Current Genetics (2022) 68:605–617
https://doi.org/10.1007/s00294-022-01250-2

ORIGINAL ARTICLE

A novel Zn_2-Cys_6 transcription factor *clcA* contributes to copper homeostasis in *Aspergillus fumigatus*

Yoko Kusuya[1] · Cai Bian[1] · Daisuke Hagiwara[2,3] · Sayaka Ban[1] · Hiroki Takahashi[1,4,5]

Received: 12 April 2022 / Revised: 29 July 2022 / Accepted: 30 July 2022 / Published online: 16 August 2022

Abstract

The filamentous fungus *Aspergillus fumigatus* is the most important pathogenic fungus among *Aspergillus* species associated with aspergillosis. *A. fumigatus* is exposed to diverse environmental stresses in the hosts during infection such as an excess of essential metal copper. To gain further insights into copper homeostasis, we generated an *A. fumigatus* laboratory evolved strain with increased fitness in copper stress, and identified the mutation in a Zn_2-Cys_6 type transcription factor *clcA*. We examined the role of *clcA* using the evolved and Δ*clcA* strains. The Δ*clcA* strain exhibited defective growth on minimal medium, PDA and copper-repleted medium, and defective conidiogenesis and conidial pigmentation. We found that *clcA* was required for the expressions of genes involved in conidiogenesis, conidial pigmentation, and transporters *cdr1B* and *mfsB* related to azole resistance. *clcA* was dispensable for the virulence in silkworm infection model. We report here that *clcA* plays an important role in hyphal growth, conidiogenesis, and copper adaptation.

Keywords *Aspergillus fumigatus* · Copper · *clcA* · Sporulation · Pigment biosynthesis · Virulence

Introduction

The filamentous fungus *Aspergillus fumigatus* is the most important pathogenic fungus among *Aspergillus* species associated with aspergillosis (Bodey and Vartivarian 1989; Latgé and Chamilos 2019). Metals such as copper (Cu) and iron (Fe) are essential micronutrients for all organisms including fungi (Andreini et al. 2008); copper serves as a critical cofactor for numerous proteins (Robinson and Winge 2010; Festa and Thiele 2011). Thus, copper homeostasis plays a critical role in a broad range of biological processes such as virulence, conidiogenesis, and secondary metabolite production in fungi including *A. fumigatus* (Park et al. 2014; Cai et al. 2017; Kusuya et al. 2017; Wiemann et al. 2017; Raffa et al. 2019). The fundamental mechanisms for copper homeostasis such as copper acquisition, utilization, and regulation have been discovered in fungi (Smith et al. 2017). Three copper-binding transcription factors (TFs) have been examined in *A. fumigatus*; Wiemann et al. (2017) reported that a TF AceA plays a critical role in defense mechanism against macrophages. Besides, Kusuya et al. (2017) reported that a TF AfMac1 plays a role in copper acquisition, contributing to the biosynthesis of conidia-specific melanin and the late stage of conidiogenesis (Wiemann et al. 2017; Park et al. 2018). CufA has been examined, but its function is still unknown (Wiemann et al. 2017; Raffa et al. 2019).

Upon bacterial and fungal infection, macrophages elevate copper levels inside the phagosome by increasing the expression of copper importer Ctr1 and locating the P-type copper ATPase pump (ATP7A) to the phagosomal membrane (White et al. 2009; Achard et al. 2012; Ding et al. 2013). It is thus conceivable that the copper stress contributes to killing *A. fumigatus*. Against the copper accumulation, *A. fumigatus* scavenges copper and detoxifies ROS through AceA-dependent response (Wiemann et al. 2017; Anabosi

✉ Hiroki Takahashi
hiroki.takahashi@chiba-u.jp

1 Medical Mycology Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan

2 Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan

3 Microbiology Research Center for Sustainability, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan

4 Molecular Chirality Research Center, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

5 Plant Molecular Science Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8675, Japan

et al. 2021). Although 10 genes as the copper-responsive genes have been functionally characterized in *A. fumigatus* (Blatzer and Latgé 2017), copper homeostasis is not fully understood.

To gain further insight into copper homeostasis, we here generated a strain adapted to copper-repleted conditions, Af293-G10, through a laboratory passage experiment. The Af293-G10 strain exhibited greater growth than the parent strain Af293 on not only AMM and PDA but also copper-repleted and -depleted medium. We identified the mutation resided within *clcA* in Af293-G10. Unlike the initial purpose, the disruption of *clcA* had a broad range of effects on the growths on AMM, PDA and copper-repleted medium, and the conidiogenesis and conidial pigmentation. In addition, *clcA* was dispensable for the virulence in the silkworm infection model. Exploring the prevalence of *clcA* in fungi, *clcA* is conserved only across Ascomycota. Taken together, we suggest that *clcA* plays an important role in hyphal growth, conidiogenesis and copper adaptation.

Material and methods

Strains and culture conditions

The strains used in this study are listed in Table 1. *A. fumigatus* Af293 was used as the parent strain. Strains were grown at 37 °C in *Aspergillus* minimal medium (AMM) supplemented with Clive Roberts trace elements solution described in the Culture Medium of Protocols at Fungal Genetics Stock Center (http://www.fgsc.net/Aspergillus/protocols/MediaForAspergillus.pdf).

Serial passaging of *A. fumigatus* Af293 under the copper-repleted condition

A serial passage experiment was performed with slight modifications of Kowalski et al. (2016) and Toyotome et al. (2021). We used an *A. fumigatus* Af293 strain as the parental strain. The conidia of Af293 as the starting material were grown on a PDA plate for 7 days at 37 °C, followed by conidia collection. Passage experiment was followed; first, to expose the strains to copper-repleted condition, the conidia were inoculated on an AMM plate containing 100 µM $CuSO_4$, as copper-repleted condition, at 37 °C for 4 days. Next, the conidia were inoculated on a PDA plate for 3 days. Following 10 passages of 7 day-culture, we obtained the laboratory evolved strain.

Table 1 *A. fumigatus* starains used in this study

Strain	Genotype	Reference
Af293	FGSC A1100, wild-type	From FGSC
Af293-G10	The laboratory passage strain from Af293	This study
Δ*clcA*	Af293 *clcA*::Hph	This study
Co-clcA	Af293 *clcA*::Hph, *clcA-ptrA*	This study

Construction of gene deletion and reconstituted strains

All primers used in this study are presented in Table S1. The *clcA* gene was disrupted by using CRISPR/Cas9 system (Umeyama et al. 2018). First, the *hph* (hygromycin B resistance cassette) was amplified from the pHph plasmid constructed from pSK397 (Krappmann et al. 2006; Umeyama et al. 2018). Next, the fragments A and B corresponding to about 1 kbp upstream and downstream regions of *clcA*, respectively, were amplified from the genomic DNA of Af293 by using the primer sets of 2g08040UF-2g08040 + HphUR and Hph + 2g08040DF-2g08040DR, respectively (Fig. S1a). Both 2g08040 + HphUR and Hph + 2g08040DF primers contained a 24 bp complementary to *hph* sequences at the 5′ end. Then, recombinant PCR was performed to fuse fragments by overlap extension PCR. Subsequently, the transformation was performed (Fig. S1b). For the complemented *clcA* strain (*Co-clcA*), the *ptrA* (pyrithiamine resistance gene) was amplified from the pPTRI plasmid (Takara Bio, Shiga, Japan). Similarly to disruption of *clcA*, two fusion fragments were transformed into the Δ*clcA* strain.

Transformation of *A. fumigatus*

Transformation of *A. fumigatus* was performed according to Umeyama's method for genome editing using CRISPR/Cas9 (Umeyama et al. 2018). The culture and protoplast solution were prepared according to Szewczyk et al. (2006), and Umeyama et al. (2018). Briefly, conidia were incubated at 37 °C in 10 mL of YG medium (5 g yeast extract (BD Difco, MD, USA), 20 g D-glucose and 400 µL of trace elements in 1 L distilled water) for 5–6 h. 10 mL of protoplast solution containing 4 g VinoTaste Pro (Novozymes, Bagsvaerd, Denmark) was supplemented in the culture for generating protoplasts. The transformants were screened by CZAPEK DOX BROTH (CDB: Duchefa Biochemie, Haarlem, Netherlands) agar supplemented with 1 M sucrose containing 400 µg/mL hygromycin B or AMM containing 0.1 µg/mL pyrithiamine. The successful transformants were selected by PCR of the inserted fragments, followed by verification of the sequences of all amplified fragments of i, ii, and iii (Fig. S2) by ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, USA) (data not shown).

