Copper Ions are Required for *Cochliobolus heterostrophus* in Appressorium Formation and Virulence on Maize

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ABSTRACT

Cochliobolus heterostrophus is the causal agent of southern corn leaf blight, a destructive disease on maize worldwide. However, how it regulates virulence on maize is still largely unknown. Here, we report that two copper transporter genes, *ChCTR1* and *ChCTR4*, are required for its virulence. *chctr1* and *chctr4* mutants showed attenuated virulence on maize compared with the wild-type strain TM17 but development phenotypes of those mutants on media with or without infection-related stress agents were the same as the wild-type strain. Moreover, *ChCTR1* and *ChCTR4* play critical roles in appressorium formation and mutation of *ChCTR1* or

ChCTR4 suppresses the appressorium formation. Furthermore, copper-chelating agent ammonium tetrathiomolybdate suppressed the appressorium formation and virulence of *C. heterostrophus* on maize, whereas copper ions enhanced the appressorium formation and virulence on maize. The results indicate that copper ions are required for appressorium formation and virulence of *C. heterostrophus* on maize and are acquired from the environment by two copper transporters: *ChCTR1* and *ChCTR4*.

Keyword: mycology

Cochliobolus heterostrophus Drechsler is the causal agent of southern leaf blight (SLB), a destructive disease on corn worldwide (Lim and Hooker 1971). In the 1970s, *C. heterostrophus* race T, specifically pathogenic to cms-T cytoplasm maize, destroyed 17% of maize in the United States (Tatum 1971). Subsequent studies found that the T-toxin produced by race T binds specifically to URF13, a protein located in the inner membrane of maize mitochondria (Levings 1990; Pring and Lonsdale 1989). The binding of URF13 by T-toxin blocks the function of maize mitochondria and enhances susceptibility to *C. heterostrophus* (Balint-Kurti and Johal 2009; Pring and Lonsdale 1989; Wise et al. 1999). After the cms-T maize was discarded by breeders, *C. heterostrophus* race O has become the major strain to cause SLB in the maize field throughout the world (Carson et al. 2004). Compared with other races, race O can infect maize with any type of cytoplasm and cause up to 40% or more yield loss (Byrnes et al. 1989). However, fighting against *C. heterostrophus* is challenged by the deficiency in understanding its pathogenicity mechanisms.

Under high humidity conditions, spores of *C. heterostrophus* germinate to produce long germ tubes, and appressoria form on the tip of the germ tubes (Braun and Howard 1994). Then, the pathogen penetrates into plant tissues directly or through stomata (Mendgen et al. 1996; Shanmugam et al. 2010). Although the appressoria generated by *C. heterostrophus* are much smaller than those of the model pathogen *Magnaporthe oryzae* (Horwitz et al. 1999), it is critical for the full virulence of *C. heterostrophus*. Mutation of *CGA1* gene encoding a G-protein α ($G\alpha$) subunit blocks appressorium formation and suppresses its virulence on maize (Degani et al. 2004; Horwitz et al. 1999). The G-protein β ($G\beta$)

subunit CGB1 required for appressorium formation also plays positive roles in virulence on maize (Ganem et al. 2004). In addition to the G protein pathway, the mitogen-activated protein kinase (MAPK) cascades also are required for the formation of appressoria and virulence on maize. Mutation of a putative MAPK kinase gene *ChSte11* shows defects in appressorium formation and pathogenicity, and the same phenotype was observed by blocking the *HOG1* gene or *CHK1* gene encoding MAPKs (Igbaria et al. 2008; Izumitsu et al. 2009; Lev et al. 2009). Melanin, required for the appressorium formation and pathogenicity of *M. oryzae*, was found not to be required for *C. heterostrophus* in conidium germination, appressorium formation, and virulence on maize under laboratory conditions, because mutation of the *CMR1* gene in *C. heterostrophus* blocks melanin biosynthesis but the conidia of the *cmr1* mutant germinate normally, form regular appressoria, and cause disease symptoms the same as the wild-type strain (Braun and Howard 1994; Eliahu et al. 2007). This means *C. heterostrophus* may deploy different mechanisms to regulate appressorium formation and cause disease on maize. Except for genes regulating appressorium formation, some other genes in *C. heterostrophus* have been identified to be required for virulence on maize. *NPS6*, encoding a nonribosomal peptides synthetase, is responsible for biosynthesis of intracellular siderophores, and deletion of *NPS6* attenuates virulence on maize (Condon et al. 2014; Lee et al. 2005; Oide et al. 2006). *Ppt1*, encoding an Sfp-type 4'-phosphopantetheinyl transferase, regulates resistance to oxidative stress, iron depletion, and virulence to maize (Zainudin et al. 2015). ChToxA protein, a host-specific ToxA-like protein generated by *C. heterostrophus*, can induce necrosis specifically on maize (Lu et al. 2015). Compared with the well-studied *M. oryzae*, *C. heterostrophus* should have more virulence factors unidentified. Overall, our understanding of *C. heterostrophus* virulence on maize is limited.

Here, we report that two copper transporter proteins, *ChCTR1* and *ChCTR4*, are required for *C. heterostrophus* virulence on maize. Mutation of *ChCTR1* or *ChCTR4* showed not only reduced virulence on maize but also deficiency in appressorium formation. Furthermore, copper ions were identified to be critical for *C. heterostrophus* in appressorium formation and virulence on maize.

