a clear decrease in the binding activity of the upper complex. In comparison, srbA probe 2 (position: -195) also exhibited weak binding to CBC complex. These data suggested that the binding of CBC to the srbA promoter is dependent on the CCAAT motif.
FIGURE 5 Lack of HapB prolongs nuclear retention of SrbA and increases the protein levels of the nuclear form of SrbA. (a) The WT GFP–SrbA and ΔhapB GFP–SrbA strains were grown in MM for 12 h and then shifted into MM with 16 μg/ml ITC for 2, 4, and 6 h. The GFP signals were observed using a fluorescence microscope. The scale bar is 5 μm. (b) The indicated strains were grown in MM at 37°C for 24 h, and then 16 μg/ml ITC was added to the media for 4 h. The protein samples extracted by the nucleoprotein extraction kit were examined by immunoblotting. (c) The transcript levels of srbA in the wild-type and ΔhapB strains grown in MM for 16 h and 24 h. Statistical significance was determined by Student’s t-test. **p < 0.01. Values are means ± SD from three independent replicates. (d) EMSA analysis of CBC binding to Cy5-labeled promoter fragments of srbA. Two specific probes (1 and 2) were designed for srbA. srbA probe 1/2 contains the CCAAT motif located at position 986/195 bp (−986/−195) upstream of the srbA translational start site. The specificity of EMSA binding was validated by using a mutant probe or adding specific competitors/cold probe (unlabeled probe).
3.6 | Upregulation of SppA and the Dsc ubiquitin E3 ligase complex induced by the lack of HapB are responsible for proteolytic cleavage of SrbA

It has been reported that the cleavage of SrbA requires the rhomboid protease RbdB, the Dsc ubiquitin E3 ligase complex composed of five subunits (DscA-E), and the signal peptide peptidase SppA, all of which are critical for translocation of SrbA from the ER into the nucleus and then SrbA could be transcriptionally activated (Figure 6-a) (Bat-Ochir et al., 2016; Dhingra et al., 2016; Vaknin et al., 2016; Willger et al., 2012). We wondered whether the increase in the nuclear forms of SrbA in ΔhapB might be related to the expression of RbdB, the Dsc complex, and SppA. We therefore performed the qRT-PCR to detect the mRNA level of these genes. As shown in Figure 6-b, except for dscB, the expression level of rbdB, sppA, and dscA/C/D/E was increased in the ΔhapB mutant compared to that of the wild-type. As the sequence analysis showed that the promoter regions of all these upregulated genes contain at least one CBC binding motif CCAAT, we next selected dscE, rbdB, and sppA to perform EMSA analysis to test whether CBC binds to their promoters, the results showed that CBC can bind to the CCAAT motifs of dscE (position −1467), rbdB (position −600) and sppA (position −170) promoter regions with different degrees, but fails to bind other CCAAT regions of dscE (positions −728, −862) and rbdB (position −300) in vitro (Figure 6-c and Figure A3). To further verify whether CBC could affect the protein expression of RbdB, SppA, and DscA/C/D/E, we tagged these proteins with GFP at the C-terminus under the control of their respective promoters in the wild-type and ΔhapB backgrounds, respectively. The phenotypic analysis confirmed that the tagged proteins are fully functional, as the resulting strains exhibited a similar growth phenotype to their respective parental strains (Figure A4). In line with the mRNA level, western blotting analysis showed that, except for the undetected DscA and RbdB expressions, DscC/D/E and SppA fusion proteins in the ΔhapB strain were increased compared to those in the parental wild-type strain (Figure 6-d), indicating that CBC represses the expression of SppA and the DscC/D/E at the protein level. Collectively, these results suggest that CBC represses the expression of SppA, RbdB, and the Dsc ubiquitin E3 ligase complex by binding the promoters of the corresponding genes.