Growth assays

Conidia were harvested from a 5-day-old culture on AMM plate at 37 °C. The numbers of conidia in the suspensions were counted using a hemocytometer (Watson, Kobe, Japan). A total of 3×10^3 conidia of Af293, Af293-G10, and Δ*clcA* strains were grown on the plates of PDA, AMM, AMM supplemented with 100 μM $CuSO_4$ (copper-repleted), and AMM supplemented with 100 μM bathocuproinedisulfonic acid (BCS) (copper-depleted) at 37 °C for 3–7 days. The assays were performed in triplicate. Statistical analysis was performed using Tukey–Kramer test by the R programming language (https://www.r-project.org/).

Microscopic observation of conidiophores

Conidiophores were observed by using the slide culture method and photographed with a Nikon ECLIPSE Ni (Nikon, Tokyo, Japan). AMM agar media was sliced into the thin agar blocks, and conidial suspensions were streaked on the sliced AMM agar blocks. The agar blocks were sandwiched between cover glasses and glass slides, and incubated at 25 °C for 5 days under moist conditions.

Phylogenetic analysis

The amino acid sequences were aligned with MAFFT (ver. 7.475) (Katoh et al. 2002; Katoh and Standley 2013). A phylogenetic tree was constructed using multithreaded RAxML (ver. 8.2.12) (Stamatakis 2014), the PROTGAMMAWAG model, and 100 bootstrap replicates, and visualized by iTOL (Letunic and Bork 2019). The graphical representation of ClcA was depicted by IBS (ver. 1.0.3) (Liu et al. 2015).

RNA extraction and quantitative real-time RT-PCR

Mycelia of Af293, Af293-G10, and Δ*clcA* were harvested after growth in AMM liquid media at 37 °C for 24 h and inoculated on AMM, AMM supplemented with 100 μM CuSO4, and AMM supplemented with 100 μM BCS plate. After cultivation, fungal cells were harvested at 24 h. Total RNA was prepared by using a RNeasy Mini Kit (Qiagen, Hilden, Germany), and a cDNA was synthesized by reverse transcription using a RevaTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan).

Quantitative real-time RT-PCR analysis was performed on a LightCycler 96 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland) with a THUNDERBIRD SYBER qPCR Mix (TOYOBO). The *act1* gene was used as an internal control for quantification of the target gene expression. The relative expression ratio relative to that of WT mycelia grown in AMM liquid media after 24 h post-inoculation was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study are listed in Table S2.

Evaluation of virulence against silkworms

We infected silkworm (*Bombyx mori*) larvae with *A. fumigatus* with slight modifications of Majima et al. (2021) and Yu et al. (2021). Briefly, fifth star silkworms were purchased from Ehime Sansyu (Ehime, Japan). They were raised at 25 °C for 2–3 days. Inoculum of 0.05 mL diluted to 3.0×10^6 CFU/mL was injected into the hemolymph of silkworms using a 1 mL Terumo Myjector 29G insulin syringe (Terumo, Tokyo, Japan). After the infection, they were maintained at 34 °C and their survival was evaluated by Kaplan–Meier method using log-rank test with survival package of the R programming language. The experiment was performed in duplicate, and the results were shown as the mean values.

Antifungal susceptibility testing

Antifungal susceptibilities of the strains were determined by Minimum Inhibitory Concentrations (MICs) using broth microdilution method based on the Clinical and Laboratory Institute (CLSI) M38-E3 using Dry Plate Eiken (Eiken Chemical Co., Ltd., Tokyo, Japan) (Kikuchi et al. 2014; CLSI 2017). The tests were performed in duplicate.

Whole-genome sequencing analysis

Genome DNA was extracted from overnight cultured mycelia by the phenol–chloroform method as described previously (Takahashi-Nakaguchi et al. 2015). Genomic DNA libraries of *A. fumigatus* strains were constructed using an NEBNext Ultra DNA Library Prep Kit (New England BioLabs, Massachusetts, USA) according to the manufacturer's protocol. A 150-bp paired-end sequencing on a HiSeq X Ten system (Illumina) was carried out by GENEWIZ (New Jersey, USA). Raw genomic reads were quality-controlled and trimmed by fastp (ver. 0.20.1) (Chen et al. 2018). Filtered reads were aligned against the Af293 reference genome (retrieved from FungiDB) using BWA-MEM (ver. 0.7.17-r1188) (Li 2013). SNP analysis was performed by samtools (ver. 1.10) (Li et al. 2009), and in-house python-script (Hagiwara et al. 2014).

Prediction of the tertiary structure of ClcA

The tertiary structures of ClcA and $ClcA_{L781F}$ were predicted by AlphaFold (ver. 2.0) with default parameters (Jumper et al. 2021). The tertiary structures of ClcA and $ClcA_{L781F}$ were depicted and aligned with PyMol (DeLano 2002).

Results

Generation of the strain adapted to copper-repleted conditions

To investigate further insight into copper homeostasis, we generated the strains adapted to the copper-repleted conditions through the laboratory evolution approach. Following 10 passages, the resulting strain, hereafter called Af293-G10, was obtained.

To evaluate the fitness of Af293-G10, we measured the colony diameters on the plates of AMM, PDA, AMM containing 100 μM $CuSO_4$ (copper-repleted conditions), and AMM containing 100 μM BCS (copper-depleted conditions). The growths of Af293-G10 were significantly greater than those of the parental strain Af293 on not only PDA and AMM, but also copper-repleted and -depleted conditions (Fig. 1a). Similarly, the dry weights of Af293 and Af293-G10 were quantified in the cultures for AMM and AMM containing 100 μM $CuSO_4$. The biomass of Af293-G10 was significantly lower than that of Af293 in AMM (Fig. 1b), while the biomass of Af293-G10 was comparable with that of Af293 in AMM containing 100 μM $CuSO_4$. By calculating the ratio of biomass in copper-depleted conditions to biomass in AMM, the fitness ratio of Af293-G10 was significantly higher than that of Af293 (Fig. 1c). These data suggest that Af293-G10 could evolve to adapt to copper-repleted conditions by our passage experiment.

Identification of a nonsynonymous mutation in Zn_2-Cys_6 transcription factor clcA

To identify causal mutations for the phenotypic change in Af293-G10, whole-genome sequencing of Af293 and Af293-G10 was performed. By comparing the genomes, we found two mutations in Af293-G10 (Table 2). One was a nonsynonymous mutation of L781F in *clcA* (Afu2g08040), and the other was found in the intron region of Afu4g06610. *clcA* is annotated as C6 finger domain protein according to FungiDB (Amos et al. 2022). ClcA harbors "Fungal Zn(2)-Cys(6) binuclear cluster domain" (PF00172) in the N-terminus, belonging to the largest class of fungal-specific domains (Shelest 2008), and the mutation L781F resides within "fungal transcription factor regulatory middle homology region" (cd12148) (Fig. 2a). The taxonomic group of genes in the ortholog group OG6_143678 including *clcA* were Ascomycota such as *A. niger* and *A. flavus* (Fig. 2b). Among the genes in OG6_143678, a total of 61 genes harbor both Zn(2)-Cys(6) binuclear cluster domain" (PF00172) and "fungal transcription factor regulatory middle homology region" (cd12148) in OG6_143678 (Fig. 2b), indicating that *clcA* could be broadly conserved across the species including Eurotiomycetes. To infer the structural changes of ClcA with L781F, refereed as $ClcA_{L781F}$, the tertiary structures of ClcA and $ClcA_{L781F}$ were predicted with AlphaFold 2.0 (Jumper et al. 2021). Interestingly, "Fungal Zn(2)-Cys(6) binuclear cluster domain" of $ClcA_{L781F}$ was not aligned with ClcA (Fig. S3), suggesting that the structural change of $ClcA_{L781F}$ might contribute to the adaptation to copper-repleted conditions in Af293-G10.

ClcA is required for hyphal growth in AMM, PDA and under copper-repleted conditions

To investigate the physiological functions of *clcA*, we constructed Δ*clcA* strain from Af293, and performed the growth assays on PDA and AMM plates. The Δ*clcA* strain exhibited a slow growth on the PDA and AMM plates, while Af293-G10 exhibited a greater growth (Fig. 1a). The Δ*clcA* strain exhibited a marked growth defect under copper-repleted conditions. Furthermore, we observed the pigmentation in the Δ*clcA* strain under copper-depleted conditions, although the growth was comparable to that of Af293 (Figs. 1a and S4). When cultured in liquid media, the biomass of Δ*clcA* strain in AMM was significantly increased, while the biomass in copper-repleted conditions was comparable with those of Af293 and Af293-G10 (Fig. 1b). As a result, the fitness ratio of biomass of Δ*clcA* strain was significantly lower than those of Af293 and Af293-G10 (Fig. 1c). Furthermore, we constructed the complemented strain *Co-clcA*. The growths of *Co-clcA* strain were comparable to those of Af293 on both PDA and AMM containing 100 μM $CuSO_4$ (Fig. S5). These findings indicated that *clcA* could be required for hyphal growth on not only AMM and PDA, but also copper-repleted conditions.

