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Laccases Involved in 1,8-Dihydroxynaphthalene Melanin Biosynthesis in *Aspergillus fumigatus* Are Regulated by Developmental Factors and Copper Homeostasis

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Aspergillus fumigatus **produces heavily melanized infectious conidia. The conidial melanin is associated with fungal virulence and resistance to various environmental stresses. This 1,8-dihydroxynaphthalene (DHN) melanin is synthesized by enzymes encoded in a gene cluster in *A. fumigatus*, including two laccases, Abr1 and Abr2. Although this gene cluster is not conserved in all aspergilli, laccases are critical for melanization in all species examined. Here we show that the expression of *A. fumigatus* laccases Abr1/2 is upregulated upon hyphal competency and drastically increased during conidiation. The Abr1 protein is localized at the surface of stalks and conidiophores, but not in young hyphae, consistent with the gene expression pattern and its predicted role. The induction of Abr1/2 upon hyphal competency is controlled by BrlA, the master regulator of conidiophore development, and is responsive to the copper level in the medium. We identified a developmentally regulated putative copper transporter, CtpA, and found that CtpA is critical for conidial melanization under copper-limiting conditions. Accordingly, disruption of CtpA enhanced the induction of *abr1* and *abr2*, a response similar to that induced by copper starvation. Furthermore, nonpigmented *ctpA*Δ conidia elicited much stronger immune responses from the infected invertebrate host *Galleria mellonella* than the pigmented *ctpA*Δ or wild-type conidia. Such enhancement in eliciting *Galleria* immune responses was independent of the *ctpA*Δ conidial viability, as previously observed for the DHN melanin mutants. Taken together, our findings indicate that both copper homeostasis and developmental regulators control melanin biosynthesis, which affects conidial surface properties that shape the interaction between this pathogen and its host.**

Aspergillus fumigatus is an opportunistic pathogen that causes life-threatening invasive disease in immunocompromised hosts (1). Its asexual spores, named conidia, are the initial source of inocula for infection and are the vehicles for dispersal and survival. Under favorable conditions, conidia break dormancy and grow as elongated, highly polarized hyaline hyphae. Upon achieving competency and exposure to air, hyphae produce aerial conidiophore stalks and develop elaborate multicellular conidiophores that generate chains of melanized uninucleate conidia (2, 3) (Fig. 1A and B). BrlA, a C_2H_2 zinc finger transcription factor, is the master regulator that controls the initiation of conidiophore development in *Aspergillus* (4, 5). AbaA and WetA, two other important regulators that function downstream of BrlA, are required for proper progression of conidiation (6). The *abaA* gene is activated during the middle stage of conidiophore development, while the *wetA* gene is required for the activation of late conidiation-specific genes.

The 1,8-dihydroxynaphthalene (DHN) melanin coating *A. fumigatus* conidia gives them their characteristic bluish green color (Fig. 1B). Melanin is an amorphous polymer that is produced by a variety of microbes and higher eukaryotes. Melanin not only helps fungi to fend off various environmental insults (e.g., UV irradiation) but also helps the survival in hosts of different animal and plant pathogens such as *A. fumigatus*, *Botrytis cinerea*, *Cryptococcus neoformans*, *Exophilia dermatitidis*, and *Magnaporthe grisea* (7–13). Melanin is required for full virulence in *Cryptococcus*, and melanized *Cryptococcus* cells are found in infected host tissues (8, 14–17). Many phytopathogenic fungi, such as *Colletotrichum lagenarium*, *Colletotrichum lindemuthianum*, and *Magnaporthe grisea*, require melanized appressoria to penetrate host cells (18–20).

The DHN melanin and the L-3,4-dihydroxyphenylalanine (L-dopa) melanin are the two major types of melanin synthesized by fungi. The DHN melanin is synthesized *de novo* using endogenous substrates through the DHN intermediate. The final product is produced by a series of enzymatic reactions involving a polyketide synthase and other enzymes such as scytalone dehydratase, hydroxynaphthalene reductase, and laccases (21, 22). The genes encoding these enzymes are organized in a cluster in some fungi, such as *A. fumigatus*, *Aspergillus niger*, *Aspergillus clavatus*, and *Cochliobolus heterostrophus* (22, 23). However, such clustered organization of melanin biosynthetic genes is not conserved even among some close relatives such as *Aspergillus flavus*, *Aspergillus nidulans*, and *Aspergillus terreus* (21, 24). In contrast, the L-dopa melanin is synthesized by laccases, and its production requires exogenously supplied phenolic precursors such as L-dopa (15, 16, 21). L-Dopa melanin is the well characterized in *C. neoformans*, and the deletion of the laccase gene attenuates cryptococcal virulence in animal models (8, 17).

Regardless of the organisms or the type of melanin (DHN or L-dopa), laccases are the required and conserved factors for melanin biosynthesis. Although laccases have been among the earliest and most intensively studied enzymes, factors that regulate their activities are not defined in the vast majority of microbes. What is clear is that melanization is associated with development in some

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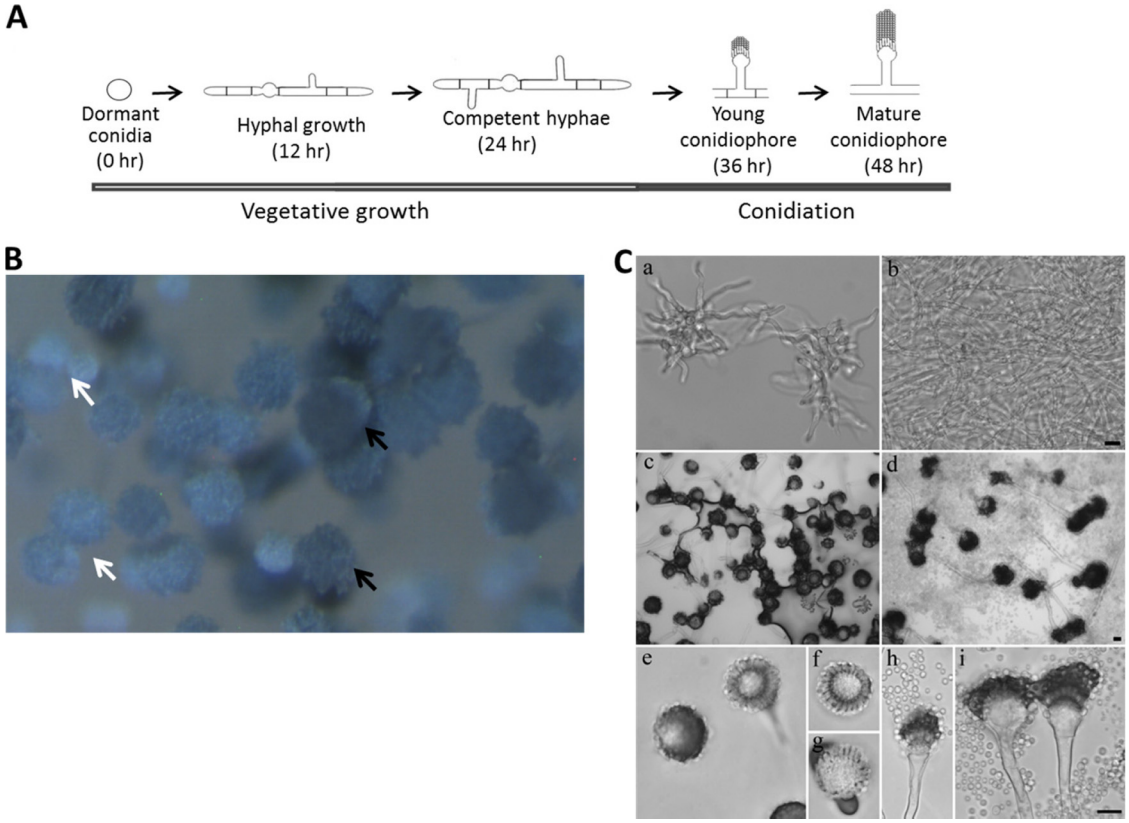


FIG 1 Different developmental stages of *A. fumigatus*. (A) Asexual reproductive life cycle of *A. fumigatus*. (B) Melanization increases with conidial maturation. Black arrows point to mature conidiophores with a darker color, and white arrows point to young conidiophores with a lighter color. (C) *A. fumigatus* of different developmental stages collected at the following time points: 12 h (vegetative hyphae) (a), 24 h (competent hyphae) (b), 36 h (early conidiophores) (c), and 48 h (conidiophores with mature conidia) (d). (e, f, and g) Thirty-six-hour sample at higher magnification. (h and i) Forty-eight-hour sample at higher magnification. Scale bars, 10 μm (b and d) and 5 μm (i); each scale bar shown applies to all panels within the same row of images.

filamentous fungi. For instance, in the model filamentous fungus *A. nidulans*, the laccase level drastically increases during conidiation (25). Consistently, the expression of the *wA* gene encoding a polyketide synthase and that of the *yA* gene encoding a *p*-diphenol oxidase in *A. nidulans* are both upregulated during conidiation (22, 26). Similarly, in *A. fumigatus*, the DHN gene cluster, including the laccase-encoding *abr1* and the *abr2* genes, is expressed during conidiation but not in vegetative hyphae (22, 27–29). However, the role of the central regulators of conidiation (BrlA, AbaA, and WetA) in melanization is still unclear.