4 | DISCUSSION

In fungi, the transcriptional regulator SrbA and the CBC complex have been reported to transcriptionally regulate the expression of the azole target Erg11A and therefore play critical roles in azole resistance and sterol biosynthesis (Gsaller et al., 2016). It has been reported that both SrbA and CBC can bind to the TR34 region of the erg11A promoter, and they perform opposing actions to govern sterol biosynthesis and azole tolerance. The absence of any of the CBC subunits results in increased tolerance of A. fumigatus to azoles mainly due to the increased mRNA expression of erg11A. In contrast, the increased azole susceptibility in the srbA null mutant strain is the result of erg11A transcript insufficiency. In this study, we identified that the non-erg11A azole-resistant isolate 415-2 harbors a mutation in the hapB gene that leads to the premature transcription termination of hapB. Importantly, we revealed a potential regulatory mechanism by which the CBC negatively regulates SrbA expression by directly binding to srbA promoter, and represses SrbA cleavage by down-regulating the expression of the rhomboid protease RbdB, the Dsc ubiquitin E3 ligase complex, and the signal peptide peptidase SppA.

In mammals, low sterol activates ER-localized SREBP by triggering its protein cleavage accompanied by its N-terminal translocation to the nucleus (Bat-Ochir et al., 2016; Brown & Goldstein, 1997, 1998, 1999). A similar mechanism exists in SREBP homolog, SrbA, in Aspergillus species under hypoxic conditions (Willger et al., 2012). We found that azole also induces the cleavage of A. fumigatus SrbA and its translocation into the nucleus (Figure 3-a) (Song et al., 2017). In addition, expression of the putative nuclear forms of SrbA in the wild-type strain increased Erg11A protein expression and tolerance to azole (Figure 4c-e). These data suggest that azole exerts a similar influence on SrbA cleavage and nuclear translocation. The rhomboid protease RbdB, Dsc ubiquitin E3 ligase complex in A. fumigatus, and signal peptide peptidase SppA in A. nidulans are required for the cleavage of SrbA precursor protein (Bat-Ochir et al., 2016; Dhingra et al., 2016; Willger et al., 2012). We found that the lack of HapB increased the nuclear forms of SrbA and prolonged the retention time of SrbA in the nucleus under azole treatment conditions (Figure 5a,b) may arise as a consequence of the upregulation of RbdB, SppA, and the Dsc complex (Figures 6-b,d and 7). In addition, the HapB also negatively regulated the expression of SrbA, and loss of HapB increased the content of the total SrbA protein, which could also relatively increase nuclear forms of SrbA (Figures 5-b and 7). According to previous reports, more than 2000 gene promoters are occupied by the CBC, and the high abundance of CBC binding motifs CC(G)AAAT are identified in eukaryotic promoters (Furukawa, Scheven, et al., 2020), suggesting that CBC plays a role as a global regulator. Therefore, these data raise a possibility that HapB can negatively mediate the expression and cleavage of SrbA to regulate azole resistance besides repressing erg11A expression. In line with this hypothesis, our EMSA

**FIGURE 6** HapB regulates the expression of the proteins associated with the proteolytic cleavage of SrbA. (a) The putative schematic diagram of cleavage of A. fumigatus SrbA. (b) qRT-PCR showed the mRNA expression of dscA-E, rbdB, sppA in the wild-type and ΔhapB backgrounds. The indicated strains were grown in MM at 37°C for 16, 20, and 24 h. Statistical significance was determined by Student’s t-test: *p < 0.05; **p < 0.01; ns, not significant. Values are means ± SD from three independent replicates. (c) EMSA analysis of CBC binding to Cy5-labeled promoter fragments of dscE, rbdB, and sppA. Probes of dscE/rbdB/sppA contain the CCAAT motif located at −1467/−600/−170 position of their promoters, respectively. (d) Western blotting shows the protein expression of DscC-E and SppA in the wild-type and ΔhapB backgrounds. The indicated strains were grown in MM at 37°C for 24 h.
analysis validated that CBC can specifically bind to srbA, dscE, rbdB, and sppA in vitro via the conserved CCAAT motifs (srbA: position −986 and −195, dscE: position −1467, rbdB: position −600 and sppA: position −170). Previous ChIP-seq analysis has also revealed that the CBC-binding peaks were located at position 849 (−849) and 318 (−318) base pairs upstream of the srbA and dscE translational start.
sites, respectively (Furukawa, Scheven, et al., 2020), however, no typical CCAAT or CGAAT motif was observed in these regions. This may be because the ChIP peaks may result from the indirect binding events via intermediary partners, as the previous study showed that the transcription factor HapX forms a complex with CBC and CBC/HapX complex recognizes other DNA motifs than CC(G)AAT such as 5′-RWT-3′ and 5′-TKAN-3′ motifs (Furukawa, Scheven, et al., 2020). Nevertheless, our qRT-PCR and western blotting experiments further showed that loss of CBC resulted in increased expression of SrbA and the majority of its cleavage-related genes. In addition, since azole drugs and CBC deficiency have been reported to change the sterol profile (Gsaller et al., 2016; Shapiro et al., 2011), we therefore cannot rule out the potential role of altered sterol profile in the regulation of SrbA expression and nuclear retention, which needs further investigation and analysis.