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MATERIALS AND METHODS

Fungal strains and growth conditions. *C. heterostrophus* strain TM17 and other derived strains described in this article were maintained and cultured on the V8 medium plates (V8) (36% V8, 0.2% CaCO₃, and 2% agar) at 25°C. Cultures for genomic DNA isolation and RNA isolation were grown on potato dextrose agar (PDA) plates (200 g of potato, 2% glucose, and 1% agar) at 25°C for 6 to 8 days. Protoplast preparations were grown in the complete medium (CM) (0.6% yeast extract, 0.6% casein hydrolysate, and 1% sucrose) in a 150-rpm shaker at 25°C for 1 day. The selective top agar medium was supplemented with hygromycin B at 30 µg/ml, depending on the selection marker in the plasmid vector.

Targeted gene deletion. Targeted gene deletion was used to replace the target gene by the hygromycin B resistance gene *hygromycin phosphotransferase* (*HPH*) and it was done according to the approach previously described (Lu et al. 2015; Turgeon et al. 2010). The 500-bp fragment amplified from the genomic sequence 5' upstream of the target gene open reading frame was amplified with the primer pair F1 + R1 and then fused with the HP region of the *HPH* gene amplified from plasmid pTFCM with the primer pair PTM39 + PH. The 500-bp fragment amplified from the genomic sequence 3' downstream of the target gene open reading frame was amplified with the primer pair F3 + F4 and then fused with the PH region of the *HPH* gene amplified from plasmid pTFCM with the primer pair HP + PTM40.

Protoplast preparation and transformation were processed using procedures described by Turgeon et al. (1987). Conidia were cultured in CM liquid medium in a shaker at 28°C for 17 h. The mycelium pellet was collected into the enzymatic hydrolysate (20 ml) containing Driselase at 0.01 g/ml, lysing enzymes from *Trichoderma harzianum* (Sigma L1412) at 0.01 g/ml, and 0.7 M NaCl in a shaker at 70 rpm/min and 28°C for 2 h to release enough protoplasts. Then, the two PCR fragments were cotransformed into protoplasts using PEG4000, pipetted into the above mixture, dripped onto molten regeneration (MR) plates without any antibiotics, and incubated for 14 h. Then, the MR medium was covered with hygromycin at 30 µg/ml. After 2 days, the colonies that grew on the PDA plate with hygromycin at 30 µg/ml were removed. The transformants grown on PDA plates (hygromycin at 30 µg/ml) were continuously transferred for three generations to obtain stable genetic transformants.

Plant infection assays. Corn cultivar B73 was used for infection assays. Conidial suspensions (2.5 × 10⁴ conidia/ml in 0.008% Tween solution) were prepared from V8 medium for drop inoculation and spray infection assays. For drop inoculation, 10 µl of conidial suspension was dropped on the leaf surface and the inoculated plants were covered for 3 days.

For spray inoculation, each 3-week-old plant was inoculated with 0.5 ml of spore suspension and hydrated for 14 h. Lesion formation was examined at 3 days postinoculation (dpi) on corn.

Analysis of conidial germination and appressorial formation. Conidia were prepared from 10-day-old V8 medium. Aliquots (150 µl) of conidial suspensions (1 × 10⁵ conidia/ml) were applied on the microscope cover glass (CAS 12-540-A; Thermo Fisher). The conidial droplets were incubated in a moist chamber at 25°C. Conidial germination and appressorial formation were examined at 0.5, 1, 1.5, 2, 2.5, and 3 h after incubation. Five fields were found at random, containing more than 200 spores, and observed. Mean and standard deviation were calculated using *t* test for significant differences between strains.

RNA isolation, reverse-transcription PCR, and real-time PCR analysis. RNA was isolated by using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. The total RNA was treated with DNase I; then, the first-strand cDNA was synthesized with the M-MLV Reverse Transcription System (Promega Corp.). Quantitative real-time PCR was performed with the CFX96 Real-Time Detection System using TaKaRa SYBR

Premix Ex Taq (Takara). The relative quantification of the transcripts was calculated by the 2^{-ΔΔCt} method (Voegele and Schmid 2011).

Copper ion transport functional verification by yeast complementation assay. Yeast *ctr1Δ/ctr3Δ* mutant MPY17 was transformed with the plasmids pBD (empty vector), pBD-CTR1, pBD-CTR2, pBD-CTR3, and pBD-CTR4. Transformants were picked up and grown to exponential phase in medium without tryptophan. Cells were collected by centrifugation, washed with sterile distilled water, and diluted with water to an optical density at 600 nm of 1 and 0.1. Serial dilutions were spotted on selective medium (yeast extract and peptone with 2% ethanol and 3% glycerol [YPEG]) and on YPEG medium with 0, 10, 20, 30, and 40 µM CuSO₄. Plates were incubated at 30°C for 2 or 3 days.