The importance of copper in melanization has been highlighted in a few studies; this importance is likely due to the requirement of copper as a cofactor for laccases (30, 31). In *A. nidulans*, a mutation in the *ygA* gene that encodes a transporter produces yellow conidia at high pH and green conidia at low pH (32, 33). The defect in melanization of the *ygA* mutant is likely caused by a deficiency in copper uptake, as the defect can be remediated by the addition of copper (32). Similarly, in *Colletotrichum lindemuthianum*, the disruption of the copper transporter *clap1* renders the strain nonpathogenic and causes poor pigmentation in the spores. Such defects, again, can be remediated by the addition of copper (34). In humans, the copper transporter ATP7A appears to be critical for melanogenesis as well (35). In *A. fumigatus*, the effect of copper on laccases Abr1 and Abr2 is not known, and the identity of the copper transporter needed for the DHN melanin biosynthesis has yet to be revealed.

In this study, we examined the expression of the laccase-encoding genes *abr1* and *abr2* during different development stages and corroborated the findings with the temporal and spatial protein expression pattern of Abr1. We found that *brlA* is essential for turning on the expression of both laccases during conidiation at earlier stages, while *abaA* and *wetA* likely play a role in turning off their expression during later stages of conidiation. We also identified a copper transporter gene, *ctpA*, and demonstrated its importance in the conidial melanization under copper-limiting conditions. We further demonstrated that a melanin-defective *cptA*Δ mutant, like DHN melanin gene deletion mutants, elicits enhanced immune responses from *Galleria mellonella* independent of spore viability.

MATERIALS AND METHODS

Strains and media. *A. fumigatus* strains, plasmids, and primers used in this study are shown in Tables S1 and S2 in the supplemental material. Strains were grown on *Aspergillus* complete medium (CM) and minimal medium (MM) (36) or yeast nitrogen base medium (YNB) with appropriate supplements at 30°C in the dark for 3 days as indicated below.

Generation of GFP-tagged *abr1* and fluorescence microscopy. To generate the P_{abr1}-*abr1*::e*GFP* fusion construct, a 5.3-kb fragment that contains 2,879 bp upstream of *abr1* along with its full coding sequence except the stop codon was amplified with primers abr1GFPF1 and abr1GFPR1. Similarly, a 2,690-bp GA5-eGFP-AfpyrG fragment was amplified from pFN03 (37) using the primers abr1GFPF2 and GFP Reverse. The two amplified fragments were then fused by overlap PCR to create a

5.4-kb construct, P_{abr1}-*abr1*::e*GFP*, using the primers abr1nesF and GFP-nesR. The resulting construct of the fluorescence fusion Abr1 protein under the control of its native promoter was then transformed into the *abr1*Δ mutant to examine its ability to restore conidial pigmentation. The standard protoplasting method was used for transformation as previously described (38). The same P_{abr1}-*abr1*::e*GFP* construct was also used to transform strains CEA17 and Δabr1.2. Transformants were screened for the presence of *abr1*::e*GFP* by PCR and microscopic observations. Further, images were acquired and processed with a Zeiss Axioplan 2 imaging system with AxioCam MRm camera software (Carl Zeiss Microscopy). Green fluorescent protein (GFP) was visualized using the filter FL filter set 38 HE EGFP (Carl Zeiss Microscopy). Samples were prepared and microscopic observation was performed as previously described (39).

Identification of *ctpA* in *A. fumigatus*. The *A. fumigatus* ortholog of the *A. nidulans* copper transporter *ygA* was identified by reciprocal BLASTP searches against the *Aspergillus* genomic database hosted by the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/Blast.html). The identified *A. fumigatus* homolog, Afu4g12620, was named *ctpA* (copper transporter). The subcellular localization of the encoded protein was predicted using the WoLF PSORT program (http://www.genscript.com/psort/wolf_psort.html). The transmembrane helices and the protein structure of CtpA were predicted using the Tmpred program (http://embnet.vital-it.ch/software/TMPRED_form.html) and the I-Tasser program (http://zhanglab.ccmb.med.umich.edu/I-TASSER) (40), respectively.

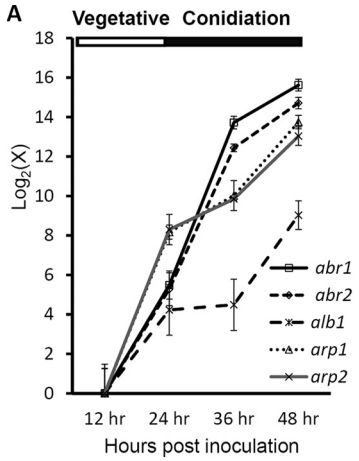
Generation of the *ctpA*Δ strain. The *ctpA*Δ deletion construct was generated using the fusion PCR protocol as previously described (37). Briefly, a 1,137-bp fragment upstream of the *ctpA* open reading frame (ORF) was amplified by PCR using primers ctpAF1 and ctpAR1. A 1,175-bp fragment downstream of the *ctpA* open reading frame (ORF) was amplified using primers ctpAF2 and ctpAR2. The 2,232-bp hygromycin marker fragment was amplified from plasmid pBHt2 (41) using the HYG-construct-F and HYG-construct-R2 primer set. These two PCR products of the *ctpA* ORF flanking sequences were then fused to the hygromycin marker using the nested primers ctpAnesF and ctpAnesR. This resulted in the final *ctpA* deletion construct of 4,418 bp. The *ctpA* deletion construct was then transformed into strain CEA17 through the standard protoplasting method as previously described (38). Transformants were selected for resistance to hygromycin. The deletion of the *ctpA* gene in selected transformants was verified by PCR and restriction enzyme digestions. The single integration event of the deletion construct at the *ctpA* locus through homologous recombination in the knockout candidates was further verified by Southern blotting analyses (see Fig. S6 in the supplemental material). For Southern blot analyses, genomic DNA from these candidates was isolated using standard methods (42) and then digested with appropriate restriction enzymes. The restriction enzymes selected recognize one digestion site in the deletion construct. The probe was amplified using primers LB-UI and AI77, and the amplified probe spanned the coding sequence of the *hph* gene. The probe was labeled with ^{32}P using the Prime-It random primer labeling kit (Stratagene, Santa Clara, CA) according to the manufacturer's instructions.

Complementation of the *ctpA*Δ mutant. To complement the defect of the *ctpA*Δ mutant, we constructed the wild-type copy of the *ctpA* gene with its native promoter fused with the GFP gene P_{ctpA}-*ctpA*::e*GFP* by fusion PCR. Briefly, a 4.2-kb fragment that contains 675 bp upstream of *ctpA* along with its full coding sequence except the stop codon was amplified with primers ctpAGFPF1 and ctpAGFPR1. Similarly, a 2,663-bp GA5-eGFP-AfpyrG fragment was amplified from pFN03 (37) using primers ctpAGFPF2 and GFP Reverse. The two amplified fragments were then fused by overlap PCR using the primers ctpAnesF and GFPnesR. The P_{ctpA}-*ctpA*::e*GFP* construct was then transformed into the *ctpA*Δ mutant. Transformants were tested for the presence of both Δ*ctpA*::*hph* and *ctpA*::e*GFP* and examined for conidial melanization under copper-limiting conditions.

Generation of the *ctpA* overexpression strains. To generate the *ctpA* overexpression construct, the *ctpA* open reading frame without the stop codon was amplified using the primer set ctpAoveF and ctpAoveR. The forward primer was designed with an overhang that contains an FseI restriction enzyme digestion site. The reverse primer was designed with an overhang that contains a PacI digestion site. The PCR amplicons were digested with PacI and FseI, and the digested products were then ligated into the overexpression vector pBCtef 27 to generate plasmid pBCtefctp. Plasmid pBCtef 27 carries the hygromycin resistance marker and also the promoter region (~1 kb) and the terminator region (582 bp) of the translation elongation factor A gene (*tefA*). The P_{tefA} and the T_{tefA} regions were fused together through overlap PCR with an *spe1* overhang at the junction of the fused product. Plasmid PBCtef 27 was generated by digesting the fused PCR amplicons with *spe1* and then ligating it into the vector pBCHygro at the *spe1* site.