**CONCLUSION**

In this study, our findings have revealed another plausible mechanism by which CBC dysfunction causes the upregulation of SrbA, and RbdB, SppA, and the Dsc complex facilitate SrbA activity, which ultimately elevates Erg11A expression andazole tolerance, and provides new insight into the molecular mechanism underlying the regulation ofazole resistance. A working model summarizing the findings of this study is depicted in Figure 7.

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**CONFLICT OF INTEREST**

None declared.

**AUTHOR CONTRIBUTIONS**

Chi Zhang: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (equal); Methodology (equal); Resources (equal); Software (equal); Validation (equal); Writing-original draft (equal). Lu Gao: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Resources (equal). Huiyu Gu: Conceptualization (equal); Methodology (equal); Project administration (equal); Supervision (equal). Yuanwei Zhang: Formal analysis (equal); Funding acquisition (equal); Methodology (equal); Project administration (equal); Supervision (equal); Writing-review & editing (equal). Ling Lu: Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing-review & editing (lead).

**ETHICS STATEMENT**

None required.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available in this published article and its appendices.

**ORCID**

Yuanwei Zhang https://orcid.org/0000-0003-0854-6123

Ling Lu https://orcid.org/0000-0002-2891-7326
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APPENDIX

A series of $2 \times 10^4$ conidia of related strains were spotted onto MM and cultured at 37°C for 2 days.

![Figure A1](image1.png)

**FIGURE A1** The growth phenotype of GFP-tagged SrbA/HapB strains on MM with or without ITC stress. A series of $2 \times 10^4$ conidia of wild-type, WT<sup>GFP-SrbA</sup>, GFP-HapB and ΔhapB and ΔhapB<sup>GFP-SrbA</sup> strains were spotted onto MM with or without ITC and cultured at 37°C for 2 or 4 days.

![Figure A2](image2.png)

**FIGURE A2** The relative srbA copy number in the related strains constructed by using the AMA1 vector. The related strains were grown in liquid MM with 220 rpm shaking at 37°C for 24 h. The respective genome was extracted from the resulting mycelium. Then qRT-PCR was used to detect the relative srbA copy number, tubA gene was used as an endogenous control. Statistical significance was determined by Student’s t-test. ns, not significant. Values are means ± SD from three independent replicates.
**Figure A3** Electrophoretic mobility shift assays analysis of CBC binding to Cy5-labeled promoter fragments of *dscE* and *rbdB*. The probe of *dscE* contains a CCAAT motif located at the −862/-728 position of its promoter. The probe of *rbdB* contains a CCAAT motif located at the −300 position of its promoter.