Infection-related stress adaptation assays. The assays for adaptation to different stress agents were done as previously described (Liu et al. 2019). Mycelial plugs of *C. heterostrophus* wild-type strain TM17, *chc1* mutants, and *chc4* mutants were inoculated on V8 medium plates with different stress agents, including NaCl, KCl, H₂O₂, sodium dodecyl sulfate (SDS), and 2,2-bipyridyl (2DP). Those plates were incubated at 24°C for 7 days. Then, the colony diameter of each plate was measured.

RESULTS

RNA sequencing analysis to compare strong virulent strain TM17 and weak virulent strain PT180. In the field at

Fig. 1. Comparing virulence and gene expression between two *Cochliobolus heterostrophus* strains. **A**, Disease phenotypes of *C. heterostrophus* strains TM17 and PT180 at 3 days after dropping inoculation on maize inbred line B73. **B**, Lesion size of *C. heterostrophus* strains TM17 and PT180 at 3 days after dropping inoculation on maize inbred line B73; the quantitative lesion size is mean ± standard error (SE) from at least 10 disease lesions. Mean values followed by different letters are significantly different from each other ($P < 0.01$, Student's *t* test). **C**, Gene ontology analysis based on RNA sequencing data to compare the gene expression difference between two *C. heterostrophus* strains (TM17 and PT180). **D**, Quantitative reverse-transcription PCR to check the 10 gene expressions in transition metal ion transmembrane transporter activity between two *C. heterostrophus* strains (TM17 and PT180); data represent mean values ± SE from three technical replicates. Experiments were repeated at least three times with similar results. Mean values followed by different letters are significantly different from each other ($P < 0.01$, Student's *t* test).

Henan province, China, we isolated two *C. heterostrophus* strains (TM17 and PT180) showing different virulence on maize inbred line B73. Two days after drop inoculation, the lesion size caused by TM17 was approximately twice that caused by PT180 (Fig. 1A and B). In order to understand the difference in virulence between them, we isolated RNA from mycelia cultured on V8 medium for 7 days and did RNA sequencing analysis (SRA accessions SRR9970439 and SRR9970438) to compare the gene expression difference between TM17 and PT180. Gene ontology analysis of the differently expressed genes revealed that the most prominent biological processes were oxidoreductase activity and transition metal ion transmembrane (TM) transporter activity (Fig. 1C). In the group involved in transition metal ion TM transporter activity, there are only 10 genes in *C. heterostrophus*: COCC4DRAFT_148665 (copper transporter 1, *ChCTR1*), COCC4DRAFT_159511 (ZIP zinc transporter 1, *ChZIP1*), COCC4DRAFT_164047-D2 (cation efflux 2, *ChCE2*), COCC4DRAFT_168512 (copper transporter 2, *ChCTR2*), COCC4DRAFT_168867 (cation efflux 1, *ChCE1*), COCC4DRAFT_176420 (ferrous iron transporter 1, *ChFTR1*), COCC4DRAFT_31949 (copper transporter 3, *ChCTR3*), COCC4DRAFT_32662 (copper transporter 4, *ChCTR4*), COCC4DRAFT_40955 (nickel cation TM transport, *ChNico1*), and COCC4DRAFT_57093-D2 (ATPase-associated gene, *ChAAA*). We performed quantitative reverse-transcription PCR (qRT-PCR) to check the gene expression between TM17 and PT180. The results showed that no difference was found in *ChCE2*, *ChNico1*, *ChFTR1*, and *ChAAA* between TM17 and PT180; the expression levels of *ChZIP1* and *ChCE1* in TM17 were lower than those in PT180; and

the expression levels of four copper transporter genes (*ChCTR1*, *ChCTR2*, *ChCTR3*, and *ChCTR4*) in PT180 were lower than in TM17 (Fig. 1D). Therefore, we assumed that copper transporter genes might have some virulence function in *C. heterostrophus* on maize.

***ChCTR1* and *ChCTR4* are required for virulence of *C. heterostrophus* on maize.** All characterized CTR proteins contain multiple TM domains and Met-rich copper-binding motifs (Met-X_n-Met, *n* = 1 to 5) (Puig et al. 2002; Yuan et al. 2018). *ChCTR1* contains three TM domains and three Met-X_n-Met motifs, *ChCTR2* contains three TM domains and two Met-X_n-Met motifs, *ChCTR3* has two TM domains and one Met-X_n-Met motif, and *ChCTR4* has three TM domains and four Met-X_n-Met motifs (Fig. 2A).

In order to understand the function of those four *ChCTR* genes in *C. heterostrophus*, deletion mutants *chctr1*, *chctr2*, *chctr3*, and *chctr4* were generated by replacing the *ChCTR1*, *ChCTR2*, *ChCTR3*, and *ChCTR4* genes individually in *C. heterostrophus* TM17 with a hygromycin resistance gene (*HPH*) (Fig. 2B). For each mutant, two independent single spores from two independent transformants were selected for future assays. Successful knockout mutants were identified by PCR using three different pairs of primers targeting different fragments (Fig. 2B and C), and qRT-PCR results confirmed the loss of gene expression in those corresponding mutants (Fig. 2D). To examine their virulence, those mutants were inoculated on maize inbred line B73 by spray inoculation and the wild-type strain TM17 was taken as the control. At 4 dpi, plants inoculated with TM17 showed extensive necrotic lesions whereas