Phenotypic assays. For phenotypic characterizations, 1×10^4 conidia of the wild type, the gene deletion mutant, the complemented strain, and the gene overexpression strain were used. The copper-limiting condition was generated by the addition of the copper chelator bathocuproine disulfonate (BCS; 150 μM) to the media, while the copper-replete condition was generated by the addition of $CuSO_4$ (500 μM) to the media. We also tested the effect of iron by adding bipyrimidine (50 μM), the iron chelator bathophenanthroline disulfonic acid (BPS; 100 μM), and $FeCl_3$ (500 μM) to the growth media. Strains were also tested for their sensitivity to oxidative stresses using hydrogen peroxide (6 mM) and $NaNO_2$ (25 mM) and to osmotic stress using NaCl (0.6 M), KCl (0.6 M), KOH (25 mM), sucrose (1 M), and sorbitol (1 M). Strains were also tested for their resistance to the antifungal drug caspofungin (10 μg/ml) and the detergent SDS (50 μg/ml).

Gene expression studies using quantitative real-time PCR. For gene expression studies, experiments were conducted and samples were collected as previously described (39). The wild-type strain and the *ctpA*Δ mutant were grown in 100 ml of complete liquid medium with 1.2 M sorbitol at 30°C with or without the addition of BCS or $CuSO_4$. Five-milliliter samples of fungal tissues were collected at 12 h and 24 h after conidial inoculation and were labeled as samples from these time points (Fig. 1C). At 24 h postinoculation, 25-ml samples of the cultures were filtered by vacuum through Whatman filter paper no. 2. The fungal mycelial mats were then transferred to solid media and incubated at 30°C for an additional 12 h or 24 h (Fig. 1C; see also Fig. S1 in the supplemental material). These samples were labeled as samples from the 36-h or 48-h time point, respectively. For the *brlA*Δ, *abaA*Δ, and *wetA*Δ mutant samples, only samples from the 24-h, 36-h, and 48-h time points were collected. These mutant strains were grown for 12 h in complete medium at 30°C, then transferred to complete medium with 1.2 M sorbitol, and incubated for another 12 h. Samples were collected at this time point and labeled as being from the 24-h time point. The rest of the experiment and the collection of the mutant samples from the 36-h and 48-h time points were carried out as described above for the wild type and the *ctpA*Δ mutant strains. Three biologically independent experiments were performed. Total RNA from collected samples was extracted using an RNA purification kit (Invitrogen, CA) by following the instructions from the manufacturer. The RNA samples were treated with TURBO DNA FREE (Invitrogen) to remove any contamination from genomic DNA. The integrity of the RNA samples was examined by gel electrophoresis at multiple steps. The lack of genomic DNA contamination of DNase-treated RNA samples was verified by PCR. The first-strand cDNA synthesis was carried out using a SuperScript III kit (Invitrogen) according to the manufacturer's instructions. KAPA SYBR FAST qPCR master mix was used for the preparation for real-time PCR, and the reaction was performed in an Eppendorf RealPlex 2 machine according to the manufacturer's instructions (KAPA Biosystems). Gene expression levels were normalized based on the *tefA* expression level of the same samples. Primers ctpArtpcrF and ctpArtpcrR were used for *ctpA*, primers crtArtpcrF and crtArtpcrR were used for *crtA*, primers abr1rtpcrF and abr1rtpcrR were used for



B

Strains	Genes	24 hr	36 hr	48 hr
WT	*alb1*	1.0 ± 1.0	1.6 ± 1.1	28.9 ± 8.6
	arp1	1.0 ± 0.3	4.0 ± 1.9	49.0 ± 11.6
	arp2	1.0 ± 0.5	2.9 ± 0.2	27.7 ± 9.2
	abr1	1.0 ± 2.7	307.6 ± 69.5	1242.1± 25.6
	abr2	1.0 ± 0.5	144.8 ± 20.7	698.9 ± 134.3
brlAΔ	*alb1*	0.8 ± 0.4	2.2 ± 0.8	4.3 ± 3.6
	arp1	1.4 ± 0.4	0. 6 ± 0.1	1.2 ± 0.5
	arp2	0.5 ± 0.2	0.2 ± 0.03	0.2 ± 0.1
	abr1	0.5 ± 0.1	0.6 ± 0.3	1.8 ± 0.5
	abr2	1.5 ± 0.2	2.2 ± 1.3	6.6 ± 3.3
abaAΔ	*alb1*	2.5 ± 0.4	5.7 ± 2.3	10.0 ± 7.1
	arp1	141.0 ± 30.9	73.0 ± 16.0	33.1 ± 18. 9
	arp2	13.1 ± 5.2	20.1 ± 11.9	40.8 ± 24.6
	abr1	6566.7 ± 2066.7	1082.7 ± 131.1	695.4 ± 202.8
	abr2	962.2 ± 360.7	352.2 ± 24.9	202.4 ± 82.0
wetAΔ	*alb1*	13.0 ± 1.9	46.5 ± 34.9	107.4 ± 45.3
	arp1	91.4 ± 30.1	70.0 ± 41.6	81.2 ± 49.2
	arp2	42.1 ± 21.0	139.8 ± 52.8	90.4 ± 31.2
	abr1	2477.1 ± 1291.1	2171.7 ± 192.2	1587.7 ± 183.4
	abr2	914.9 ± 484.1	638.7 ± 357.5	461.8 ± 136.9

FIG 2 The expression of *abr1* and *abr2* and other melanin genes is developmentally regulated and subject to the control of master developmental regulators. (A) Expression of *alb1*, *arp1*, *arp2*, *abr1*, and *abr2* during development. All gene expression levels were normalized based on the *tefA* expression levels. The 12-h time point was used as the reference for the subsequent time points. (B) Melanin gene expression levels in the wild type and the *brlA*Δ, *abaA*Δ, and *wetA*Δ mutants. All gene expression levels were normalized based on the *tefA* expression levels. The expression level of each gene at 24 h was used as the reference for the subsequent time points (fold changes with standard deviations are shown).

abr1, primers abr2rtpcrF and abr2rtpcrR were used for *abr2*, primers alb1rtpcrF and alb1rtpcrR were used for *alb1*, primers arp1rtpcrF and arp1rtpcrR were used for *arp1*, primers arp2rtpcrF and arp2rtpcrR were used for *arp2*, and primers tefrtpcrF and tefrtpcrR were used for *tefA*. The sequences of these primers are included in Table S2 in the supplemental material.

Statistical analyses. Statistical significance of gene expression among different groups was assessed by one-way analysis of variance (ANOVA) nonparametric tests. Pairwise two-tailed *t* test nonparametric analysis was used to analyze the differences between two groups. All statistical analyses were performed using the Graphpad Prism 5 program, with *P* values lower than 0.05 considered statistically significant.

Inoculation of *G. mellonella* larvae with A. fumigatus conidia. Larvae of the great wax moth, *G. mellonella*, in the final instar larval stage were obtained from Vanderhorst, Inc. (St. Marys, OH). Larvae of about 0.3 to 0.4 g were used for inoculation as previously described (43). Briefly, 1×10^6 *A. fumigatus* conidia in 5 μl of phosphate-buffered saline (PBS) were injected into the hemocoel of each wax moth via the last left proleg. After injection, the larvae were incubated in plastic containers at 37°C and photographed at various time points. For each experiment, 10 to 15 larvae per group were infected. All experiments were replicated at least twice. Heat-killed conidia were prepared by heating the conidia in PBS suspension at 100°C for 10 min. UV-killed conidia were prepared by irradiating 1×10^8 conidia in 10 ml of water in petri dishes for an hour using the default time mode function of an XL-1500 UV cross-linker with an intensity of ~6,000 μW/cm^2 (Spectronics Corporation, NY). The killing of the conidia by the heat or the UV treatment was confirmed by plating treated conidia on complete medium and culturing them for an additional 3 days at 30°C. The conidial suspension was then washed twice with PBS prior to injection. Larvae were infected with live conidia, UV-killed conidia, heat-killed conidia, or mixtures of live and dead spores of different combinations as indicated below. The mixtures were prepared using equal numbers of dead spores and live spores of indicated strains.

The hemolymph of *G. mellonella* was collected as previously described (44), with minor modifications. Briefly, *G. mellonella* larvae were made to bleed by making a small incision on one of the prolegs. A fresh hemolymph drop was then squeezed out by applying gentle pressure and collected directly onto a glass slide containing 10 to 20 μl of anticoagulant solution (100 mM sodium phosphate buffer, pH 7). A glass coverslip was overlaid gently on top of the hemolymph. Before putting coverslips on top of the hemolymph, spacers (made from coverslips) were placed on both sides of the hemolymph to reduce any turbulence to hemocytes by the overlaid coverslips. These freshly prepared slides were then used immediately for microscopic examination.