**Figure A4** The growth phenotype of the GFP-tagged SppA, DscA, DscC-E, and RbdB strains on MM.
| Strains | Genotype | Source |
|---------|----------|--------|
| ZC03/WT | Δku80; pyrG1; AMA1::pgpdA::Cas9::pyr4; hapB::hph | Zhang et al., 2016 |
| 415-2   | Δku80; pyrG1; ΔalgA::pyr4; hapB::hph | From Lu Lab |
| 415-2ΔhapB | Δku80; pyrG1; ΔalgA::pyr4; hapB::hph; hapB::hph | This study |
| ΔhapB   | ZC03; ΔhapB::hph | Ren et al., 2021 |
| hapBΔ | Δku80; pyrG1; ΔhapB::hph; hapB::pyr4 | From Lu Lab |
| ΔhapC   | ZC03; ΔhapC::hph | Ren et al., 2021 |
| ΔhapE   | ZC03; ΔhapE::hph | Ren et al., 2021 |
| Erg11A-GFP | ZC03; erg11A::gfp::ptrA | This study |
| ΔhapBΔErg11A-GFP | ZC03; ΔhapB::hph;erg11A::gfp::ptrA | This study |
| ΔsrB ΔsrBΔ | Δku80; pyrG1; ΔsrB::pyr4; erg11A- gfp::ptrA | This study |
| ΔhapBΔsrBΔerg11A-GFP | Δku80; pyrG1; ΔsrB::pyr4; ΔhapB::hph; erg11A-gfp::ptrA | This study |
| ΔhapBΔerg11A | ZC03; ΔhapB::hph;Δerg11A::ptrA | This study |
| Δerg11A | ZC03; Δerg11A::ptrA | This study |
| ΔsrB Δ | Δku80; pyrG1; ΔsrB::pyr4 | This study |
| ΔhapBΔsrBΔ | Δku80; pyrG1; ΔsrB::pyr4; hapB::hph | This study |
| WTΔFNR | Δku80; pyrG1; AMA1::pgpdA::gfp::srbA::pyr4 | This study |
| GFP·HapB | ZC03; gfp::hapB::hph | Ren et al., 2021 |
| GFP·SrbARFP−H2A | Δku80; pyrG1; AMA1::pgpdA::gfp::srbA::pyr4; p:pgpdA::rfp::H2A::pyr4 | This study |
| GFP·SrbAT·RFP−H2A | Δku80; pyrG1; AMA1::pgpdA::gfp::srbAT380::p:pgpdA::rfp::H2A::pyr4 | This study |
| SrbAΔ | Δku80; pyrG1; AMA1::pgpdA::gfp::srbA·pyr4 | This study |
| SrbAΔ | Δku80; pyrG1; AMA1::pgpdA::gfp::srbA·pyr4 | This study |
| SrbAT·Erg11A-GFP | Δku80; pyrG1; erg11A-gfp::ptrA; AMA1::pgpdA::gfp::srbA·pyr4 | This study |
| ΔhapBΔSrbAΔErg11A-GFP | Δku80; pyrG1; ΔhapB::hph; erg11A-gfp::ptrA | This study |
| ΔSPP−GFP | ZC03; sppA::gfp::ptrA | This study |
| ΔhapBΔSPP−GFP | ZC03; ΔhapB::hph; sppA::gfp::ptrA | This study |
| DscA·GFP | ZC03; dscA::gfp::ptrA | This study |
| ΔhapBΔDscA·GFP | ZC03; ΔhapB::hph; dscA::gfp::ptrA | This study |
| DscB·GFP | ZC03; dscB::gfp::ptrA | This study |
| ΔhapBΔDscB·GFP | ZC03; ΔhapB::hph; dscB::gfp::ptrA | This study |
| DscC·GFP | ZC03; dscC::gfp::ptrA | This study |
| ΔhapBΔDscC·GFP | ZC03; ΔhapB::hph; dscC::gfp::ptrA | This study |
| DscD·GFP | ZC03; dscD::gfp::ptrA | This study |
| ΔhapBΔDscD·GFP | ZC03; ΔhapB::hph; dscD::gfp::ptrA | This study |
| DscE·GFP | ZC03; dscE::gfp::ptrA | This study |
| ΔhapBΔDscE·GFP | ZC03; ΔhapB::hph; dscE::gfp::ptrA | This study |
| RbdB·GFP | ZC03; rbdB::gfp::ptrA | This study |
| ΔhapBΔRbdB·GFP | ZC03; ΔhapB::hph; rbdB::gfp::ptrA | This study |
| Name             | Sequence                                                                 | Intention                                                                 |
|------------------|--------------------------------------------------------------------------|--------------------------------------------------------------------------|
| sgRNA-R          | AAAAAAGCACCAGCTCGGTGCC                                                   | for the DNA template of sgRNA                                              |
| T7-hapB-sgRNA2-F | TAATACGACCTCTATAGGGATGTTGGTGCCCATATTGT TTAGAGCTAGAATAAGC                | for the DNA template of hapB-sgRNA2 (RNA); for constructing hapB<sup>165</sup> |
| hapB<sup>T</sup>-hph-R | CTCATACTGAGGTCATGGGACATTTTGGGTCAGAG TGGAGTTGAGTGGG                      | for the repair template of hapB truncation                                |
| hapB<sup>T</sup>-hph-F | AGATTTCCAGACAGATG CCC ATG GTA CTA TAA                                    | for the repair template of hapB truncation                                |
| hapB-seq-F       | ACTGACGCTTTACTTGATTTGAAG                                                 | diagnostic primer for hapB<sup>165</sup>                                  |