ClcA contributes to sporulation and pigment biosynthesis

To confirm the defective conidiogenesis in the Δ*clcA* strain (Figs. 1a and S4), we observed the conidiophores in the Af293, Af293-G10, and Δ*clcA* strains by microscopy. Indeed, the Δ*clcA* strain exhibited defective conidiogenesis and hyphal growth (Fig. 3). These data suggest that *clcA* could be required for both hyphal growth and conidiogenesis.

To further investigate the conidia formations in the Δ*clcA* strain, we compared the expression levels of the genes involved in conidiation and DHN-melanin biosynthesis, *brlA*, *abaA*, *wetA*, *pksP*, *abr1*, and *abr2* at 24 h (Fig. 4a–c). While the expression of *brlA* in the Δ*clcA* strain was higher than that in Af293, the expressions of

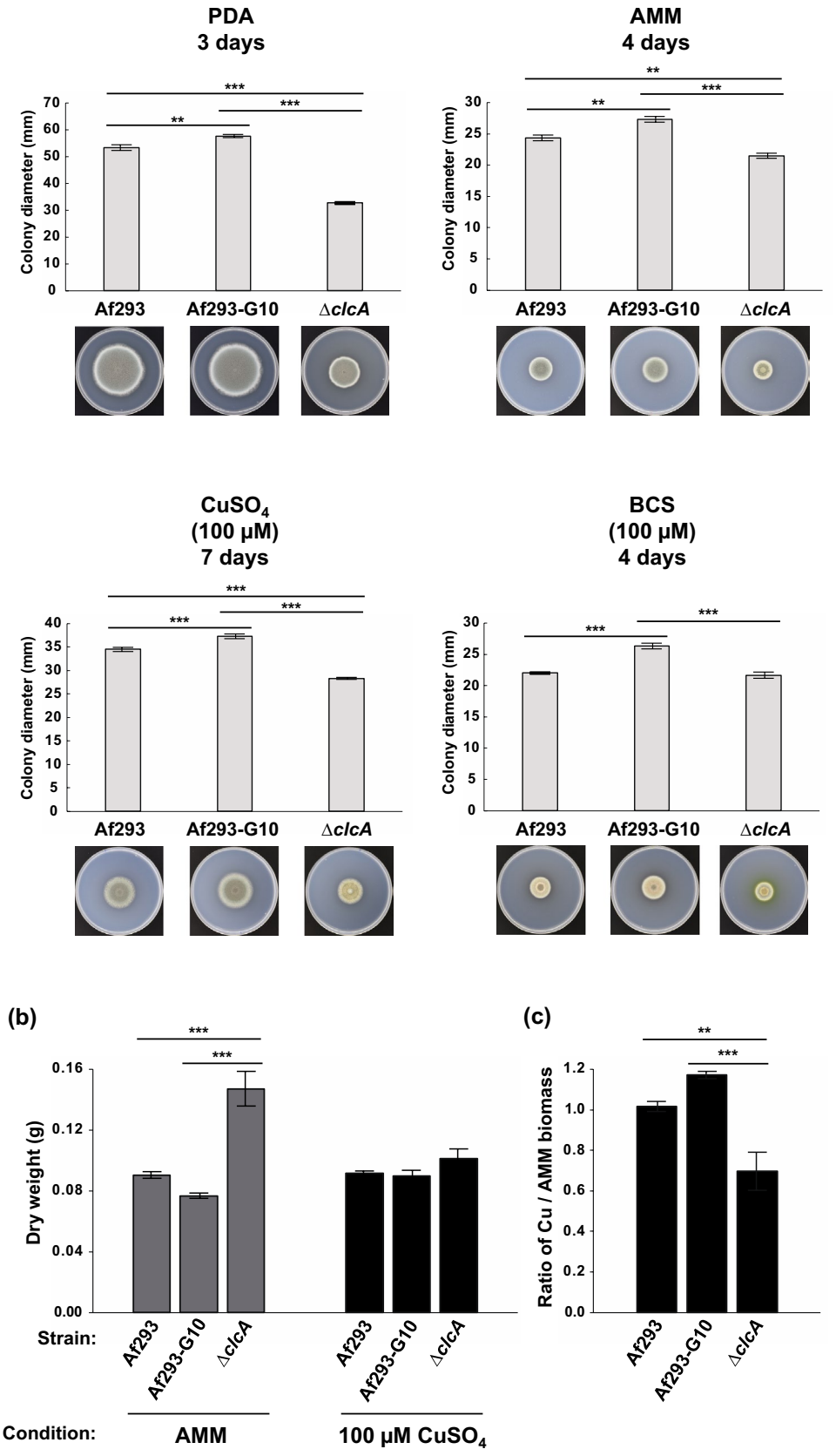

Fig. 1 Growth phenotypes of Af293, Af293-G10 and Δ*clcA* strains. (**a**) Growth assays of Af293, Af293-G10 and Δ*clcA* strains. The radial growth of Af293, Af293-G10, and Δ*clcA* strains were measured. The colony diameters and microscopic images are shown. A total of 3×10^3 conidia of strains were inoculated, and grown on PDA for 3 days, AMM and AMM containing 100 μM BCS for 4 days, and AMM containing 100 μM $CuSO_4$ for 7 days. (**b**) Biomass from liquid cultures grown in AMM and AMM containing 100 μM $CuSO_4$ at 48 h. Ratio of biomass in AMM containing 100 μM $CuSO_4$ to biomass in AMM. Statistical analysis was performed using Tukey–Kramer test (**p-value < 0.01, ***p-value < 0.001)

Table 2 Detected mutations in Af293-G10

Gene	Variant type
$clcA_{L781F}$ (Afu2g08040)	Missense
Afu4g06610 $_{G\rightarrow T\ (1227nt)}$	Intron

abaA and *wetA* were lower than those, possibly leading to smaller amount of conidia and pigmentation in the Δ*clcA* strain (Figs. 1a, 4c and S4). The expressions of *pksP*, *abr1*, and *abr2* were markedly decreased in the Δ*clcA* strain compared with those in Af293 (Fig. 4c). These expression profiles other than *pksP* were comparable with those under copper-repleted and -depleted conditions (Fig. S6). Taken together, these results suggested that the disruption of *clcA* could impair sporulation and pigmentation, and the L781F mutation of ClcA could lead to the defect in conidia formation.

The Δ*clcA* strain is susceptible to azoles

The antimicrobial susceptibilities of Af293, Af293-G10, and Δ*clcA* strains were determined by testing with micafungin (MCFG), amphotericin B (AMPH-B), and 5-flucytosine (5-FC), as well as the azole drugs fluconazole (FLCZ), itraconazole (ITCZ), voriconazole (VRCZ), and miconazole (MCZ). The Δ*clcA* strain exhibited increased susceptibility to ITCZ and MCZ, while the susceptibility to VRCZ in the Δ*clcA* strain was comparable with that in Af293, indicating that *clcA* could be involved in the ITCZ and MCZ resistance (Table 3).

Apart from alternations in *cyp51A* (Fisher et al. 2018; Toyotome et al. 2018), an ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters are the causes of azole resistance in fungi (Lupetti et al. 2002; Fraczek et al. 2013; Paul and Moye-Rowley 2014; Hagiwara et al. 2017; Toyotome et al. 2021). To examine whether the transporters participate in azole susceptibility in the Δ*clcA* strain, we measured the expressions of three transporters, *cdr1B* encoding ABC transporter, *mfsD* and