RESULTS

The expression of laccase genes *abr1* and *abr2* and other melanin genes is developmentally regulated and is controlled by master regulators of conidiation. To facilitate the examination of gene expression at different developmental stages, we classified the growth cycle of *A. fumigatus* as follows. Dormant conidia break dormancy and germinate when environmental conditions become favorable. Germinated conidia then switch to polar growth to produce elongated vegetative hyphal cells, which develop further into interconnected mycelia that are competent for conidiation (Fig. 1A). Upon exposure to air, competent hyphae produce aerial stalks to initiate the development of conidiophores with elaborate multicellular structures that subsequently generate chains of uninucleate conidia (Fig. 1A). The conidia become heavily pigmented during maturation due to increased deposition of the DHN melanin (Fig. 1B) (21, 45, 46). To examine the expression pattern of *abr1* and *abr2* genes, we collected wild-type *A. fumigatus* samples at 12 h (vegetative hyphae), 24 h (competent hyphae), 36 h (early conidiophores), and 48 h (conidiophores with mature conidia) (Fig. 1C; see also Fig. S1 in the supplemental material). As expected, the expression of both the *abr1* and *abr2* genes was low during vegetative hyphal growth, but it was dramatically increased during conidiation (Fig. 2A). We further examined the expression pattern of other genes from the melanin gene cluster (*alb1*, *arp1*, and *arp2*) and found that their expression was also increased during conidiation (Fig. 2B). The expression dynamics of the *abr1*, *abr2*, *alb1*, *arp1*, and *arp2* genes during development were consistent with previous studies showing that the DHN melanin gene cluster is highly expressed during conidiation (22, 28, 29).

To assess if the gene expression pattern correlates with the protein expression pattern, we constructed GFP-fused Abr1 controlled by the *abr1* native promoter (P_{abr1}-Abr1-GFP). The P_{abr1}-

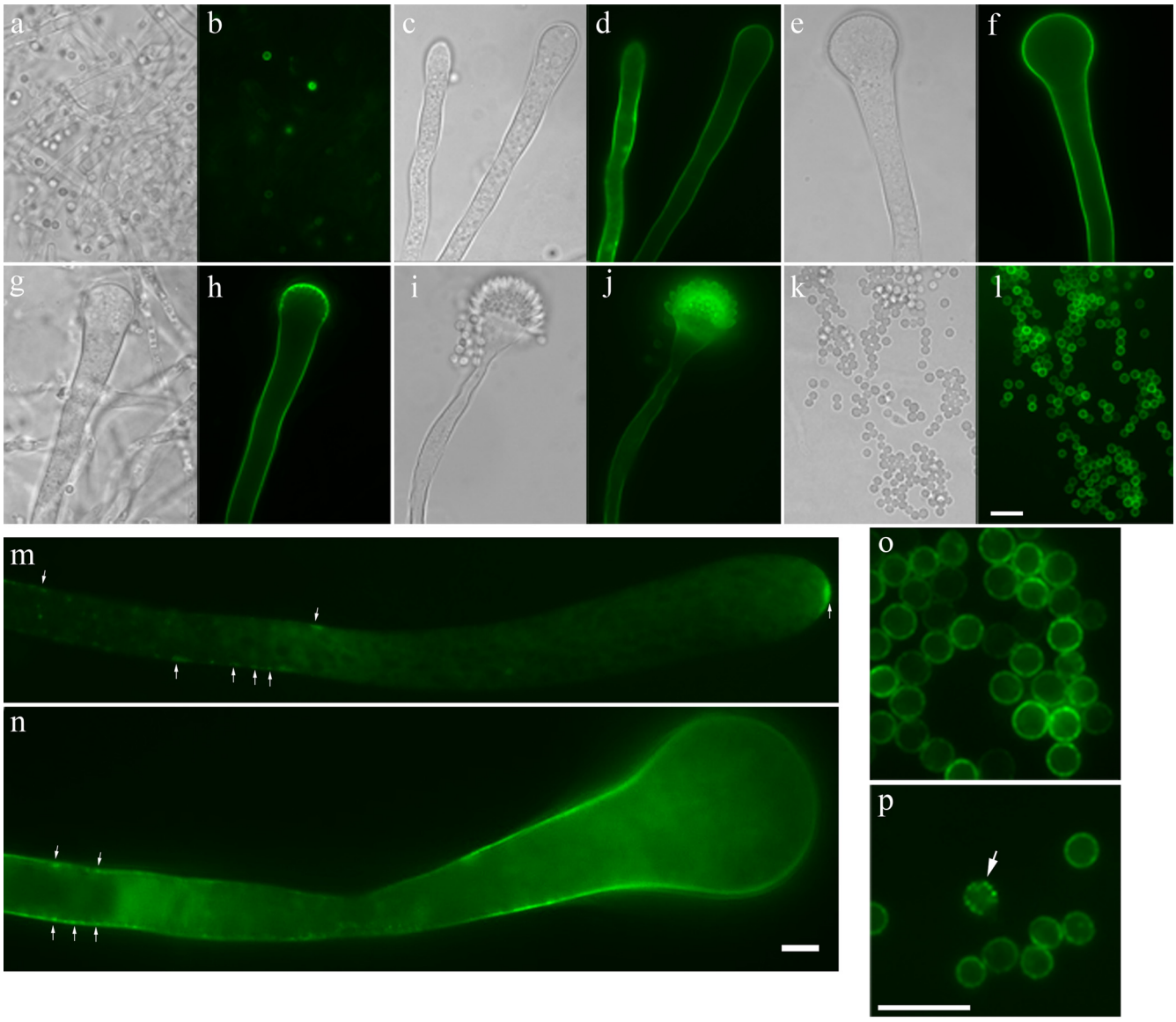


FIG 3 Localization of Abr1-GFP. Abr1-GFP is observed on the cell surface of aerial hyphae, vesicles, phialides, and conidia. No GFP is observed in vegetative hyphae. (a and b) Vegetative hyphae; (c and d) aerial hyphae; (e and f) young vesicles; (g and h) bright spots observed in the area of vesicles from which phialides would emerge; (i and j) vesicles with phialides; (k and l) conidia. (m and n) Abr1-GFP was observed as small patches (arrows) decorating the lines of the cell surface and the tip of the aerial stalk. Scale bars (a to n), 10 μm. (o and p) In conidia, Abr1-GFP is observed at the cell surface. The white arrow shows small patches. Scale bar, 5 μm.

Abr1-GFP construct, when introduced into the brownish *abr1*Δ mutant, was able to restore the mutant conidial pigmentation to the wild-type color (see Fig. S2 in the supplemental material), indicating the functionality of this fluorescence-tagged Abr1. Consistent with the gene expression pattern, the Abr1-GFP fluorescence signal was not detected in vegetative hyphae, but it was detected in aerial hyphae, vesicles, phialides, and young conidia (Fig. 3). The fluorescence signal became stronger in mature aerial stalks, vesicles, phialides, and conidia than that in young aerial stalks. The distribution of Abr1-GFP was not even along the cell surface and was more concentrated at the apical region in the newly generated aerial stalks (Fig. 3m), at the area of vesicles where phialides would emerge (Fig. 3g and h), and at the apex of phialides where conidia would appear. This protein was found as small bright patches decorating along the cell surface of stalks, vesicles, and conidia (Fig. 3m, n, o, and p). The fluorescence-tagged Abr1 displayed similar expression and subcellular localization patterns when it was induced to the *abr1*Δ mutant or the wild type.

The induction of the expression of the laccase genes commenced at hyphal competency that precedes the conidiation event, and their expression reached exceptionally high levels during conidiation (Fig. 2A and 3). A similar expression pattern was also observed for all other tested genes, *alb1*, *arp1*, and *arp2*, from the DHN melanin gene cluster (Fig. 2B). This led us to hypothesize that master regulators of *Aspergillus* development are likely involved in their regulation. The genes *brlA*, *abaA*, and *wetA* are known to be required for early, middle, and late conidiation, re-

spectively, in *Aspergillus* (5). The deletion of *brlA* blocks *Aspergillus* development at the stage of vesicle development prior to the formation of the conidiophore (4, 5), *abaA* is required for the generation of conidia from phialides (analogous to stem cells for conidia), and *wetA* is important for conidial maturation (6, 34). Therefore, we examined the impact of the disruption of these regulators on the expression of *abr1* and *abr2*, *alb1*, and *arp1* and *arp2*. The deletion of the *brlA* gene abolished the induction of the expression of *abr1* and *abr2*, *alb1*, and *arp1* and *arp2*. The expression levels of these genes in the *brlA*Δ mutant remained low at all the time points examined (Fig. 2A and B). This result is in accord with the essential role of BrlA in hyphal competency and conidiation (2, 5). Surprisingly, the expression pattern of the melanin genes was drastically different in the *abaA*Δ and the *wetA*Δ mutants. Specifically, the expression levels of *abr1* and *abr2* and all other melanin genes tested (*alb1*, *arp1*, and *arp2*) at 24 h (hyphal competency) and 36 h (early conidiophore development) were exceedingly high in the *abaA*Δ and *wetA*Δ mutants (Fig. 2B). At 48 h, the expression levels of these melanin genes in the *abaA*Δ and *wetA*Δ mutants were more or less at high levels comparable to those observed in the wild type at the stage of conidiophore maturation. The high levels of expression observed for these melanin genes are consistent with the fact that colonies of the *abaA*Δ and *wetA*Δ mutants are still melanized, while the *brlA*Δ mutant is not (see Fig. S3 in the supplemental material). These results suggest that *abaA* and *wetA* are not necessary for the induction of the expression of *abr1* and *abr2* and other melanin genes. Rather, *abaA* and *wetA* are necessary for the suppression of their expression at earlier stages of development prior to conidiophore maturation. Given the importance of these central regulators in development, it is not clear if these regulators control the expression of these melanin genes directly or indirectly due to their impact on fungal development.