| hapB-seq-R       | ATGAGCGCAGTCCACAAAGGGTAG                                                  | diagnostic primer for hapB<sup>165</sup>                                  |
| SrbAP1           | AAATGGGTTCGTGGATATG                                                      | for deleting srbA                                                         |
| SrbAP2           | TCTCCAGGTTGATATTGTC                                                      | for deleting srbA                                                         |
| SrbAP3           | CGATTAAGTTGGTACGCCACTTGAGGTGAGTGGTAGA                                    | for deleting srbA                                                         |
| SrbAP4           | ATAAGTACGATCCCCAGAAAAGCCACAAAAGTAGTGCGCTCAA                               | for deleting srbA                                                         |
| SrbAP5           | GATGATGTCCAGGTTGAGTCC                                                    | for deleting srbA                                                         |
| SrbAP6           | GATCCCTCACCTACCA                                                    | for deleting srbA                                                         |
| Pyr4-F           | TGGCGTTACCCACACTTAAATCG                                                 | for amplifying pyr4                                                       |
| Pyr-R            | GCTTTCCGGAACCTGCTACTTAT                                                  | for amplifying pyr4                                                       |
| SrbA-F           | AAACCTCAAAAAAGCTCATC                                                   | diagnostic primer for ΔsrbA                                              |
| SrbA-R           | CTGCCGGCCAACATCATC                                                      | diagnostic primer for ΔsrbA                                              |
| T7-erg11A-sgRNA-F | TAATACGACCTCTATAGGGATGTTGGTGCCCATATTGT TTAGAGCTAGAATAAGC                | for the DNA template of erg11A-sgRNA (RNA); for deleting and tagging erg11A |
| erg11-deletionptrA-F | TCTAATCCGGTACCCCTCCTGTGCTCTCAGAACCT AGATGGGTCCTGTGACAT                 | for the repair template of deleting and tagging erg11A                    |
| erg11-deletionptrA-R | CTCAGAGGGGGCTGAATAGTTAATACATACACCTACCTAGGGGAC                          | for the repair template of deleting erg11A                                |
| gfp-erg11A-F     | CGGCTGGGAGAAGCGCAGTTGGGACAAACATCCAAAGGAGCT GGTGCCAGGCTTGG             | for the repair template of tagging erg11A                                  |
| erg11A-seq-F     | CATTCCCCCTACATCCTCAACTC                                                  | diagnostic primer for Δerg11A and erg11A-gfp                            |
| nerg11A-seq-R    | GTATAGCGCCACAAACACTCAGG                                                   | diagnostic primer for Δerg11A and erg11A-gfp                            |
| Ama1-srbA-F      | CGGTATAGGCTACAGTGCAGATGGCTACCTAGAGTTAAGTAGTGGAGTAC                      | for amplifying P<sub>srbA</sub>S<sub>b</sub>-srbA fragment              |
| Ama1-srbA-R      | AATACGCGCTAGTGACGCAGTGCAGATGGCATGAGCT                                    | for amplifying P<sub>srbA</sub>S<sub>b</sub>-srbA fragment              |
| gpd-srbA-R       | CGTCCGGTCCTCGCGGCTACGACTGACTCAGGAGAC                                     | for amplifying P<sub>srbA</sub>S<sub>b</sub>-srbA fragment (coupled with Ama1-srbA-F) |
| gpd-F            | GCTAGCGGAGAAGCGCAGG                                                     | for amplifying P<sub>srbA</sub>S<sub>b</sub>-H2A fragment               |
| Ama1-trpC-R      | AATCACGCTTACTGCTAGTGCAGATGGCATGAGCT                                    | for amplifying P<sub>srbA</sub>S<sub>b</sub>-H2A fragment               |