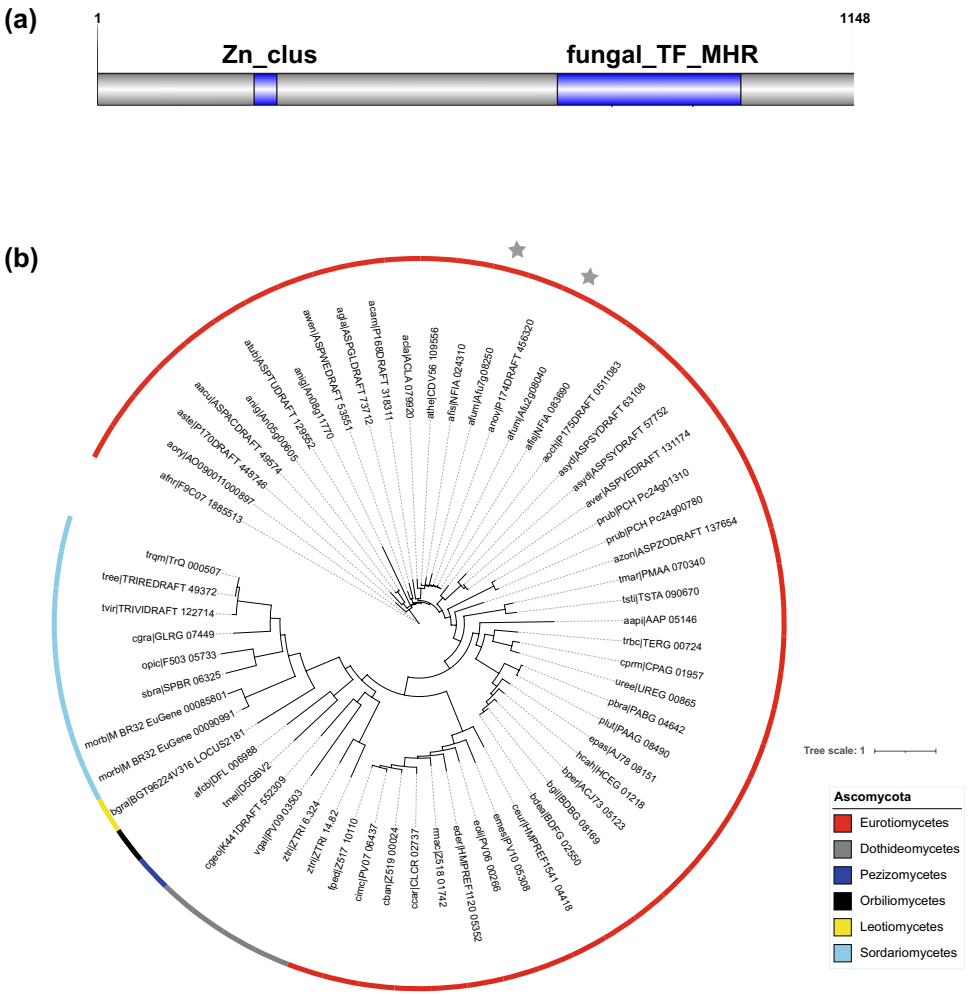

Fig. 2 Protein structure of ClcA and phylogenetic tree. (**a**) Protein structure of ClcA. ClcA harbors "Fungal Zn(2)-Cys(6) binuclear cluster domain" (PF00172) and "fungal transcription factor regulatory middle homology region" (cd12148). (**b**) Phylogenetic tree of 61 amino acid sequences among Ascomycota. Color strips along the tree correspond to taxonomy, i.e., Eurotiomycetes (red), Dothideomycetes (grey), Pezizomycetes (blue), Orbiliomycetes (black), Leotiomycetes (yellow), and Sordariomycetes (sky blue). *clcA* (Afu2g08040) and Afu7g08250 in *A. fumigatus* were marked as the star (grey)

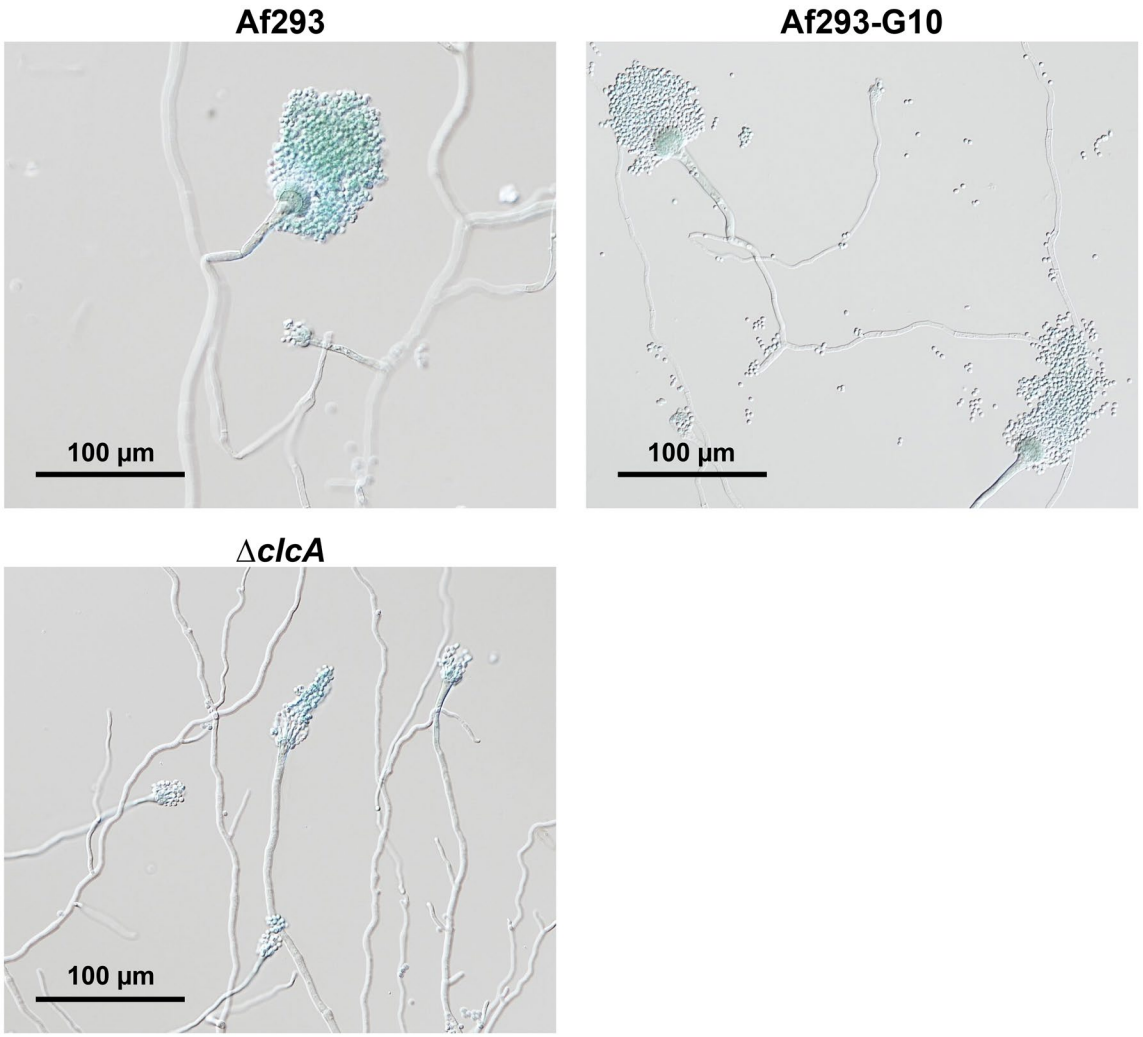

Fig. 3 Conidia formation in Af293-G10 and Δ*clcA*. Conidia were harvested after 5 days of incubation at 25 °C on a sliced AMM agar culture sandwiched between a slide glass and a covered glass. Conidiogenesis developments on a covered glass were observed

mfsB encoding MFS transporters. The expression of *mfsB* was markedly lower in the Δ*clcA* strain (Fig. 5a). These results suggest that the increased susceptibility to ITCZ in the Δ*clcA* strain may be caused by decreased expression of *mfsB*. ClcA could be required for the regulation of expressions of *cdr1B* and *mfsD* in addition to *mfsB*.

Besides, we measured the expression of *crpA* encoding Cu efflux transporter regulated by AceA (Wiemann et al. 2017; Anabosi et al. 2021). The expression of *crpA* was upregulated in Af293-G10, whereas the expression of *crpA* in the Δ*clcA* strain was comparable to that in Af293 (Fig. 5b). Interestingly, the L781F mutation of ClcA may lead to the upregulation of *crpA*.

ClcA is dispensable for the virulence of *A. fumigatus* in the silkworm infection model

Finally, we evaluated the virulence of Af293, Af293-G10, and Δ*clcA* strains by using the silkworm infection model. There were no differences in the survival rates of Af293-G10 and Δ*clcA* strains in comparison with that of Af293 (Fig. 6). These results suggested that *clcA* is dispensable for the virulence of *A. fumigauts*.