The induction of the expression of Abr1 and Abr2 at hyphal competency is responsive to copper limitation, and CtpA is a developmentally regulated putative copper transporter. Copper has been suggested to stimulate laccase gene expression in *C. neoformans* (47, 48). To examine if copper regulates the expression of the laccase genes *abr1* and *abr2* in *A. fumigatus*, we examined the expression pattern of *abr1* and *abr2* under copper-rich and copper-limiting conditions. The expression pattern of *abr1* and *abr2* was not affected by excessive copper present in the growth media (Fig. 4A and B). However, under the copper-limiting condition, we observed modestly enhanced induction of *abr1* and *abr2* expression during the hyphal competent stage (Fig. 4A and B). Thus, copper starvation appears to augment the induction of the expression of *abr1* and *abr2*. The presence or the lack of copper in the media did not improve or exacerbate the pigmentation defects in the mutants in which the melanin genes had been deleted (Fig. 4E).

To identify the copper transporter that is likely involved in conidial melanization in *A. fumigatus*, we performed reciprocal BLASTP searches of the *A. fumigatus* genome using *A. nidulans ygA* and *C. lindemuthianum clap1* as the queries. These copper transporters are shown to be involved in melanization (32, 34). The gene Afu4g12620 was identified as the homolog that shares the highest similarity with the query sequences, and we named it *ctpA*. The ORF of this gene is 3,564 bp, and it is predicted to encode a protein of 1,167 amino acids (see Fig. S4 in the supplemental material). The amino acid sequence of CtpA is 72% identical and 82% similar to that of *A. nidulans* YgA, and it shares 76% similarity to *C. lindemuthianum* Clap1. All the signature motifs found in the Clap1 and YgA, including the copper ion binding domains defined by the sequence GMTCGAC and ATP-binding sites, are conserved in CtpA and are present in the same order (see Fig. S4 and S5A in the supplemental material). Furthermore, CtpA likely carries 8 transmembrane helices, as predicted by the Tmpred program and the I-Tasser program (see Fig. S4 and S5), and consistently, CtpA is predicted to be a membrane protein by the WoLF PSORT program. The best-matched structural homolog from the protein database is 3rfuA, which is a copper transporting PIB-type ATPase (see Fig. S5B).

We speculate that if *ctpA* is involved in DHN melanization in conidia, its activity might be upregulated during hyphal competency and/or conidiation. Indeed, the *ctpA* expression was low during vegetative hyphal growth, but it peaked at the hyphal competency developmental stage (Fig. 4C). *ctpA* expression then decreased after hyphal competency, although it was still maintained at a higher level during conidiation than during vegetative hyphal growth. Surprisingly, the addition of copper or the copper chelator BCS to the growth media did not significantly alter *ctpA* expression (Fig. 4C), suggesting that *ctpA* is not regulated by extracellular copper at the transcript level. As the expression of *ctpA* is developmentally regulated, we decided to examine if the master development regulators control its expression. We analyzed the expression of *ctpA* in the *brlA*Δ, *abaA*Δ, and *wetA*Δ mutants and compared it to that of the wild-type control. As shown in Fig. 4D, *ctpA* expression failed to diminish during conidiation in the *brlA*Δ mutant, suggesting that BrlA is involved in regulating the expression of *ctpA* after hyphal competency. The effect of the deletion of *abaA* or *wetA* on *ctpA* expression was not conclusive because of less obvious effect and large variations.

Disruption of *ctpA* leads to defective conidial pigmentation that can be remediated by introducing a wild-type copy of *ctpA* or by the addition of copper. To examine the possible role of CtpA in conidial melanization, we deleted the *ctpA* gene in *A. fumigatus*. The replacement of the *ctpA* gene with the hygromycin marker through homologous replacement was verified by Southern blotting (see Fig. S4C to E in the supplemental material). We also generated a *ctpA* overexpression (*ctpA*^{oe}) strain by placing the wild-type copy of the *ctpA* gene under the control of the promoter of the housekeeping gene *tefA*. We selected the *tefA* promoter based on our observation that *tefA* was constitutively expressed at high levels in this fungus throughout its life cycle (data not shown).

Although the wild-type strain, the *ctpA*Δ mutant, and the *ctpA*^{oe} strain displayed normal vegetative hyphal growth on a variety of culture media, the *ctpA*Δ mutant showed a severe defect in conidial melanization under certain conditions. For instance, the *ctpA*Δ mutant grown on typical growth media was poorly melanized, resembling the wild-type strain grown under extremely copper-limiting conditions (Fig. 5A and B). Addition of copper to the growth media restored the conidial pigmentation of the *ctpA*Δ mutant to the wild-type color (Fig. 4E and 5A and B).

To further confirm that the defect in conidial pigmentation observed in the *ctpA*Δ mutant was indeed caused by the lack of the *ctpA* gene, we constructed GFP-fused CtpA controlled by the *ctpA* native promoter (P_{ctpA}-CtpA-eGFP). The P_{ctpA}-CtpA-GFP construct, when introduced into the *ctpA*Δ mutant, was able to confer the wild-type conidial pigmentation on the mutant under both the typical and the copper-limiting conditions (Fig. 5C). Thus, *ctpA* is