Fig. 2. Deletion of *ChCTR* genes by homologous recombination **A**, Schematic representation of the domains of the four *ChCTR* proteins in *Cochliobolus heterostrophus*. The transmembrane domains (TM) are indicated by the black boxes and the Met-rich copper-binding motifs are indicated by MxxM, MxxxM, or MxxMxxMxxMxxM; aa = amino acids. **B**, Diagram of strategy used for homologous recombination of the *hygromycin phosphotransferase* (*HPH*) gene. Primer pair F1 + F2 was used to amplify the left flanking region of target genes, primer pair F3 + F4 was used to amplify the right flanking region of target genes, primer pair C1 + C2 was used to amplify the genomic sequence of the target genes, and primer pair PTM39 + PTM40 was used to amplify the full length of *HPH* gene. Primers HP and PH, targeting in the middle region of *HPH*, were used for detecting mutants; primers PTM39, PTM40, HP, and PH are common primers. The other primers (F1, F2, C1, C2, F3, and F4) are designed based on specific target genes. **C**, PCR screening of deletion mutants. For each mutant, genomic DNA isolated from two independent transformants were used as the templates. Primer pair F1 + PH was used to indicate that the *HPH* gene was inserted in the correct position in the genome, primer pair C1 + C2 was used to indicate the deletion of the target gene, and primer pair PTM39 + PTM40 was used to indicate the successful insertion of the *HPH* gene in the genome. **D**, Reverse-transcription PCR to confirm the deletion of *ChCTR* genes in their corresponding mutants.

chctr1 and *chctr4* mutants produced fewer and limited lesions (Fig. 3A and B). Also, plants inoculated with *chctr1* and *chctr4* mutants accumulated much less fungal biomass than plants inoculated with TM17, measured by qRT-PCR amplification of *C. heterostrophus* *Actin* mRNA relative to maize *ZmUBQ* mRNA (Fig. 3D and E). Therefore, *ChCTR1* and *ChCTR4* are required for virulence of *C. heterostrophus* on maize. We also observed that plants inoculated with *chctr2* or *chctr3* mutants showed disease phenotype very similar to those of plants inoculated with the wild-type strain TM17 (Fig. 3C). Those data indicate that *ChCTR1* and *ChCTR4* positively regulate the pathogenicity of *C. heterostrophus* on maize but *ChCTR2* and *ChCTR3* are dispensable for it.

In order to understand the function of *ChCTR* genes during infection, the gene expression of those four *ChCTR* genes was checked before and after TM17 infection on maize, and RNA isolated from TM17 conidia was taken to check gene expression level before inoculation. *ChCTR1*, *ChCTR2*, and *ChCTR4* were

expressed well in conidia (at 0 h) and the expression levels of those three genes were reduced following infection (Fig. 3F to I), while the expression level of *ChCTR3* was reduced at 6 h after inoculation and was enhanced at 16 h after inoculation. This indicates that *ChCTR1*, *ChCTR2* and *ChCTR4* may mainly function in conidia and the early infection period, whereas *ChCTR3* functions in conidia and all infection stages.

ChCTR1 and ChCTR4 have copper transporter activity.

Because *ChCTR1*, *ChCTR2*, *ChCTR3*, and *ChCTR4* are annotated as copper transporters, we assumed that the four proteins might function in transporting copper ions. In order to test this hypothesis, we tested the role of *ChCTR1*, *ChCTR2*, *ChCTR3*, and *ChCTR4* in copper transport by functional complementation assay using the yeast *Saccharomyces cerevisiae* $\Delta ctr1\Delta ctr3$ double mutant, which lacks the copper transporter function of *CTR1* and *CTR3* and cannot grow on YPEG medium without copper ion supplement (Peña et al. 2000; Yuan et al. 2010). Although none of the four genes could

Fig. 3. Disease phenotype of *chctr* mutants on maize inbred line B73. **A**, Virulence assay of *chctr1* mutants and the wild-type strain TM17 on maize at 4 days postinoculation (dpi). Conidia of strain TM17 and *chctr1* mutants were inoculated on maize inbred line B73 and the disease phenotype was checked at 4 dpi. **B**, Virulence assay of *chctr4* mutants and strain TM17 on maize at 4 dpi. Conidia of strain TM17 and *chctr4* mutants were inoculated on maize inbred line B73 and the disease phenotype was checked at 4 dpi. **C**, Virulence assay of the *chctr2* mutant, *chctr3* mutant, and strain TM17 on maize at 4 dpi. Conidia of strain TM17 and mutants *chctr2* and *chctr3* were inoculated on maize inbred line B73 and the disease phenotype was checked at 4 dpi. **D**, Quantitative reverse-transcription PCR (qRT-PCR) to compare the fungal biomass on B73 at 4 days after TM17 and *chctr1* mutant inoculation; data represent mean values $\pm$ standard error (SE) from three technical replicates. Experiments were repeated at least three times with similar results. Mean values followed by different letters are significantly different from each other ($P < 0.01$, Student's *t* test). **E**, qRT-PCR to detect the fungal biomass on B73 at 4 days after TM17 and *chctr4* mutant inoculation; data represent mean values $\pm$ SE from three technical replicates. Experiments were repeated at least three times with similar results. **F**, qRT-PCR to detect the gene expression of *ChCTR1* before and after infection on maize B73 with strain TM17; data represent mean values $\pm$ SE from three technical replicates. Experiments were repeated at least three times with similar results. **G**, qRT-PCR to detect the gene expression of *ChCTR2* before and after infection on maize B73 with strain TM17; data represent mean values $\pm$ SE from three technical replicates. Experiments were repeated at least three times with similar results. **H**, qRT-PCR to detect the gene expression of *ChCTR3* before and after infection on maize B73 with strain TM17; data represent mean values $\pm$ SE from three technical replicates. Experiments were repeated at least three times with similar results. **I**, qRT-PCR to detect the gene expression of *ChCTR4* before and after infection on maize B73 with strain TM17; data represent mean values $\pm$ SE from three technical replicates. Experiments were repeated at least three times with similar results.