| Ama1-srbA<sup>T</sup>-R | AATACGCGCTAGTGACGCAGTGCAGATGGCATGAGCT                                    | for amplifying P<sub>srbA</sub>S<sub>b</sub>-srbA<sup>T</sup>/P<sub>srbA</sub>-srbA<sup>T</sup> fragment (coupled with Ama1-srbA-F) |
| gpd-srbA<sup>T</sup>-R | CGTCCGGTCCTCGCGGCTACGACTGACTCAGGAGAC                                     | for amplifying P<sub>srbA</sub>S<sub>b</sub>-srbA<sup>T</sup> fragment (coupled with Ama1-srbA-F) |
| T7-sppA-sgRNA-F  | TAATACGACCTCTATAGGGATGTTGGTGCCCATATTGT TTAGAGCTAGAATAAGC                | for the DNA template of sppA-sgRNA (RNA); for tagging sppA                |
| sppA-gfp-F       | CAAGGCCAGCATTGGTACGGAGCGCTGGCAGAGCT                                     | for the repair template of tagging sppA                                   |
| sppA-ptrA-R      | GTTCTCTCTGAGGAGCGCTCGGCCACACTAGGATGACGTC GTCAGGGCGCTG                     | for the repair template of tagging sppA                                   |
| sppA-seq-F       | CCCTTAAGGGTCATGGGAGAGAGAGAGGAGAGGAGAGGACG                               | diagnostic primer for SppA-GFP; qRT-PCR primer                             |
| sppA-seq-R       | GCACCCTGAGTATGGTATGCC                                                  | diagnostic primer for SppA-GFP                                            |
| sppA-RT-R        | GGAAGGAAAGATCAAAATCGGAG                                                   | qRT-PCR primer                                                             |
| Name         | Sequence Intention                                                                 |
|--------------|-------------------------------------------------------------------------------------|
| T7-dscA-sgRNA-F | TAATACGACTCACTATAGGGATGTGTACGACTGATGTTT TAGAGCTAGAAATAGC for the DNA template of dscA-sgRNA (RNA); for tagging dscA |
| dscA-gfp-F   | CCCATTGGTCGGGAGTCAATCCCT CCGTGGAGACTGGCGGAGCGT for the repair template of tagging dscA |
| dscA-ptrA-R  | TTTTGTTCTGGAAGTTGTACGACTGATCTAGGAGA TCGTTCCGGGAGTG for the repair template of tagging dscA |
| dscA-seq-F   | GGTACGTTGCAACGTCG for diagnostic primer for DscA-gfp; qRT-PCR primer |
| dscA-seq-R   | TCGCCAAGATCGAGGTCAGTG for diagnostic primer for DscA-GFP |
| dscB-RT-F    | CGGTATAGGATCACGAC for qRT-PCR primer |
| dscB-RT-R    | AACTCGCCAGAGCCACGT for qRT-PCR primer |
| T7-dscC-sgRNA-F | TAATACGACTCACTATAGGGTTGTTGATAATGACATTAGTTTTAG AGCTAGAAATAGC for the DNA template of dscC-sgRNA (RNA); for tagging dscC |
| dscC-gfp-F   | GGTACGTTGCAACGTCG for diagnostic primer for DscC-GFP; qRT-PCR primer |
| dscC-ptrA-R  | TCGTATAGGATCACGAC for qRT-PCR primer |
| dscC-seq-F   | CGAAGAGGGTTGGAATGGAT for diagnostic primer for DscC-GFP; qRT-PCR primer |
| dscC-seq-R   | CAGAGTGAAGATGATGAGTAGAC for diagnostic primer for DscC-GFP |
| dscC-RT-R    | CCTCAACCACATGGCCACAC for qRT-PCR primer |
| T7-dscD-sgRNA-F | TAATACGACTCACTATAGGGTTGTTGATAATGAC for the DNA template of dscD-sgRNA (RNA); for tagging dscD |
| dscD-gfp-F   | GGTACGTTGCAACGTCG for diagnostic primer for DscD-GFP; qRT-PCR primer |
| dscD-ptrA-R  | CCAACCGTGAGAGCTACAGGTATG for the repair template of tagging dscD |
| dscD-seq-F   | TGCCGGTGAGGAGGAAAGCAGGAAGT for diagnostic primer for dscD-gfp; qRT-PCR primer |
| dscD-seq-R   | GATGTGCTTGAGGAACCTCT for diagnostic primer for DscD-GFP |
| dscD-RT-R    | CCGTCTGTTCCGAAGGTAT for qRT-PCR primer |
| T7-dscE-sgRNA-F | TAATACGACTCACTATAGGGTTGTTGATAATGAC for the DNA template of dscE-sgRNA (RNA); for tagging dscE |
| dscE-gfp-F   | GGTACGTTGCAACGTCG for diagnostic primer for DscE-GFP; qRT-PCR primer |
| dscE-ptrA-R  | TCCGGGCGGAGTGCGGCGGTCTTCAGCTAGAGATCGTCCGCGATG for the repair template of tagging dscE |
| dscE-seq-F   | ACGACATTTGGCGCAATGGGAAG for diagnostic primer for DscE-GFP; qRT-PCR primer |