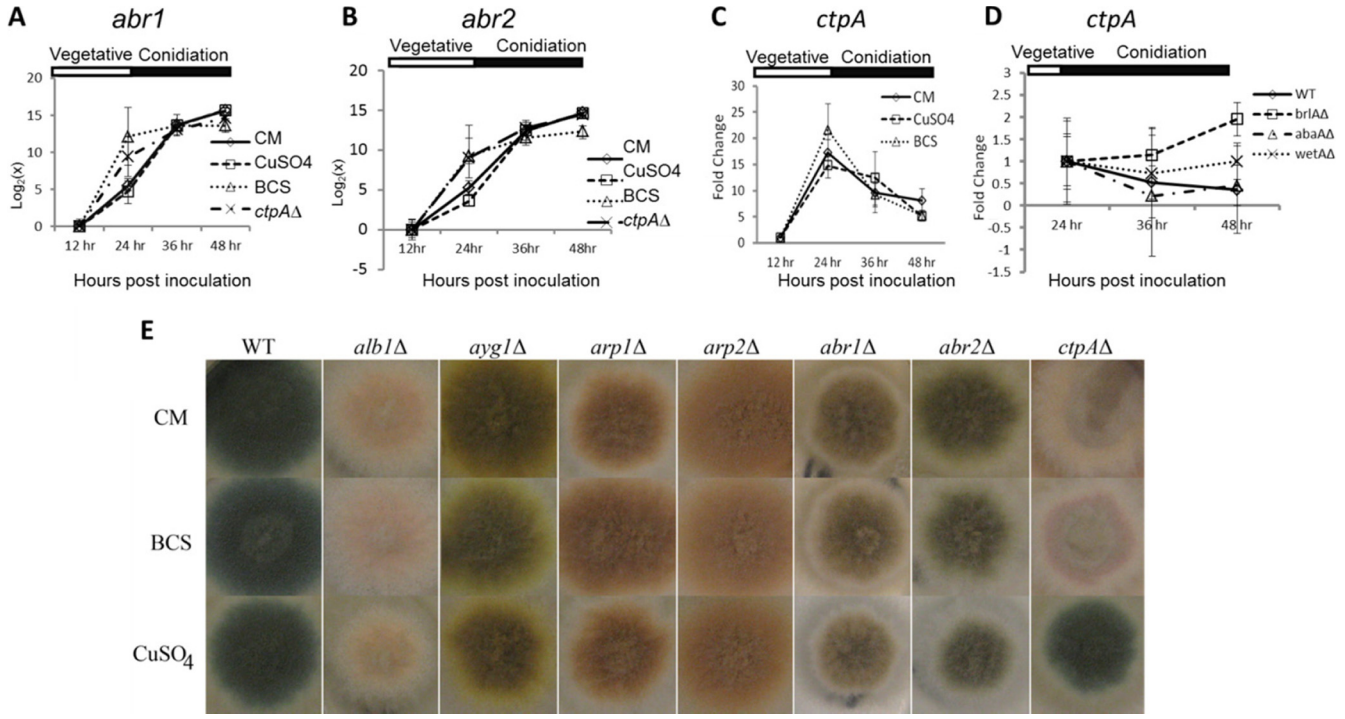


FIG 4 Identification of a developmentally regulated copper transporter, *ctpA*. (A and B) The induction of the expression of *abr1* and *abr2* is significantly enhanced at hyphal competency stage as well as late conidiation stage by copper starvation in the wild type. The *P* value for the *abr1* expression level among all the groups at the 24-h time point was 0.0059, indicating statistical significance. The *P* values for the pairwise comparisons of *abr1* expression for the 24-h time point were 0.1422 (CM and $CuSO_4$), 0.0383 (CM and BCS), and 0.0255 (CM and *ctpA*Δ). The *P* value for the *abr1* expression level among all the groups at the 36-h time point was 0.4701 and was not considered significantly different. The *P* value for the *abr1* expression level among all the groups at the 48-h time point was 0.0002, indicating statistical significance. The *P* values for the pairwise comparisons for the 48-h time point were 0.8892 (CM and $CuSO_4$), 0.0035 (CM and BCS), and 0.0424 (CM and *ctpA*Δ). Similarly, the *P* value for the *abr2* expression level among all the groups at the 24-h time point was 0.0145, indicating statistical significance. The *P* values for the pairwise comparisons of *abr2* expression for the 24-h time point were 0.0899 (CM and $CuSO_4$), 0.0922 (CM and BCS), and 0.0293 (CM and *ctpA*Δ). The *P* value for the *abr2* expression level among all the groups at the 36-h time point was 0.4692 and was not considered significantly different. The *P* value for the *abr2* expression level among all the groups at the 48-h time point was 0.0071, indicating statistical significance. The *P* values for the pairwise comparisons for the 48-h time point were 0.9942 (CM and $CuSO_4$), 0.0104 (CM and BCS), and 0.1043 (CM and *ctpA*Δ). Thus, the expression of both *abr1* and *abr2* is significantly enhanced at the hyphal competency stage in the *ctpA*Δ mutant compared to the wild type and also in the wild type under copper starvation conditions compared to that under normal conditions. The *ctpA*Δ mutant was grown in CM. RNA extracted from fungal cultures at indicated developmental stages was used to measure gene expression levels. All gene expression levels were normalized based on the *tefA* expression levels. The 12-h time point was used as the reference for the subsequent time points. (C) Expression pattern of *ctpA* in the wild-type *A. fumigatus* during development and in media with varied levels of copper. The expression of *ctpA* is enhanced significantly only compared to that under the $CuSO_4$ andBCS conditions at 24 h (*P* value, 0.0080). WT, wild type; $CuSO_4$, CM with addition of copper; BCS, CM with addition of the copper chelator. The 12-h time point was used as the reference for the subsequent time points. (D) Expression pattern of *ctpA* in the wild type and the *brlA*Δ, *abaA*Δ, and *wetA*Δ mutants. The *ctpA* expression pattern was statistically significantly different between the wild type and the *brlA*Δ mutant only at the 48-h time point (*P* value, 0.0160). The 24-h time point was used as the reference for the subsequent time points. Error bars indicate standard deviations. (E) The strains were cultured on CM and media with $CuSO_4$ or BCS at 30°C for 2 days.

required for conidial pigmentation under normal and copper-limiting conditions.

CtpA-GFP fluorescence was detected in all development stages, with a slightly weaker signal in vegetative hyphae and a stronger signal in conidiophores (Fig. 6). The fluorescence signal of CtpA-GFP has tubular or punctate shapes, resembling secretion vesicles. CtpA protein expression appears to follow development, as fluorescence was brighter in actively growing cells, like vesicles and phialides in young conidiophores. In more mature conidiophores, fluorescence in vesicles became dimmer but remained bright in phialides (stem cells) and conidia (Fig. 6).

Previous studies have implied interconnection between copper and iron homeostasis (49, 50). Hence, we also tested conidial melanization of the *ctpA*Δ mutant in response to iron limitation. The *ctpA*Δ mutant was much more severely impaired in conidial pigmentation when grown on media that contained bipyrimidine, a chemical that has a high affinity for iron but also chelates copper (51) (Fig. 7). Since the effect of bipyrimidine on pigmentation of the *ctpA*Δ mutant could be due to a lower copper level, we decided to test the effect of the iron chelator bathophenanthroline disulfonic acid (BPS), which has been used to specifically study iron regulation in a variety of organisms, including another fungal pathogen, *Cryptococcus neoformans* (52). As shown in Fig. 7, the *ctpA*Δ mutant pigmented at a reduced but similar level when grown on media with or without BPS, indicating that BPS, or iron limitation, does not as significantly affect conidial pigmentation of the *ctpA*Δ mutant as copper limitation. Consistently, the addition of $FeCl_3$ to the growth media failed to significantly improve *ctpA*Δ mutant pigmentation (Fig. 7). In contrast, the addition of $CuSO_4$ to the growth media fully restored the melanin defects of the *ctpA*Δ mutant (Fig. 5). Taken together, these results suggest that *ctpA* encodes a copper-specific transporter involved in melanization.

The expression pattern of *abr1* and *abr2* in the *ctpA*Δ mutant resembles that under copper-limiting growth conditions. As

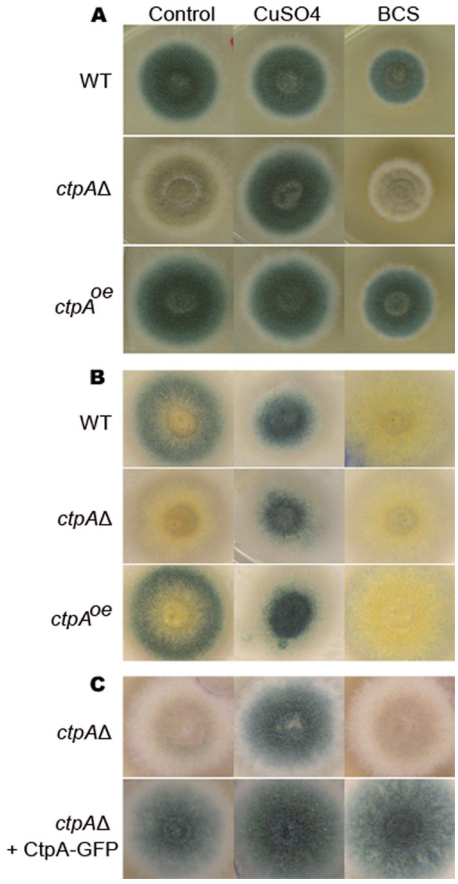


FIG 5 *ctpA* is critical for conidial melanization under normal and copper-limiting growth conditions. (A) The wild type, the *ctpA*Δ mutant, and the *ctpA*^{oe} strain were grown in complete sorbitol medium, with or without $CuSO_4$ or BCS. (B) The wild type, the *ctpA*Δ mutant, and the *ctpA*^{oe} strain were grown in YNB sorbitol medium, with or without $CuSO_4$ or BCS. The different media have different levels of copper availability. (C) The *ctpA*Δ mutant and the complemented strain (*ctpA*Δ plus P_{ctpA}-CtpA-GFP) were grown in complete medium, with or without $CuSO_4$ or BCS.

copper limitation modestly enhances the expression of *abr1/2* at the hyphal competency stage (Fig. 3A and B), we speculated that the disruption of the copper transporter CtpA would resemble copper starvation and may similarly affect the expression of these laccase genes. Indeed, the induction of the expression of both *abr1* and *abr2* in the *ctpA*Δ mutant strain grown under normal conditions was significantly enhanced at the stage of hyphal competency, mimicking the pattern observed in the wild-type strain grown under copper-limiting conditions (Fig. 4A and B). Thus, the lack of *ctpA* in *A. fumigatus* creates a response similar to the one toward copper starvation. This finding is consistent with the poor conidial melanization of the wild-type *A. fumigatus* under copper-limiting conditions and of the *ctpA*Δ mutant under both normal and copper-limiting growth conditions (Fig. 5B).

Pigment-defective conidia of the *ctpA*Δ mutant stimulate host immune responses in the *Galleria mellonella* infection model. We showed previously that conidia of all the DHN melanin biosynthesis mutants, including *abr1* and *abr2* mutants, cause rapid darkening of infected *G. mellonella* larvae (53). Such darkening is most likely due to the formation of melanotic capsules by the host, a key immune defense mechanism in insects (44, 54). We hypothesize that the lack of melanin in these mutants exposes immunogenic molecules on the *A. fumigatus* cell wall and consequently elicits strong immune responses from this insect (53). As the copper transporter *ctpA*Δ mutant exhibited a provisional defect in conidial melanization under normal and copper-limiting growth conditions (Fig. 5), we speculated that pigment-defective *ctpA*Δ conidia collected under such growth conditions would elicit immune responses from the larvae as strong as those induced by the DHN melanin mutants. Indeed, *G. mellonella* larvae rapidly darkened after being inoculated with pigment-defective conidia of the *ctpA*Δ mutant (Fig. 8A). In contrast, the normal-pigmented conidia of the *ctpA*Δ mutant collected under copper-excessive growth conditions did not cause increased darkening in the infected larvae compared to those inoculated with the wild-type pigmented conidia (Fig. 8A). The pigmented *ctpA*Δ mutant conidia and wild-type conidia both induced only modest darkening in the larvae compared to that in the PBS-inoculated larvae (Fig. 8A).