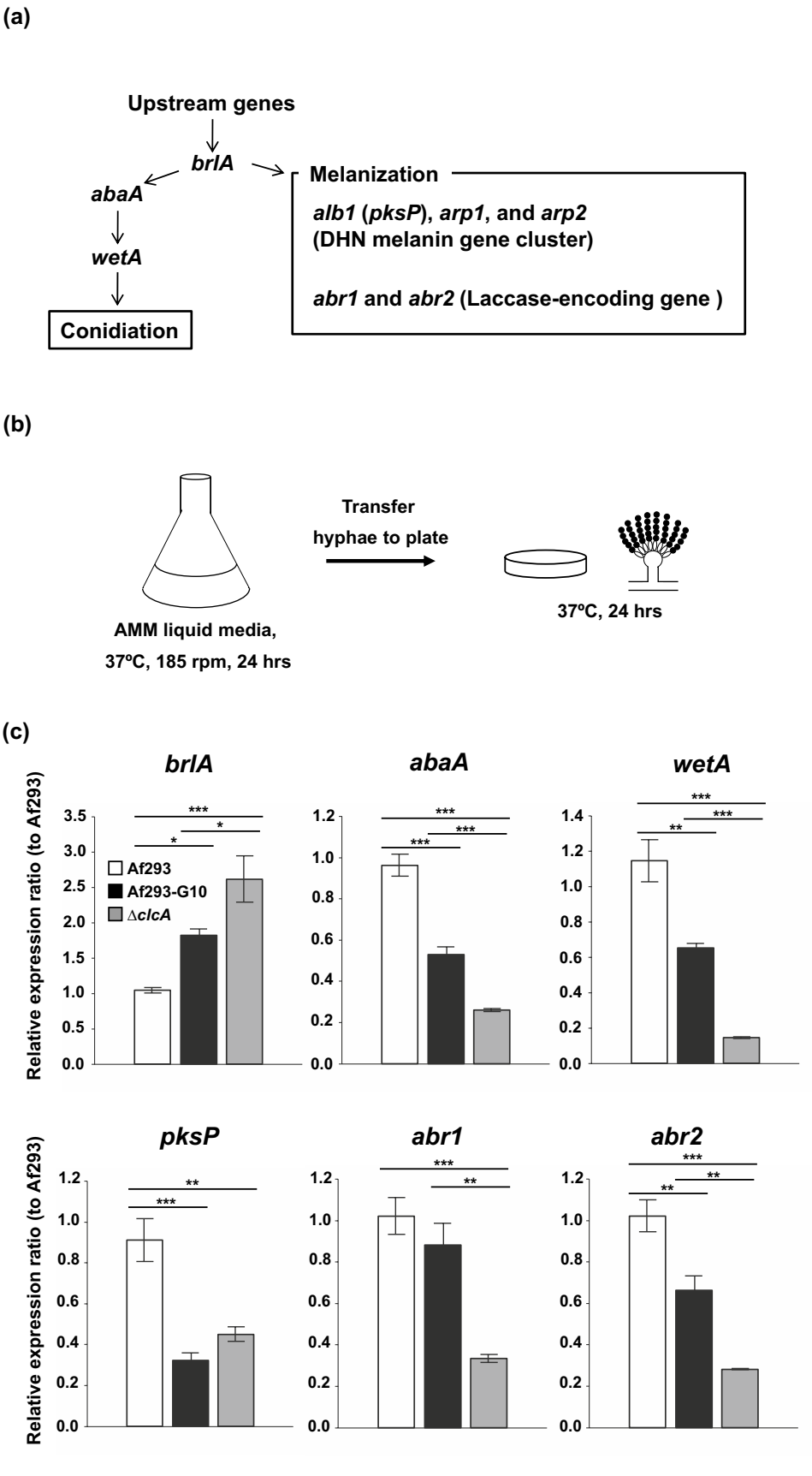

Fig. 4 Expression analysis in the genes involved in the BrlA pathway. (**a**) The transcriptional regulation of conidiation and melanization in *A. fumigatus*. BrlA regulates the expressions of *abaA* and melanization genes including the DHN-melanin gene cluster, i.e., *alb1* (*pksP*), *arp1*, and *arp2*, laccase-encoding genes, i.e., *abr1* and *abr2*. Following the activation of the expression of *wetA* by AbaA, the conidiation is induced. (**b**) Schematic diagram of the experimental procedure. Following cultivating the conidia of Af293, Af293-G10, and Δ*clcA* in AMM liquid media for 24 h at 37 °C, the collected hyphae were transferred to AMM and incubated for 24 h at 37 °C. Total RNA in the hyphae was extracted. (**c**) Quantitative real-time RT-PCR analyses of *brlA*, *abaA*, *wetA*, *pksP*, *abr1*, and *abr2*. All relative ratios to Af293 were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was performed using Tukey–Kramer test (**p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001)

Table 3 MICs of antifungals against Af293, Af293-G10, and Δ*clcA*

Strain	MIC (mg/L)							
	MCFG	CPFG	AMPH	5-FC	FLCZ	ITCZ	VRCZ	MCZ
Af293	< 0.015	0.25	1–2	64	> 64	1	1–2	8
Af293-G10	< 0.015	0.25	0.5–2	64	> 64	0.5–1	2	4–8
Δ*clcA*	< 0.015	0.25	0.5	64	> 64	0.25	2	4

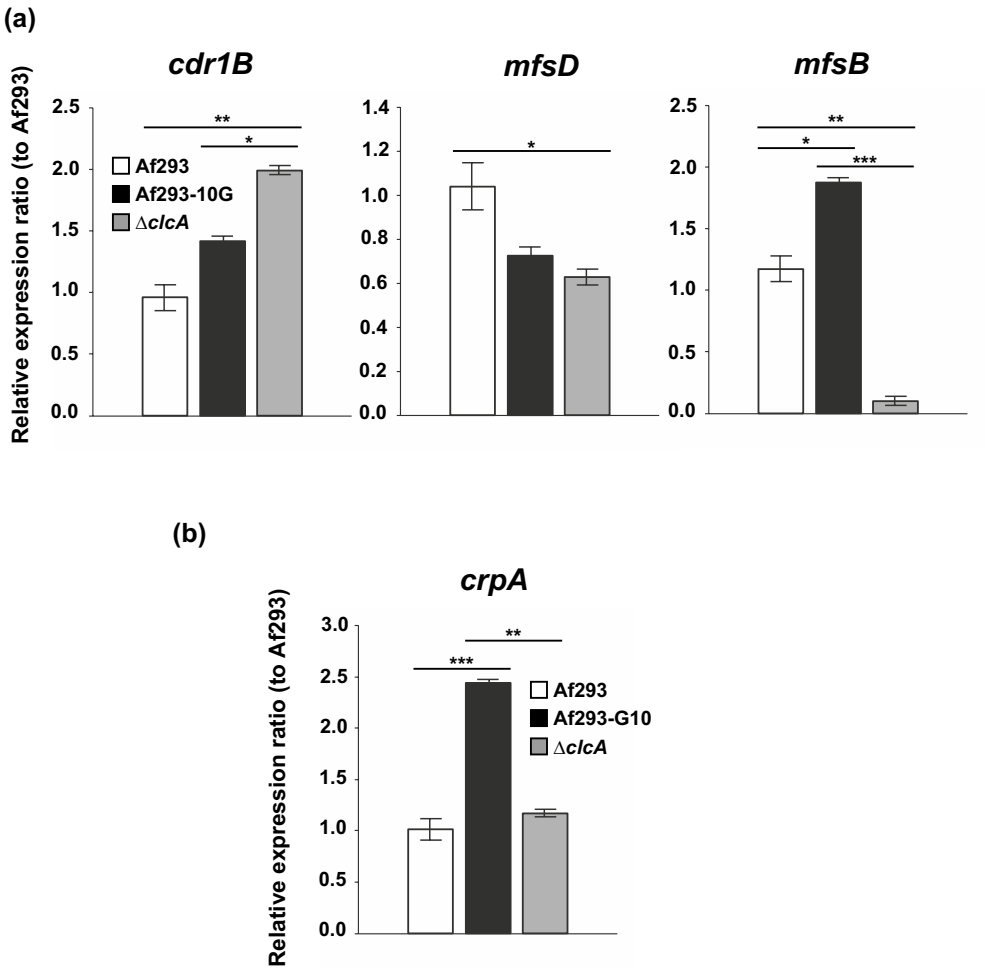

Fig. 5 Expression analysis in four transporters in Af293, Af293-G10, and Δ*clcA* strains. (**a**) The expressions of *cdr1B* encoding ABC transporter, *mfsD* and *mfsB* encoding MFS transporters were determined by quantitative real-time RT-PCR analyses. (**b**) The expression of *crpA* encoding Cu efflux transporter. Conidia were inoculated and cultivated in AMM liquid media at 37 °C for 24 h. The collected mycelia were transferred to AMM plates and incubated at 37 °C for 24 h. All relative ratios to harvested Af293 were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed using Tukey–Kramer test (**p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001)

Discussion

In the present study, we identified a novel Zn_2-Cys_6 transcription factor *clcA* that plays an important role in hyphal growth and conidiogeneis in *A. fumigatus*. Through the passage experiment, we generated Af293-G10 that exhibited greater growth in copper-repleted and -depleted conditions (Fig. 1). We found the mutation L781F of ClcA in Af293-G10, and investigated the physiological functions of *clcA* by using the Δ*clcA* strain. Our results indicate that *clcA* is involved in hyphal growth, conidiogenesis and copper-homeostasis, but dispensable for the virulence in *A. fumigatus*.