complement the yeast mutant phenotype without adding copper ions, expression of *ChCTR1* or *ChCTR4* alone complemented the yeast mutant phenotype and helped the growth of yeast on YPEG medium plus 40 or 60 μM copper sulfate whereas expression of *ChCTR2* or *ChCTR3* alone could not complement the phenotype of the yeast mutant on YPEG medium plus 40 and 60 μM copper sulfate (Fig. 4). Because *S. cerevisiae* requires CTR1 and CTR3 to uptake copper ions for survival on YPEG medium, the complementation phenotype by expression of ChCTR1 and ChCTR4 indicated that ChCTR1 and ChCTR4 are involved in copper uptake, while the failure of the complementation assay by expression of *ChCTR2* and *ChCTR3* indicated that ChCTR2 and ChCTR3 might not have copper transporter function or might not function in copper import. Because only *ChCTR1* and *ChCTR4* are required for virulence and copper uptake, we focus on *ChCTR1* and *ChCTR4* for further analysis.

***ChCTR1* and *ChCTR4* are dispensable for the growth of mycelia and abiotic stress adaptation.** In order to understand the function of *ChCTR1* and *ChCTR4* during the infection process, we tested the growth phenotypes of the wild-type strain TM17, *chctr1* mutants, and *chctr4* mutants on V8 medium containing different stress chemicals such as the osmotic stress agents NaCl and KCl, the oxidative-stress agent H_2O_2 , the cell-wall-disturbing agent SDS, and the iron-chelating agent 2DP (Liu et al. 2019). At 7 dpi of mycelial plugs, no difference was observed on V8 medium and on V8 with different agents between wild-type strain TM17, *chctr1* mutants, and *chctr4* mutants (Fig. 5), suggesting *ChCTR1* and *ChCTR4* are dispensable for the mycelium growth and the adaptation of *C. heterostrophus* to those infection-related stresses.

***ChCTR1* and *ChCTR4* are required for the appressorium formation.** Because appressoria were reported to be required for virulence of *C. heterostrophus*, we tested TM17, *chctr1* mutants, and *chctr4* mutants in the appressorium formation rate. Conidial suspensions of TM17, *chctr1* mutants, and *chctr4* mutants were dropped on the microscope cover glass; then, the appressorium formation from conidia was checked. At 150 min after dropping on

the glass, more than 66 to 75% of TM17 conidia generated typical appressoria (Fig. 6A, B, and C), whereas approximately 29% of conidia of the *chctr1-1* mutant, 23% of *chctr1-2*, 21% of *chctr4-1*, and 38% of *chctr4-2* generated typical appressoria (Fig. 6A and B). This indicated that *ChCTR1* and *ChCTR4* are required for appressorium formation and mutation of *ChCTR1* or *ChCTR4* significantly attenuates the formation of appressoria.

To avoid the possibility that the low appressorium formation rate of *chctr1* and *chctr4* mutants was caused by the low germination rate, we also checked the germination rates of wild-type strain TM17, *chctr1* mutants, and *chctr4* mutants at different time points. It was observed that almost all conidia germinated at 120 min after dropping on the glass and no difference was observed in the germination rate between TM17, *chctr1* mutants, and *chctr4* mutants at 120, 150, and 180 min after dropping on the glass (Fig. 6D and E).