Microscopic observation of the hemolymph (equivalent of blood in higher animals) isolated from larvae infected by the pigment-defective *ctpA*Δ conidia and those infected by the normal-pigmented *ctpA*Δ conidia revealed differences in melanized hemocytic aggregates (Fig. 9). Melanization accompanying phagocytosis and hemocyte aggregation (melanotic capsules) is one main means for *G. mellonella* and other insects to contain foreign invaders (44, 54). Much larger melanotic capsules were observed in the hemolymph isolated from larvae challenged with the pigment-defective conidia harvested from the *ctpA*Δ mutant grown under copper-limiting conditions than those on copper-rich media (Fig. 9). No difference was observed between pigmented *ctpA*Δ mutant conidia and pigmented wild-type conidia in eliciting the formation of melanotic capsules in larvae.

If the rapid and enhanced immune responses of larvae toward the melanin-defective mutants are caused by structural changes in the conidial cell wall, such responses should be independent of conidial viability. To test this idea, we heat killed the conidia of the *ctpA*Δ mutant harvested under different growth conditions and used them for the inoculation. Similar to what we observed with live conidia, the heat-killed pigment-defective conidia of the *ctpA*Δ mutant caused severe darkening in the larva cuticle (Fig. 8). We carried out similar experiments using UV-killed pigment-defective conidia of the *ctpA*Δ mutant, and the results were similar to those for the heat-killed conidia (see Fig. S6 in the supplemental material). In contrast, heat-killed pigmented conidia of the *ctpA*Δ mutant did not elicit enhanced larva darkening or the formation of melanotic capsules compared to the wild-type conidia (Fig. 9; see also Fig. S7 in the supplemental material). When a mixture of dead pigment-defective *ctpA*Δ conidia and live pigmented *ctpA*Δ conidia or vice versa was used to inoculate larvae, a modest enhancement in larva darkening was observed (Fig. 7B). Furthermore, coinoculation of live wild-type conidia with heat-killed pigment-defective *ctpA*Δ conidia modestly increased larva darkening compared to the live wild-type conidia, with the same total fungal cell number (dead and live) (data not shown). These results suggest that an active metabolism or active secretion of proteins or small molecules exhibited by viable cells is not responsible for this enhanced larva immune response. Rather, alterations in the conidial surface of the pigment-defective *ctpA*Δ mutant might be responsible. This is in accordance with our previous finding that rapid and intense cuticle darkening of *G. mellonella* larvae in response to the DHN melanin mutants is independent of conidial

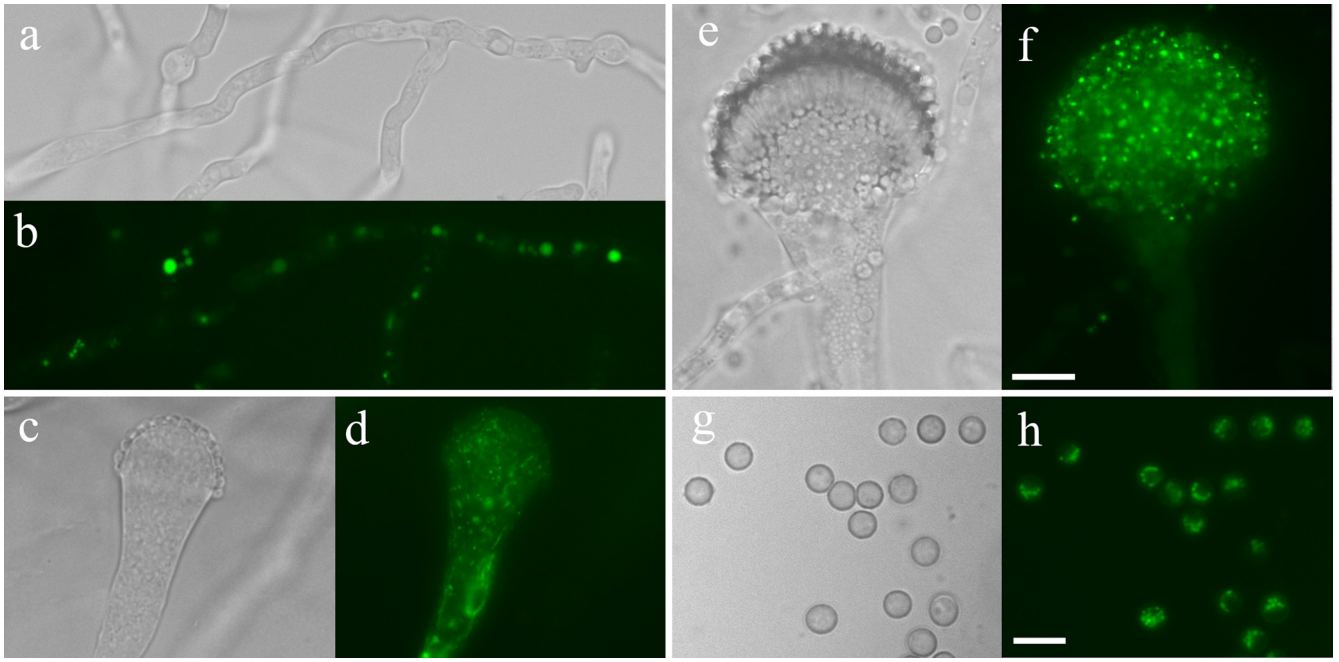


FIG 6 Localization of CtpA-GFP. Fluorescence of CtpA-GFP was detected in the vegetative hyphae (a and b), young conidiophore (c and d), mature conidiophore with conidia (e and f), and conidia (g and h). CtpA fluorescence signals take shapes resembling secretion vesicles. Scale bars, 10 μm (a to f) and 5 μm (g to h).

viability. In this regard, the *ctpA*Δ mutant grown under copper-limiting conditions behaves similarly to the DHN melanin mutants.

DISCUSSION

Melanin biosynthesis is one of the most universal phenomena, observed in a variety of life forms, including plants, animals, bacteria, and fungi. However, the regulation of melanin production is

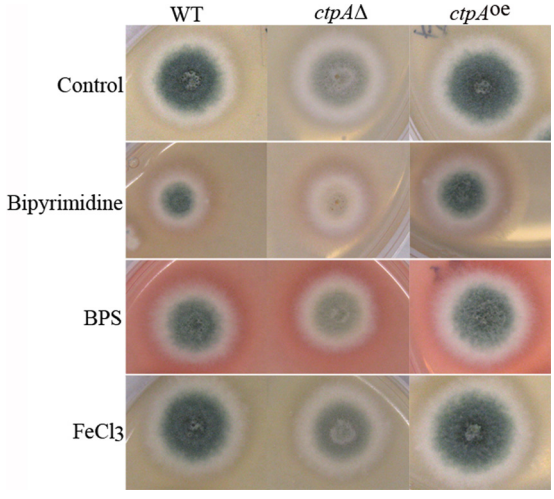


FIG 7 The conidial pigmentation in the *ctpA*Δ mutant is not significantly affected by iron limitation or excess in the medium. The wild type, the *ctpA*Δ mutant, and the *ctpA*^{oe} strain were grown in CM with bipyrimidine, the iron chelator BPS, and $FeCl_3$. The wild-type strain produced melanized conidia under these conditions. The *ctpA*Δ mutant showed a severe defect in conidial pigmentation when grown in bipyrimidine, but its pigmentation was not significantly affected by BPS or $FeCl_3$.