| dscE-yz- R   | CAGTCGGAAGGACGACGAGAG for diagnostic primer for DscE-GFP |
| dscE-RT-R    | GCCCTGTGCAAGCTTGCAGAAC for qRT-PCR primer |
| sbA-RT-F     | CCGTCTGAGCTGACGACGAC for qRT-PCR primer |
| sbA-RT-R     | GTGCAGAAGCTTCTTGCGACATCG for qRT-PCR primer |
| tubA-RT-F    | TGACTGCTTCAGGGGCTTCC for qRT-PCR primer |
| tubA-RT-R    | GCGTGTAAAGCTGCAACG for qRT-PCR primer |
| rbdB-RT-F    | CTACTGCTGGGCTTCGAAG for qRT-PCR primer, diagnostic primer for RbdB-GFP |
| rbdB-RT-R    | CGAGACCGAGAGATAAGCC for qRT-PCR primer |
| Name          | Sequence                                                                 | Intention                                                                                      |
|---------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| T7-rbdB-F     | TAATACGACCTCTATAGGGAGTGGTTCAAGGACCGGT TTAGAGCTAGAAATAGCA                  | for the DNA template of rbdB-sgRNA (RNA); for RbdB-GFP                                         |
| rbdB-gfp-F    | CTATCTGGGCTGAATCAGCAGCCTGGTCTGGAGCAGGTTGAGGCGCTGGG                        | for the repair template of tagging rbdB (RbdB-GFP)                                            |
| rbdB-ptrA-R   | CATCGCCGAGGAGCTCACCTTCTACCTCGTCAAGCAG GAGAGATG                             | for the repair template of tagging rbdB (RbdB-GFP)                                            |
| rbdB-seq-R    | CCATGCCTAGTTGTTAGTG                                                       | diagnostic primer for RbdB-GFP                                                                |
| Pet30A-F      | GAATTCGCCCTGCAACAGCTTGGCAGGCGCAGCTCGAG                                    | Linearization of Pet30A                                                                       |
| Pet30A-R      | CATATGATATCTCTTCTTAAATGTTAAC                                               | Linearization of Pet30A                                                                       |
| EmsA-hapB-F   | GAAGGAGATATACATATGATGGAATACCCCTCCAACAATATCAC                             | Amplification of hapB cDNA                                                                    |
| EmsA-hapB-R   | GTCGACGGAGGCTGAATACCCCTATCTACCTGAGGGTTGTTTC                               | Amplification of hapB cDNA                                                                    |
| EmsA-hapC-F   | GAAGGAGATATACATATGATGCGCCCTCCCTCCCTGAAAAG                                 | Amplification of hapC cDNA                                                                    |
| EmsA-hapC-R   | GTCGACGGAGGCTGAATACCCCTCCCTCCCTGCTC                                     | Amplification of hapC cDNA                                                                    |
| EmsA-hapE-F   | GAAGGAGATATACATATGAGGGAGATGTTTGGCGATACCTGTCG                             | Amplification of hapE cDNA                                                                    |
| EmsA-hapE-R   | GTCGACGGAGCCTGGAATTCAAGGATTGGCGATACCTGTCG                                | Amplification of hapE cDNA                                                                    |
| EmsA-srbA2-F  | AGATGCAGCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for srbA probe 2                                                                             |
| EmsA-srbA2-MF | AGATGCAGCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for srbA mutant probe 2                                                                       |
| EmsA-srbA2-R  | AGATGCAGCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for srbA probe 2                                                                             |
| EmsA-srbA1-F  | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for srbA probe 1                                                                             |