Copper deficiency attenuates appressorium formation and virulence of *C. heterostrophus*. Because *ChCTR1* and *ChCTR4* are involved in acquiring copper ions and are required for virulence of *C. heterostrophus* on maize, we assumed that copper ions might also be required for virulence of *C. heterostrophus* on maize. At first, copper-chelating agent ammonium tetrathiomolybdate (TTM) was used to reduce free copper ions in the environment. We suspended the conidia of *C. heterostrophus* strain TM17 in different concentrations of TTM and sprayed those conidia on maize plants. At 4 dpi, plants inoculated by conidia without TTM showed extensive necrotic lesions while plants inoculated by conidia with different concentrations of TTM (1, 3, or 5 μM) showed limited lesions (Fig. 7A). Plants infected by conidia without TTM accumulated much more fungal biomass than plants infected by conidia with TTM (Fig. 7B). This indicated that copper deficiency attenuates virulence of *C. heterostrophus* on maize. Next, the germination rate and the rate of appressorium formation were checked by dropping conidial suspensions with different concentrations of TTM. Conidia of the wild-type strain TM17 with different concentrations of TTM showed the same level of germination rate as those without TTM, although 5 μM TTM slightly suppressed the germination rate (Fig. 7C). Therefore, copper deficiency does not significantly affect conidium germination of wild-type strain TM17, whereas we observed that the rate of appressorium formation was significantly suppressed by TTM. At 150 min after dropping on cover glass, 59% conidia of TM17 without TTM generated appressoria but only 20%, 3%, and 1% conidia of TM17 treated with 1, 3, or 5 μM TTM, respectively, generated appressoria (Fig. 7D). Also, we did observe the same phenotype that TTM suppressed appressorium formation at 180 and 210 min after dropping on the glass. This indicates that copper ions are specifically required for appressorium formation (Fig. 7D). In order to avoid the possibility that TTM affects the growth of mycelia, we inoculated the wild-type strain TM17 on V8 medium containing TTM. At 7 dpi, the diameter of all colonies reached approximately 5.5 cm and no big difference was observed on the growth of *C. heterostrophus* on V8 medium containing 0, 1, 5, and 10 μM TTM (Fig. 7E and F). This indicates that TTM does not affect the mycelium growth of *C. heterostrophus*. All of this evidence indicates that deficiency of copper ions significantly attenuates virulence and appressorium formation of *C. heterostrophus* during infection.

Copper ions enhance the appressorium formation and virulence of *C. heterostrophus*. To further confirm the function of copper ions, we tried to test whether copper ion treatment could enhance virulence and appressorium formation of *C. heterostrophus*. The conidia of TM17 suspended in different concentrations of CuSO_4 were inoculated on maize plants. Because the disease phenotype was too strong and no phenotype difference between those treatments was observed at 4 dpi, we analyzed the disease phenotype at 3 dpi. Unfortunately, we did not observe any different disease phenotype after inoculation of the wild-type strain TM17 with different concentrations of CuSO_4 at 3 dpi (Fig. 8A) and the fungal biomass

Fig. 4. Copper transporter activity of ChCTR proteins. To detect the role of ChCTR proteins in copper transport, plasmids containing the four *ChCTR* genes were individually expressed in *Saccharomyces cerevisiae* $\Delta ctr1\Delta ctr3$ mutant MPY17 to test whether expression of *ChCTR* genes could complement the mutant phenotype with or without adding CuSO_4 . YPEG = yeast extract, peptone, glycerol, and ethanol medium. In this assay, empty vector was taken as the negative control.

accumulation checked by qPCR also showed no difference between maize plants inoculated by TM17 with 0, 0.3, and 0.5 μM CuSO_4 (Fig. 8B). Moreover, the function of copper ions on the germination rate and appressorium formation was detected. The conidia of wild-type strain TM17 showed the same germination rate without or with different concentrations of CuSO_4 treatment at 120, 150, 180, and 210 min after dropping on cover glass (Fig. 8C), suggesting CuSO_4 did not affect the germination of *C. heterostrophus* conidia. At 150 min after dropping on the glass, 42% of conidia of TM17 without added CuSO_4 generated appressoria, while 70% of conidia of TM17 generated appressoria after adding 0.3 μM CuSO_4 and 83% of conidia of TM17 generated appressoria after adding 0.5 μM CuSO_4 . At 180 and 210 min after dropping on the glass, we also observed that conidia with CuSO_4 treatment generated more appressoria than conidia without CuSO_4 treatment (Fig. 8D). These results indicate that copper ions can enhance the formation of appressoria. In order to check whether copper ions affect the growth of mycelia, the mycelia of wild-type strain TM17 were inoculated on V8 medium containing different concentrations of CuSO_4 . At 7 dpi, the diameter of all colonies reached approximately 5.5 cm and no big difference was observed in the growth of *C. heterostrophus* on V8 medium plates containing 0, 0.3, 0.5, and 1 μM CuSO_4 (Fig. 8E and F). This evidence indicates that copper ions are required for appressorium formation.

Because copper ions significantly enhance appressorium formation but did not enhance virulence of *C. heterostrophus* on maize, we inferred that the conidium concentration (1×10^5 conidia/ml) for the regular inoculation was too high and the high conidium concentration might mask the function of copper ions. Therefore, low conidium concentration (0.5×10^5 conidia/ml) was used to test the function of copper ions on virulence on maize. At 4 dpi, some green tissue was observed on leaves inoculated by conidia suspended with water but the leaves inoculated by conidia suspended by 0.3 or 0.5 μM CuSO_4 were completely dead (Fig. 8G). Those results indicated that copper ions help appressorium formation and enhance virulence of *C. heterostrophus* on maize.

DISCUSSION

SLB of corn caused by *C. heterostrophus* is a destructive disease on maize fields worldwide but how *C. heterostrophus* regulates virulence on maize is largely unknown. Here, we report that copper ions are required for *C. heterostrophus* appressorium formation and virulence on maize. The deficiency of copper ions significantly attenuated virulence and appressorium formation of *C. heterostrophus* and copper ion treatment enhanced virulence on maize and appressorium formation during infection. Furthermore, *ChCTR1* and

Fig. 5. ChCTR1 and ChCTR4 are dispensable for infection-related stress. Mycelial growth of the wild-type strain TM17, *chctr1* mutants, and *chctr4* mutants on V8 medium plates containing different stress-mimetic agents: 1 mM NaCl, 1 mM KCl, 5 μM H_2O_2 , 0.005% sodium dodecyl sulfate (SDS) or 100 μM 2,2-bipyridyl (2DP) at 7 days after inoculation with fresh mycelial plugs. Control: V8 medium without any stress-mimetic agents.