Among six fungal-specific TF families, the Zn_2-Cys_6 binuclear cluster domain family (zinc binuclear cluster) is the largest class of fungal-specific TFs (Shelest 2008; Todd and Andrianopoulos 1997). This family plays a

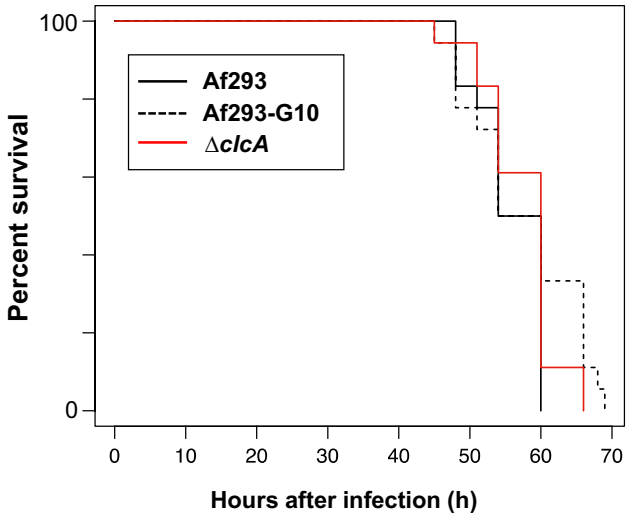

Fig. 6 Survival of silkworms after infection. Survival rates of silkworms inoculated with Af293 ($n=23$), Af293-G10 ($n=23$), and Δ*clcA* ($n=23$) strains were evaluated. We also made Mock (10 silkworms) as an uninfected group

role in a broad range of cell physiology including fungal-specific functions, such as secondary metabolite and mycotoxin production (Bok et al. 2006; Brakhage 2013), and azole resistance (Hagiwara et al. 2017). Valero et al. (2020) identified a Zn_2-Cys_6 binuclear cluster family AFUB_054000 involved in caspofungin resistance by screening a library of 484 *A. fumigatus* TF null mutants (Furukawa et al. 2020). Although 223 genes including *clcA* are annotated as "C6 transcription factor" in *A. fumigatus* Af293 in FungiDB (Amos et al. 2022), most of them have not yet been investigated. While we found that two paralogue genes *clcA* and Afu7g08250 exist in *A. fumigatus*, *A. niger* or *Penicillium rubens* have one or two *clcA* homologue (Fig. 2b), that is, the duplication of *clcA* varied among the species. This result suggests that the duplication of *clcA* may be involved in the physiological functions in *A. fumigatus*, possibly leading to the diversity of the Zn_2-Cys_6 binuclear cluster domain family.

The mutation L781F of *clcA* contributed to greater growth, although the disruption of *clcA* impaired the hyphal growth in AMM and copper-repleted conditions (Fig. 1a). The expression of *crpA* encoding Cu efflux transporter was upregulated in Af293-G10, but not the Δ*clcA* strain (Fig. 5b), suggesting that the upregulation of *crpA* may account for the increased fitness of Af293-G10 to copper-repleted conditions. The effect of $ClcA_{L781F}$ was not identical to the disruption of *clcA*, i.e., loss of function. Instead, $ClcA_{L781F}$ may have abnormal activity of gene regulation, supported by the upregulation of *crpA* (Fig. 5b) and the prediction of "Fungal Zn(2)-Cys(6) binuclear cluster domain" with AlphaFold 2.0 (Fig. S3). The mutation of *clcA* was reported in the clinical strains isolated from the same patient (Hagiwara et al. 2014). Interestingly, the mutation Y958C of *clcA* in IFM 59355-1 resided within "fungal transcription factor regulatory middle homology region" (cd12148) similarly to Af293-G10, suggesting that human lung could be copper abundant microenvironment, and the mutations of *clcA* could frequently occur in *A. fumigatus* upon exposure the environmental stresses, such as during infection. Besides the mutations of *cyp51A* and *hmg1* (Hagiwara et al. 2014, 2018a), the increased fitness through the mutation of *clcA* might contribute to long-term infection. It has been reported the altered expression of *clcA*, such as upregulation in conidia upon exposure to neutrophils and in hyphae upon exposure to heat stress (Sugui et al. 2008; Takahashi et al. 2017), indicating that *clcA* may participate in adaptation to environmental stresses.

In addition to the effect of *clcA* on hyphal growth, we observed the pigmentation and defective conidiogenesis in the Δ*clcA* strain (Figs. 1, 3, 4c and S4). The defective conidiogenesis could be accounted for lower expressions of *abaA* and *wetA* similarly to that in the Δ*Afmac1* strain (Kusuya et al. 2017). The disruption of *clcA* may affect the timing of expression of *brlA*, leading to the defective conidiogenesis. Although the gene expression pattern indicated that the L781F mutation of *clcA* may result in the defect in the conidiogenesis, it was unclear why Af293-G10 formed normal conidiogenesis even if lower

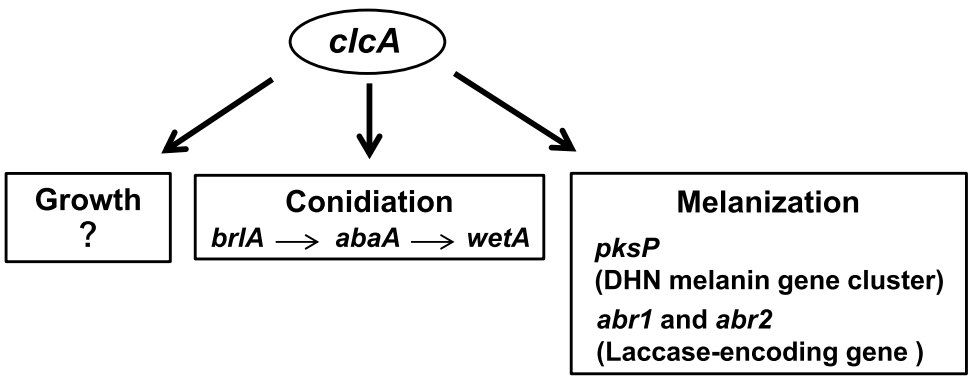

Fig. 7 Schematic diagram of proposed *clcA* regulatory mechanisms

expressions of *abaA* and *wetA*. It is conceivable that the conidiogenesis might be delayed in timing in Af293-G10. These results indicated that *clcA* plays an important role in the conidial maturation processes potentially as an activator (Fig. 7).

Although *clcA* was dispensable for the virulence in the silkworm infection model (Fig. 6), it has been reported that there were the cases that the virulence in the silkworm infection model could not reflect the virulence in mouse infections (Yu et al. 2021). Since Af293-G10 exhibited increased fitness to environmental copper stress (Fig. 1), the timeframe setting in silkworm infection model could be inappropriate, suggesting that *clcA* virulence might be underestimated.

The transporters are the causes of azole resistance in fungi (Lupetti et al. 2002; Fraczek et al. 2013; Paul and Moye-Rowley 2014; Hagiwara et al. 2017; Toyotome et al. 2021). The Δ*clcA* strain exhibited increased susceptibility to ITCZ and MCZ (Table 3). The expression of *mfsB* in the Δ*clcA* strain was significantly decreased, suggesting that *clcA* may be a regulator of *mfsB* and increased susceptibility to ITCZ in the Δ*clcA* strain may be caused by downregulation of *mfsB* (Fig. 5a). Besides, the expression of *cdr1B* was upregulated in the Δ*clcA* strain. It has been reported that TF AtrR directly regulates the expression of *cdr1B* (Hagiwara et al. 2017), indicating that *clcA* may cooperate with AtrR to regulate the expression of *cdr1B*.

Here we present the role of the Zn_2-Cys_6 transcription factor *clcA*, which contributes to a broad range of physiological functions in *A. fumigatus*. The environmental fitness of *A. fumigatus* is an important factor for virulence (Kowalski et al. 2016, 2019). Although the copper fitness of Af293-G10 was increased (Fig. 1a), Af293-G10 did not exhibit the hypervirulence phenotype (Fig. 6). Both genotypic and phenotypic heterogeneities are crucial for the virulence of *A. fumigatus* (Takahashi-Nakaguchi et al. 2015; Keller 2017; Hagiwara et al. 2018b; Ries et al. 2019; Barber et al. 2021). Further studies will be required to elucidate copper fitness, heterogeneities and virulence in *A. fumigatus*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00294-022-01250-2.