| EmsA-srbA1-MF | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for srbA mutant probe 1                                                                       |
| EmsA-srbA1-R  | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for srbA probe 1                                                                             |
| EmsA-sppA-F   | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for sppA probe                                                                               |
| EmsA-sppA-MF  | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for sppA mutant probe                                                                         |
| EmsA-sppA-R   | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC                 | for sppA probe                                                                               |
| EmsA-1467-dscE-F | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC             | for dscE probe targeting −1467 region                                                        |
| EmsA-1467-dscE-MF | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC          | for dscE mutant probe targeting −1467 region                                                   |
| EmsA-1467-dscE-R | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC          | for dscE probe and dscE mutant probe targeting −1467 region                                   |
| EmsA-862-dscE-F | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC             | for dscE probe targeting −862 region                                                         |
| EmsA-862-dscE-MF | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC          | for dscE mutant probe targeting −862 region                                                   |
| EmsA-862-dscE-R | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC          | for dscE probe and dscE mutant probe targeting −862 region                                   |
| EmsA-728-dscE-F | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC             | for dscE probe and dscE mutant probe targeting −728 region                                   |
| EmsA-728-dscE-R | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC          | for dscE probe and dscE mutant probe targeting −728 region                                   |
| EmsA-728-dscE-MR | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC          | for dscE mutant probe targeting −728 region                                                   |
| EmsA-600-RbdB-F | AGATGCACCTAGACCCGAGTGTATGTTCAAGGATGGTG TCGATACAGATACTTTC             | for rbdB probe targeting −600 region                                                         |

(Continues)
| Name              | Sequence                                                                 | Intention                                                      |
|-------------------|---------------------------------------------------------------------------|----------------------------------------------------------------|
| EmsA−600-RbdB-MF  | AGATGCAGCTAGCACGATATCTCATATATAAAGCTGTCATGATTTAATGAGCTAGAAG               | for rbdB mutant probe targeting −600 region                   |
| EmsA−600-RbdB-R   | AGATGCAGCTAGCACGATATCTCATATATAAAGCTGTCATGATTTAATGAGCTAGAAG               | for rbdB probe and rbdB mutant probe targeting −600 region     |
| EmsA−300-RbdB-F   | AGATGCAGCTAGCACGATATCTCATATATAAAGCTGTCATGATTTAATGAGCTAGAAG               | for rbdB probe targeting −300 region                           |
| EmsA−300-RbdB-MF  | AGATGCAGCTAGCACGATATCTCATATATAAAGCTGTCATGATTTAATGAGCTAGAAG               | for rbdB mutant probe targeting −300 region                    |
| EmsA−300-RbdB-R   | AGATGCAGCTAGCACGATATCTCATATATAAAGCTGTCATGATTTAATGAGCTAGAAG               | for rbdB probe and rbdB mutant probe targeting −300 region     |