ChCTR4, encoding copper transporter proteins, were identified to uptake copper ions for *C. heterostrophus* and to be required for appressorium formation and virulence on maize. Mutation of *ChCTR1* or *ChCTR4* significantly reduced the rate of appressorium formation and suppressed virulence on maize. Therefore, we conclude that *ChCTR1* and *ChCTR4* play critical roles in appressorium formation and virulence on maize through importing extracellular copper ions into *C. heterostrophus*.

This is the first report that copper ions are required for *C. heterostrophus* in appressorium formation and virulence on maize. It opens a new window to explore virulence mechanisms of *C. heterostrophus* on maize. Also, it is very useful to direct the design of new fungicides against SLB of corn. Because copper ions are

required for the development of the disease phenotype, it is better to include copper-chelating agents into fungicides and remove copper-containing agents from fungicides in order to protect maize plants from SLB of corn.

Copper ions are also an important mineral nutrient for all organisms and they function as a cofactor in many physiological processes (Smith et al. 2017). Recently, copper ions were reported to be critical for virulence of fungal pathogens *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* on humans (Mauch et al. 2013; Park et al. 2014; Smith et al. 2017; Sun et al. 2014), and copper ions act as a cofactor of multiple virulence factors such as laccases and copper- and zinc-containing superoxide dismutase (Hwang et al. 2002; Mauch et al. 2013). Also, copper ions

Fig. 6. *ChCTR1* and *ChCTR4* are required for appressorium (AP) formation. **A**, AP of wild-type strain TM17; the AP is indicated by arrows. Conidia of TM17 (1×10^5 conidia/ml) were dropped on cover glass and were checked by microscope at 180 min after dropping. **B**, Quantification of relative AP ratio between wild-type strain TM17 and *chctr1* mutants at 180 min after dropping on the glass; data represent mean values $\pm$ standard error (SE) from more than 200 spores. Experiments were repeated at least three times with similar results. Mean values followed by different letters are significantly different from each other ($P < 0.01$, Student's *t* test). **C**, Quantification of relative AP ratio between wild-type strain TM17 and *chctr4* mutants at 180 min after dropping on glass; data represent mean values $\pm$ SE from more than 200 spores. Experiments were repeated at least three times with similar results. **D**, Quantification of the germination rates between wild-type strain TM17 and *chctr1* mutants at 120, 150, and 180 min after dropping on glass; data represent mean values $\pm$ SE from more than 200 spores. Experiments were repeated at least three times with similar results. **E**, Quantification of the germination rates between wild-type strain TM17 and *chctr4* mutants at 120, 150, and 180 min after dropping on glass; data represent mean values $\pm$ SE from more than 200 spores. Experiments were repeated at least three times with similar results.

are required for hyphal formation in *Candida albicans*, and pulmonary infection and capsule formation in *Cryptococcus neoformans* (Cheng et al. 2013; Kronstad et al. 2013; Waterman et al. 2012). However, how copper ions affect virulence of plant fungal pathogens is unclear. *Cochliobolus heterostrophus* is a typical necrotrophic pathogen on maize, which generates appressoria to penetrate the cuticle to get into plants. Blocking appressorium formation suppresses its virulence on maize (Degani et al. 2004). Identification of copper ions required for appressorium formation indicates that some copper-dependent virulence factors

in *C. heterostrophus* play important roles in appressorium formation. Multiple G proteins and several MAPK proteins have been identified to play critical roles in appressorium formation but none of them were reported to require copper ions as a cofactor. Therefore, a copper-mediated pathway may be a new mechanism to regulate appressorium formation in *C. heterostrophus*. Diphenol oxidase laccase Lac1, a virulence protein in *Cryptococcus neoformans*, requires copper ions for its function in melanin biosynthesis (Walton et al. 2005). Although melanin provides resistance against oxidative stress and is required for virulence of multiple fungi such

Fig. 7. Copper-chelating agent ammonium tetrathiomolybdate (TTM) suppresses the formation of appressorium (AP) and virulence of *Cochliobolus heterostrophus*. **A**, Virulence assay of TM17 with different concentration of TTM on maize inbred line B73; the disease phenotype was checked at 4 days postinoculation. **B**, Quantitative reverse-transcription PCR to compare the fungal biomass on B73 at 4 days after TM17 inoculation with different concentrations of TTM; data represent mean values $\pm$ standard error (SE) from three technical replicates. Experiments were repeated at least three times with similar results. Mean values followed by different letters are significantly different from each other ($P < 0.01$, Student's t test). **C**, Compare the germination rate of wild-type strain TM17 treated by different concentrations of TTM at different time points after dropping on cover glass. Germination rate = number of germinated spores/total number of spores; data represent mean values $\pm$ SE from more than 200 spores. Experiments were repeated at least three times with similar results. **D**, Compare the AP formation of wild-type strain TM17 treated by different concentration of TTM at different time points after dropping on glass. AP formation ratio = number of spores forming AP/total number of germinated spores; data represent mean values $\pm$ SE from more than 200 spores. Experiments were repeated at least three times with similar results. **E**, Compare the growth of wild-type strain TM17 on V8 medium containing different concentrations of TTM. **F**, Diameter of colony growing on V8 medium containing different concentrations of TTM.

as *Wangiella dermatitidis* and *C. neoformans* (García-Santamarina and Thiele 2015), melanin is not required for virulence and development in *Cochliobolus heterostrophus* (Eliahu et al. 2007). Therefore, identification of more proteins in the copper-mediated pathway will be helpful to elucidate the molecular mechanisms of appressorium formation and virulence in *C. heterostrophus*.