Acknowledgements We would like to thank Takashi Umeyama (National Institute of Infectious Diseases) for his fruitful discussion, Ryoko Mori and Getong Dao for experimental assistance.

Author contributions YK conceived the study, participated in designing and coordinating the study, and wrote the manuscript. DH coordinated the study and wrote the manuscript. CB and SB coordinated the study. HT initiated and supervised the project, and wrote the manuscript. All authors read and approved the final manuscript.

Funding This work has been partly supported by JST SPRING (JPMJSP2109 to CB), MEXT KAKENHI (Nos. 16K18671 and 21K07001 to HT, and 18K15144 to YK), and AMED (Nos. JP19fm0208024 and 22wm0325035 to HT and DH).

Availability of data The raw data have been submitted to DDBJ Short Read Archive under accession number PRJDB13659.

Declarations

Conflict of interest The authors declare no competing interests.

References

Achard ME, Stafford SL, Bokil NJ, Chartres J, Bernhardt PV, Schembri MA, Sweet MJ, McEwan AG (2012) Copper redistribution in murine macrophages in response to *Salmonella* infection. Biochem J 444:51–57. https://doi.org/10.1042/BJ20112180

Amos B, Aurrecoechea C, Barba M, Barreto A, Basenko EY, Bazant W, Belnap R, Blevins AS, Bohme U, Brestelli J, Brunk BP, Caddick M, Callan D, Campbell L, Christensen MB, Christophides GK, Crouch K, Davis K, DeBarry J, Doherty R, Duan Y, Dunn M, Falke D, Fisher S, Flicek P, Fox B, Gajria B, Giraldo-Calderon GI, Harb OS, Harper E, Hertz-Fowler C, Hickman MJ, Howington C, Hu S, Humphrey J, Iodice J, Jones A, Judkins J, Kelly SA, Kissinger JC, Kwon DK, Lamoureux K, Lawson D, Li W, Lies K, Lodha D, Long J, MacCallum RM, Maslen G, McDowell MA, Nabrzyski J, Roos DS, Rund SSC, Schulman SW, Shanmugasundram A, Sitnik V, Spruill D, Starns D, Stoeckert CJ, Tomko SS, Wang H, Warrenfeltz S, Wieck R, Wilkinson PA, Xu L, Zheng J (2022) VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center. Nucleic Acids Res 50:D898–D911. https://doi.org/10.1093/nar/gkab929

Anabosi D, Meir Z, Shadkchan Y, Handelman M, Abou-Kandil A, Yap A, Urlings D, Gold MS, Krappmann S, Haas H, Osherov N (2021) Transcriptional response of *Aspergillus fumigatus* to copper and the role of the Cu chaperones. Virulence 12:2186–2200. https://doi.org/10.1080/21505594.2021.1958057

Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM (2008) Metal ions in biological catalysis: from enzyme databases to general principles. J Biol Inorg Chem 13:1205–1218. https://doi.org/10.1007/s00775-008-0404-5

Barber AE, Sae-Ong T, Kang K, Seelbinder B, Li J, Walther G, Panagiotou G, Kurzai O (2021) *Aspergillus fumigatus* pan-genome analysis identifies genetic variants associated with human infection. Nat Microbiol 6:1526–1536. https://doi.org/10.1038/s41564-021-00993-x

Blatzer M, Latgé JP (2017) Metal-homeostasis in the pathobiology of the opportunistic human fungal pathogen *Aspergillus fumigatus*. Curr Opin Microbiol 40:152–159. https://doi.org/10.1016/j.mib.2017.11.015

Bodey G, Vartivarian S (1989) Aspergillosis. Eur J Clin Microbiol Infect Dis 8:413–437. https://doi.org/10.1007/BF01964057

Bok JW, Chung D, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Kirby KA, Keller NP (2006) GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. Infect Immun 74:6761–6768. https://doi.org/10.1128/IAI.00780-06

Brakhage AA (2013) Regulation of fungal secondary metabolism. Nat Rev Microbiol 11:21–32. https://doi.org/10.1038/nrmicro2916

Cai Z, Du W, Zeng Q, Long N, Dai C, Lu L (2017) Cu-sensing transcription factor Mac1 coordinates with the Ctr transporter family to regulate Cu acquisition and virulence in *Aspergillus fumigatus*.

Fungal Genet Biol 107:31–43. https://doi.org/10.1016/j.fgb.2017.08.003

Chen S, Zhou Y, Chen Y, Gu J (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34:i884–i890. https://doi.org/10.1093/bioinformatics/bty560

CLSI (2017) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. In: CLSI standard M38, 3rd edn. Clinical and Laboratory Standards Institute, Wayne, PA. https://clsi.org/media/1894/m38ed3_sample.pdf

DeLano WL (2002) Pymol: An open-source molecular graphics tool. http://www.ccp4.ac.uk/newsletters/newsletter40/11_pymol.pdf

Ding C, Festa RA, Chen YL, Espart A, Palacios Ò, Espín J, Capdevila M, Atrian S, Heitman J, Thiele DJ (2013) *Cryptococcus neoformans* copper detoxification machinery is critical for fungal virulence. Cell Host Microbe 13:265–276. https://doi.org/10.1016/j.chom.2013.02.002

Festa RA, Thiele DJ (2011) Copper: an essential metal in biology. Curr Biol 21:R877–R883. https://doi.org/10.1016/j.cub.2011.09.040

Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ (2018) Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science 360:739–742. https://doi.org/10.1126/science.aap7999

Fraczek MG, Bromley M, Buied A, Moore CB, Rajendran R, Rautemaa R, Ramage G, Denning DW, Bowyer P (2013) The *cdr1B* efflux transporter is associated with non-*cyp51a*-mediated itraconazole resistance in *Aspergillus fumigatus*. J Antimicrob Chemother 68:1486–1496. https://doi.org/10.1093/jac/dkt075

Furukawa T, van Rhijn N, Fraczek M, Gsaller F, Davies E, Carr P, Gago S, Fortune-Grant R, Rahman S, Gilsenan JM, Houlder E, Kowalski CH, Raj S, Paul S, Cook P, Parker JE, Kelly S, Cramer RA, Latgé JP, Moye-Rowley S, Bignell E, Bowyer P, Bromley MJ (2020) The negative cofactor 2 complex is a key regulator of drug resistance in *Aspergillus fumigatus*. Nat Commun 11:427. https://doi.org/10.1038/s41467-019-14191-1

Hagiwara D, Arai T, Takahashi H, Kusuya Y, Watanabe A, Kamei K (2018a) Non-*cyp51A* azole-resistant *Aspergillus fumigatus* isolates with mutation in HMG-CoA reductase. Emerg Infect Dis 24:1889–1897. https://doi.org/10.3201/eid2410.180730

Hagiwara D, Miura D, Shimizu K, Paul S, Ohba A, Gonoi T, Watanabe A, Kamei K, Shintani T, Moye-Rowley WS, Kawamoto S, Gomi K (2017) A novel Zn2-Cys6 transcription factor AtrR plays a key role in an azole resistance mechanism of *Aspergillus fumigatus* by co-regulating *cyp51A* and *cdr1B* expressions. PLoS Pathog 13:e1006096. https://doi.org/10.1371/journal.ppat.1006096

Hagiwara D, Takahashi H, Takagi H, Watanabe A, Kamei K (2018b) Heterogeneity in pathogenicity-related properties and stress tolerance in *Aspergillus fumigatus* clinical isolates. Med Mycol J 59:E63–E70. https://doi.org/10.3314/mmj.18-00007

Hagiwara D, Takahashi H, Watanabe A, Takahashi-Nakaguchi A, Kawamoto S, Kamei K, Gonoi T (2014) Whole-genome comparison of *Aspergillus fumigatus* strains serially isolated from patients with aspergillosis. J Clin Microbiol 52:4202–4209. https://doi.org/10.1128/JCM.01105-14

Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Zidek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D (2021) Highly accurate protein structure prediction with AlphaFold. Nature 596:583–589. https://doi.org/10.1038/s41586-021-03819-2

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066. https://doi.org/10.1093/nar/gkf436

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780. https://doi.org/10.1093/molbev/mst010

Keller NP (2017) Heterogeneity confounds establishment of "a" model microbial strain. mBio 8:e00135-17. https://doi.org/10.1128/mBio.00135-17