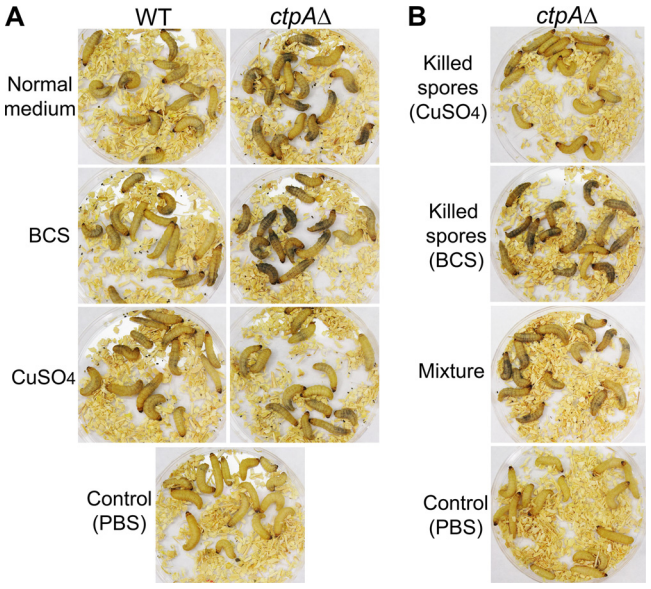


FIG 8 Melanin-defective *ctpA*Δ mutant stimulates *G. mellonella* host immune responses. (A) The *A. fumigatus* wild-type strain and the *ctpA*Δ mutant were grown on CM with or without the addition of BCS or $CuSO_4$. Conidia of the strains grown under these conditions were collected and used for larva inoculation. Larvae injected with PBS were used as a control. Images shown here are photos of the inoculated larvae taken at 16 h after inoculation. Darkening of the larvae was apparent within 4 h (data not shown). Larvae responded to the *ctpA*^{oe} conidia similarly to the wild-type conidia and thus are not shown here. (B) Heat-killed conidia of the *ctpA*Δ mutant harvested from growth media containing $CuSO_4$ or BCS were used to inoculate *G. mellonella* larvae. A mixture of heat-killed conidia of the *ctpA*Δ mutant harvested from CM-BCS media and live conidia of the *ctpA*Δ mutant harvested from CM plus $CuSO_4$ showed modest darkening of the larvae. Reversed combination yielded similar results, which are not shown here. Combination of the *ctpA*Δ mutant and the wild-type conidia also yielded similar results, not shown here. Larvae injected with the PBS buffer were used as a control.

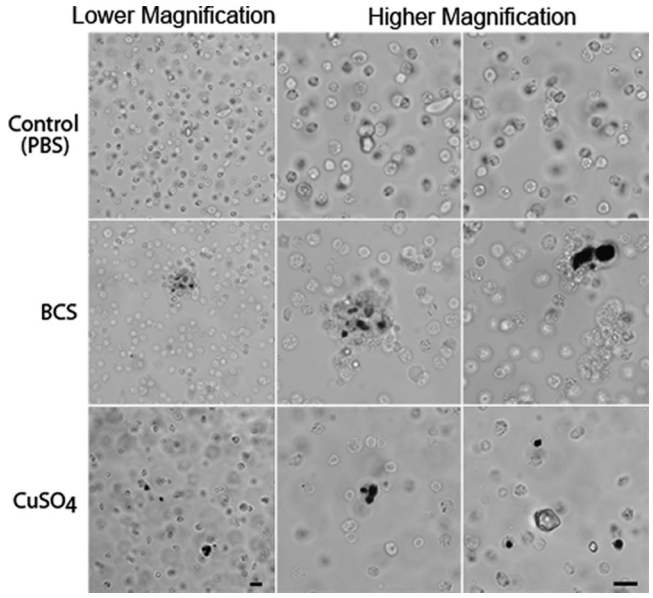


FIG 9 *G. mellonella* exhibits earlier and stronger hemocyte-mediated defense responses when infected with pigment-defective *ctpA*Δ conidia. Images are of *G. mellonella* larvae taken at 16 h after inoculation with 1.0×10^6 *ctpA*Δ mutant conidia harvested from copper-limiting or copper-rich conditions. Larger capsules produced by the larva hemolymph (aggregations composed of a variety of hemocytes to contain foreign particles) were visible when inoculated with pigment-defective *ctpA*Δ conidia harvested under copper-limiting conditions (BCS) compared to those infected by the pigmented *ctpA*Δ conidia harvested under copper-rich conditions. Some of the capsules were heavily melanized (melanotic capsule) by the larvae. Larvae injected with the PBS buffer were used as a control. Scale bar, 20 μm.

uniquely adopted by living organisms in their adaptation to variable environmental conditions and their lifestyles (55). Not surprisingly, melanin is often synthesized in a cell-type-specific manner (56): Melanin is deposited in the appressorium to provide rigidity and selective permeability to its cell wall, and it is required for pathogenicity in the plant pathogens *Colletotrichum orbiculare* and *Magnaporthe oryzae* (57, 58). The exclusion of melanin at the penetration site implies a role in determination of the correct invasion point (59). In this study, we focused on *abr1* and *abr2* in the *A. fumigatus* DHN melanin cluster, as laccases are the most conserved factors required for melanin production irrespective of the type of melanin or the organism involved. We found that the expression of both laccase genes together with other melanin genes was induced upon hyphal competency and was maintained at even higher levels during conidiation (Fig. 2). Using fluorescence-tagged Abr1, we confirmed that this laccase was in stalks and conidiophore structures (Fig. 3). The fluorescent Abr1 mostly delineated the cell periphery as small bright patches, consistent with the deposition of melanin granules in the cell wall.

In *Colletotrichum*, melanization in mycelia and appressoria is controlled by different transcription factors (60). However, the *A. fumigatus* melanin cluster does not carry any regulatory factor (10, 22), unlike some other fungi (61). As *A. fumigatus* produces hyaline vegetative hyphae and pigmented conidiophores, it is logical to reason that DHN melanin genes are upregulated during conidiation, and the key development regulators might control their expression. BrlA, AbaA, and WetA are the central development regulators in *Aspergillus* spp. What is different in *A. fumigatus* compared to its model relative *A. nidulans* is that the expression of *abaA* and *wetA* is not completely dependent on *brlA* (28). In this study, we found that BrlA, AbaA, and WetA exert different impacts on the expression of *abr1* and *abr2* and other melanin genes (Fig. 2B): BrlA is required for the induction of their expression at the hyphal competency, while AbaA and WetA appear to act to suppress the expression of these melanin genes prior to conidiophore maturation (Fig. 2B). A previous study also indicated a requirement for developmental modifier StuA in regulating the melanin gene cluster (28). Another developmental modifier, MedA, when disrupted, causes a delay in conidial melanization and a modest reduction in melanin gene expression independent of *brlA*, *abaA*, or *wetA* in *A. fumigatus* (62). Thus, the regulation of melanin genes in *A. fumigatus* is controlled by multiple developmental regulators in a complicated manner.

Laccases require copper as their cofactor, and dysregulation of copper homeostasis could affect their activity and/or their expression. When laccases are deleted, however, the pigmentation defects cannot be exacerbated by copper limitation or improved by copper repletion (Fig. 4E). In the wild-type strain, however, copper starvation augmented the induction of expression of *abr1* and *abr2* during hyphal competency (Fig. 4A and B). Interestingly, the disruption of the copper transporter *ctpA* enhanced the induction of *abr1* and *abr2* during hyphal competency, mimicking the effect of copper starvation (Fig. 4A and B). The expression of *ctpA* itself peaked at hyphal competency stage, which is consistent with its potential role in assisting the supply of copper to laccases Abr1/2. Consistent with its predicted role as the copper transporter involved in conidial melanization, *ctpA* is required for normal pigmentation under normal and copper-limiting conditions in *A. fumigatus* (Fig. 4E and Fig. 5). The observations that the defect in conidial pigmentation of the *ctpA*Δ mutant can be caused by copper but not iron limitation and that excessive copper but not iron in the media restored the melanin defect (Fig. 5) suggest that this transporter is a major factor responsible for supplying copper for these laccases. These findings also underscore the significance of the hyphal competency stage in the preparation for conidiation in *A. fumigatus*. The disruption of *ctpA* enhanced *A. fumigatus* resistance toward oxidative stress caused by hydrogen peroxide, but the mutation did not appear to affect its tolerance to other stresses, such as salts (KCl and NaCl), sucrose, SDS, antifungal caspofungin (inhibits β-glucan biosynthesis), or $NaNO_2$ (data not shown). Thus, it appears that *ctpA* exerts its primary effect on conidial pigmentation.

Wild-type *A. fumigatus* produces echinulate conidia and is weakly immunogenic. The melanin mutants have profound modification of the cell wall and display a smooth conidial surface (10, 63). As conidial surface-associated molecules represent the interface between this fungus and its host, it is not surprising that melanin plays multiple roles in modulating *A. fumigatus* interaction with its host (27, 53, 64). Here we showed that both dead and live pigment-defective *ctpA*Δ conidia caused rapid darkening of *Galleria* larvae after inoculation due to drastically enhanced formation of melanotic capsules by the larva hemocytes (Fig. 8 and 9). This reflects highly stimulated host innate immune responses in this insect, consistent with enhanced immune responses in mammalian cells elicited by melanin mutants. A very recent study has shown that melanin mutant conidia are more rapidly cleared from lungs of mice than are wild-type conidia (65). The responses of larvae to the pigment-defective *ctpA*Δ conidia are the same as

what we observed when *Galleria* larvae were inoculated with the DHN melanin mutants (53). As insects like the great wax moth provide several experimental advantages over mammal models (e.g., reduced cost, easier handling, and fewer bioethics considerations), they have become increasingly popular in assessing host innate immune responses and fungal virulence factors (43, 66–68), including melanin biosynthesis factors in *Cryptococcus* and *Aspergillus*. Collectively, our findings and those of others indicate complicated regulation of melanin in *A. fumigatus* by developmental factors and copper homeostasis and the importance of melanin in shaping the interaction between fungi and various hosts.

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