Although it was reported that copper ions are also required for development and abiotic stress resistance in fungi such as *A. fumigatus* and *Cryptococcus neoformans* (Cai et al. 2017;

Zhang et al. 2016), copper sulfate and copper-chelating agent TTM did not affect *Cochliobolus heterostrophus* growth on medium; moreover, *chctr1* and *chctr4* mutants did not show any significantly different growth phenotype from the wild-type strain TM17 on medium with or without different stress-related agents (Fig. 5). This evidence indicates that copper ions are not required for the development of *C. heterostrophus*. Therefore, our results indicate that copper ions are specifically required for appressorium formation.

Fig. 8. Copper affects the formation of appressorium (AP) and virulence of *Cochliobolus heterostrophus* on maize. **A**, Virulence assay of TM17 with different concentrations of CuSO_4 on maize inbred line B73. TM17 conidia at 1×10^5 conidia/ml suspended with 0, 0.3, and 0.5 μM CuSO_4 were sprayed on the leaves of 3-week-old maize inbred line B73. The phenotype was photographed at 3 days postinoculation (dpi). **B**, qRT-PCR to compare the fungal biomass on B73 at 4 days after TM17 inoculation with different concentrations of CuSO_4 ; data represent mean values $\pm$ standard error (SE) from three technical replicates. Experiments were repeated at least three times with similar results. Mean values followed by different letters are significantly different from each other ($P < 0.01$, Student's *t* test). **C**, Compare the germination rate of wild-type strain TM17 treated by different concentrations of CuSO_4 . Germination rate = number of germinated spores/total number of spores; data represent mean values $\pm$ SE from more than 200 spores. Experiments were repeated at least three times with similar results. **D**, Compare the AP formation of wild-type strain TM17 treated by different concentrations of CuSO_4 . AP formation ratio = number of spores forming AP/total number of germinated spores; data represent mean values $\pm$ SE from more than 200 spores. Experiments were repeated at least three times with similar results. **E**, Compare the growth of wild-type strain TM17 on V8 medium containing different concentrations of CuSO_4 ; data represent mean values $\pm$ SE from three plates. Experiments were repeated at least three times with similar results. **F**, Diameter size of wild-type strain colony on V8 medium containing different concentrations of CuSO_4 ; data represent mean values $\pm$ SE from three plates. Experiments were repeated at least three times with similar results. **G**, Compare the virulence of TM17 with different concentrations of CuSO_4 treatment on maize at 4 dpi with 0.5×10^5 conidia/ml. TM17 conidia at 0.5×10^5 conidia/ml suspended with 0, 0.3, and 0.5 μM CuSO_4 were sprayed on the leaves of 3-week-old maize inbred line B73; at 3 dpi, the phenotype was photographed.

Most of the CTR proteins are characterized by three TM domains and three CTR monomers form a homotrimer as a functional copper transporter unit (Puig et al. 2002; Smith et al. 2017). Molecular and structural analysis revealed that the extracellular N-terminal Met-rich domain, the Met-X_n-Met motif, and the cytosolic C-terminal Cys-His-Cys motif are potential copper-binding sites necessary for effective copper transport (Smith et al. 2017). All four ChCTR proteins contain the extracellular N-terminal Met-rich domain and the Met-X_n-Met motif but none of them have the cytosolic C-terminal Cys-His-Cys motif. A yeast complementation assay showed that ChCTR1 and ChCTR4 have copper-importing activity but ChCTR2 and ChCTR3 do not. Compared with ChCTR2 and ChCTR3, ChCTR1 and ChCTR4 contains more Met-X_n-Met motifs (Fig. 2A). Therefore, the number of Met-X_n-Met motifs might be critical for the copper-importing activity.

Multiple copper transporters have been identified from different fungi and they play different roles in development and stress responses. Three copper transporters (Ctr1, Ctr2, and Ctr4) in *Cryptococcus neoformans* were reported to be required for fungal pathogenesis (Ding et al. 2011; Waterman et al. 2007, 2012) but which infection stage is affected in these mutants is unclear. The reduced appressorium formation rate in *chctr1* and *chctr4* mutants indicated that copper transporters in other fungi might play the same roles in appressorium formation.

In summary, the copper ion-mediated pathway is required for the pathogenicity of *Cochliobolus heterostrophus* on maize. However, how copper affects virulence and appressorium formation in *C. heterostrophus* is still unclear. Identification of fungal proteins taking copper ions as cofactors would help explore the molecular mechanism. The candidate protein should play important roles in appressorium formation.

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