Kikuchi K, Watanabe A, Ito J, Oku Y, Wuren T, Taguchi H, Yarita K, Muraosa Y, Yahiro M, Yaguchi T, Kamei K (2014) Antifungal susceptibility of *Aspergillus fumigatus* clinical isolates collected from various areas in Japan. J Infect Chemother 20:336–338. https://doi.org/10.1016/j.jiac.2014.01.003

Kowalski CH, Beattie SR, Fuller KK, McGurk EA, Tang YW, Hohl TM, Obar JJ, Cramer RA Jr (2016) Heterogeneity among isolates reveals that fitness in low oxygen correlates with *Aspergillus fumigatus* virulence. mBio 7:e01515-16. https://doi.org/10.1128/mBio.01515-16

Kowalski CH, Kerkaert JD, Liu KW, Bond MC, Hartmann R, Nadell CD, Stajich JE, Cramer RA (2019) Fungal biofilm morphology impacts hypoxia fitness and disease progression. Nat Microbiol 4:2430–2441. https://doi.org/10.1038/s41564-019-0558-7

Krappmann S, Sasse C, Braus GH (2006) Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end-joining-deficient genetic background. Eukaryot Cell 5:212–215. https://doi.org/10.1128/EC.5.1.212-215.2006

Kusuya Y, Hagiwara D, Sakai K, Yaguchi T, Gonoi T, Takahashi H (2017) Transcription factor *Afmac1* controls copper import machinery in *Aspergillus fumigatus*. Curr Genet 63:777–789. https://doi.org/10.1007/s00294-017-0681-z

Latgé JP, Chamilos G (2019) *Aspergillus fumigatus* and aspergillosis in 2019. Clin Microbiol Rev 33:e00140-e218. https://doi.org/10.1128/CMR.00140-18

Letunic I, Bork P (2019) Interactive tree of life (iTOL) v4: recent updates and new developments. Nucleic Acids Res 47:W256–W259. https://doi.org/10.1093/nar/gkz239

Li H (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv. https://doi.org/10.48550/arXiv.1303.3997

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R (2009) The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Liu W, Xie Y, Ma J, Luo X, Nie P, Zuo Z, Lahrmann U, Zhao Q, Zheng Y, Zhao Y, Xue Y, Ren J (2015) IBS: an illustrator for the presentation and visualization of biological sequences. Bioinformatics 31:3359–3361. https://doi.org/10.1093/bioinformatics/btv362

Lupetti A, Danesi R, Campa M, Del Tacca M, Kelly S (2002) Molecular basis of resistance to azole antifungals. Trends Mol Med 8:76–81. https://doi.org/10.1016/s1471-4914(02)02280-3

Majima H, Arai T, Kusuya Y, Takahashi H, Watanabe A, Miyazaki Y, Kamei K (2021) Genetic differences between Japan and other countries in *cyp51A* polymorphisms of *Aspergillus fumigatus*. Mycoses 64:1354–1365. https://doi.org/10.1111/myc.13370

Park YS, Lian H, Chang M, Kang CM, Yun CW (2014) Identification of high-affinity copper transporters in *Aspergillus fumigatus*. Fungal Genet Biol 73:29–38. https://doi.org/10.1016/j.fgb.2014.09.008

Park YS, Kang S, Seo H, Yun CW (2018) A copper transcription factor, AfMac1, regulates both iron and copper homeostasis in the opportunistic fungal pathogen *Aspergillus fumigatus*. Biochem J 475:2831–2845. https://doi.org/10.1042/BCJ20180399

Paul S, Moye-Rowley WS (2014) Multidrug resistance in fungi: regulation of transporter-encoding gene expression. Front Physiol 5:143. https://doi.org/10.3389/fphys.2014.00143

Raffa N, Osherov N, Keller NP (2019) Copper utilization, regulation, and acquisition by *Aspergillus fumigatus*. Int J Mol Sci 20:1980. https://doi.org/10.3390/ijms20081980

Ries LNA, Steenwyk JL, de Castro PA, de Lima PBA, Almeida F, de Assis LJ, Manfiolli AO, Takahashi-Nakaguchi A, Kusuya Y, Hagiwara D, Takahashi H, Wang X, Obar JJ, Rokas A, Goldman GH (2019) Nutritional heterogeneity among *Aspergillus fumigatus* strains has consequences for virulence in a strain- and host-dependent manner. Front Microbiol 10:854. https://doi.org/10.3389/fmicb.2019.00854

Robinson NJ, Winge DR (2010) Copper metallochaperones. Annu Rev Biochem 79:537–562. https://doi.org/10.1146/annurev-biochem-030409-143539

Shelest E (2008) Transcription factors in fungi. FEMS Microbiol Lett 286:145–151. https://doi.org/10.1111/j.1574-6968.2008.01293.x

Smith AD, Logeman BL, Thiele DJ (2017) Copper acquisition and utilization in fungi. Annu Rev Microbiol 71:597–623. https://doi.org/10.1146/annurev-micro-030117-020444

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Sugui JA, Kim HS, Zarember KA, Chang YC, Gallin JI, Nierman WC, Kwon-Chung KJ (2008) Genes differentially expressed in conidia and hyphae of *Aspergillus fumigatus* upon exposure to human neutrophils. PLoS ONE 3:e2655. https://doi.org/10.1371/journal.pone.0002655

Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR (2006) Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat Protoc 1:3111–3120. https://doi.org/10.1038/nprot.2006.405

Takahashi H, Kusuya Y, Hagiwara D, Takahashi-Nakaguchi A, Sakai K, Gonoi T (2017) Global gene expression reveals stress-responsive genes in *Aspergillus fumigatus* mycelia. BMC Genomics 18:942. https://doi.org/10.1186/s12864-017-4316-z

Takahashi-Nakaguchi A, Muraosa Y, Hagiwara D, Sakai K, Toyotome T, Watanabe A, Kawamoto S, Kamei K, Gonoi T, Takahashi H (2015) Genome sequence comparison of *Aspergillus fumigatus* strains isolated from patients with pulmonary aspergilloma and chronic necrotizing pulmonary aspergillosis. Med Mycol 53:353–360. https://doi.org/10.1093/mmy/myv003

Todd RB, Andrianopoulos A (1997) Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA binding motif. Fungal Genet Biol 21:388–405. https://doi.org/10.1006/fgbi.1997.0993

Toyotome T, Hagiwara D, Takahashi H, Watanabe A, Kamei K (2018) Emerging antifungal drug resistance in *Aspergillus fumigatus* and among other species of *Aspergillus*. Curr Fungal Inf Rep 12:105–111. https://doi.org/10.1007/s12281-018-0318-9

Toyotome T, Onishi K, Sato M, Kusuya Y, Hagiwara D, Watanabe A, Takahashi H (2021) Identification of novel mutations contributing to azole tolerance of *Aspergillus fumigatus* through in vitro exposure to tebuconazole. Antimicrob Agents Chemother 65:e0265720. https://doi.org/10.1128/AAC.02657-20

Umeyama T, Hayashi Y, Shimosaka H, Inukai T, Yamagoe S, Takatsuka S, Hoshino Y, Nagi M, Nakamura S, Kamei K, Ogawa K, Miyazaki Y (2018) CRISPR/Cas9 genome editing to demonstrate the contribution of Cyp51A Gly138Ser to azole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 62:e00894-e918. https://doi.org/10.1128/AAC.00894-18

Valero C, Colabardini AC, Chiaratto J, Pardeshi L, de Castro PA, Ferreira Filho JA, Silva LP, Rocha MC, Malavazi I, Costa JH, Fill T, Barros MH, Wong SSW, Aimanianda V, Wong KH, Goldman GH (2020) *Aspergillus fumigatus* transcription factors involved in the caspofungin paradoxical effect. mBio 11:e00816–20. https://doi.org/10.1128/mBio.00816-20

White C, Lee J, Kambe T, Fritsche K, Petris MJ (2009) A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. J Biol Chem 284:33949–33956. https://doi.org/10.1074/jbc.M109.070201

Wiemann P, Perevitsky A, Lim FY, Shadkchan Y, Knox BP, Landero Figueora JA, Choera T, Niu M, Steinberger AJ, Wuthrich M, Idol RA, Klein BS, Dinauer MC, Huttenlocher A, Osherov N, Keller NP (2017) *Aspergillus fumigatus* copper export machinery and reactive oxygen intermediate defense counter host copper-mediated oxidative antimicrobial offense. Cell Rep 19:1008–1021. https://doi.org/10.1016/j.celrep.2017.04.019

Yu Y, Wolf AK, Thusek S, Heinekamp T, Bromley M, Krappmann S, Terpitz U, Voigt K, Brakhage AA, Beilhack A (2021) Direct visualization of fungal burden in filamentous fungus-infected silkworms. J Fungi (basel) 7:136. https://doi.org/10.3390/jof7020136