NADPH Oxidase CINOX2 Regulates Melanin-Mediated Development and Virulence in *Curvularia lunata*

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The role of NADPH oxidases (NOXs) in pathogenesis and development in the *Curvularia* leaf spot agent *Curvularia lunata* remains poorly understood. In this study, we identified *C. lunata* CINOX2, which localized to the plasma membrane and was responsible for reactive oxygen species (ROS) generation. Scavenging the ROS production inhibited the conidial germination and appressorial formation. The CINOX2 and CIBRN1 deletion mutants were defective in 1,8-dihydroxynaphthalene (DHN) melanin accumulation, appressorial formation, and cellulase synthesis and exhibited lower virulence. However, disruption of the CINOX2 and CIBRN1 genes facilitated hyphal growth, enhanced stress adaptation to cell-wall-disrupting agents, and promoted developmental processes such as conidiation, conidial germination, and pseudothecium and ascus formation. Interestingly, loss of CIMI, the cell wall integrity (CWI) mitogen-activated protein kinase gene in *C. lunata*, led to morphology and pathogenicity phenotypes similar to CINOX2 and CIBRN1 deletion mutants such as abnormal conidia, fewer appressoria, less melanin, increased hyphal growth, and enhanced tolerance to Congo red (CR). These results indicated that the CINOX2 gene plays an important role in *C. lunata* development and virulence via regulating intracellular DHN melanin biosynthesis. Quantitative reverse-transcription PCR revealed that the CINOX2-related ROS signaling pathway and CIMI-mediated CWI signaling pathway are cross-linked in regulating DHN melanin biosynthesis. Our findings provide new insights into how CINOX2 participates in pathogenesis and development in hemibiotrophic plant fungal pathogens.

Keywords: appressorium, *Curvularia lunata*, development and virulence, DHN melanin, NADPH oxidase 2, reactive oxygen species (ROS)

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Reactive oxygen species (ROS) are pivotal signaling molecules that play essential roles in cell–cell and host–pathogen interactions (Apel and Hirt 2004). There is growing evidence that ROS are vital for vegetative development and pathogenic processes in pathogenic fungi (Heller and Tudzynski 2011). The NADPH oxidase (NOX) complex, as a major ROS-producing enzyme system, comprises multiple subunits (Lambeth 2004). The best-characterized are NOX1 and NOX2, the membrane-associated functional subunits that form the catalytic core of the complex. A great variety of functions are ascribed to fungal NOX1 and NOX2. In *Neurospora crassa*, NOX-1 elimination results in complete female sterility, decreased asexual development, and reduction of hyphal growth (Cano-Domínguez et al. 2008). In *Verticillium dahliae*, VdNoxB/VdPls1-mediated ROS production activates VdCrz1 signaling through Ca²⁺ elevation in hyphopodia (infectious structures of *V. dahliae*) to regulate penetration peg formation during the initial colonization of cotton roots (Zhao et al. 2016).

The cell wall integrity signaling (CWIS) pathway is responsible for the remodeling and repair of damaged cell walls (Yoshimi et al. 2013). The cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) plays a prominent role in the CWIS pathway. Deletion of the CWI MAPK genes *mps1*, *cpmk2*, and *mgv1* in *Magnaporthe grisea*, *Claviceps purpurea*, and *Fusarium graminearum*, respectively, results in weakened cell walls that are more sensitive to cell wall lytic enzymes, nikkomycin Z (a chitin synthase inhibitor), and calcofluor white (Hou et al. 2002; Mey et al. 2002). CWI MAPK genes are involved in fungal development and pathogenesis. *MAK1* in *N. crassa* and *PaMpk1* in *Podospora anserina* are crucial for the formation of conidial anastomosis tubes (Chan Ho Tong et al. 2014). In *Botrytis cinerea*, the CWI MAPK gene *Bmp3* is essential for sclerotia formation, conidial production, and full virulence (Rui and Hahn 2007). In *Mycosphaerella graminicola* and *C. purpurea*, deletion of CWI MAPK fails to form appressoria or infection nail (Mehrabi et al. 2006; Mey et al. 2002).

Deletion of NOX and CWI MAPK genes causes complete or partial reduction of fungal melanin by inhibiting the expression of melanin biosynthesis genes (Egan et al. 2007; Malagnac et al. 2004). Melanin is an amorphous polymer that is produced by a variety of microbes and higher eukaryotes (Upadhyay et al. 2013). The main type of fungal melanin, 1,8-dihydroxynaphthalene (DHN) melanin, is synthesized from acetyl CoA or malonyl CoA via the polyketide pathway that involves multiple enzymes, including polyketide synthase, scytalone dehydratase (SCD1), and hydroxynaphthalene reductases (BRN1/BRN2) (Gao and Chen 2017; Kihara et al. 2004; Kimura and Tsuge 1993). The other type of melanin, 3,4-dihydroxyphenylalanine (DOPA),

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requires the activity of a polyphenol oxidase and an exogenous o-diphenolic or p-diphenolic substrate (Nosanchuk et al. 2002; Nurudeen and Ahearn 1979). Melanin is found to be located in the fungal cell wall as layers of globular particles and a structural component, or externalized from the fungal cells and often loosely bound to the cell wall exterior (Eisenman et al. 2005). Melanin not only enables organisms to survive and adapt to extreme environmental conditions but also protects the fungi against host resistance during infection. In plant-pathogenic fungi *Colletotrichum lagenarium* and *Magnaporthe grisea*, melanin has a vital role in forming melanized appressoria to penetrate the host cell (Chumley and Valent 1990; Perpetua et al. 1996).

Curvularia lunata (Wakker) Boedijn 1933 (teleomorph: *Cochliobolus lunatus*), is a known human and plant pathogen (Khan et al. 2017; Shrivastava et al. 2017). *Curvularia* leaf spot, caused by *Curvularia lunata*, is distributed in major maize-planting regions worldwide (Garcia-Aroca et al. 2018; Wang et al. 2019). *C. lunata* utilizes appressoria and pathogenicity-associated factors such as melanin, toxin, and cell-wall-degrading enzymes to infect and destroy maize leaves (Wang et al. 2019). Although NOXs have been reported to be involved in various cellular processes in plant fungal pathogens (Takemoto et al. 2007), the role of NOXs in developmental and pathogenic processes in hemibiotrophic *C. lunata* is unknown. In this study, we identified the O_2^- -generating gene *CINOX2*, which regulates the DHN melanin biosynthesis, asexual and sexual development, CWI, secondary metabolism, and virulence in *C. lunata*. Furthermore, our data showed that the regulated synthesis of DHN melanin by *C. lunata* *CINOX2* might affect cytoskeleton remodeling, thereby affecting *C. lunata* development and pathogenicity. The *CINOX2*-derived ROS signal pathway and *CIMI*-mediated CWI signaling pathway are crosslinked in regulating DHN melanin biosynthesis.

RESULTS

ROS are required for development and melanin biosynthesis in *C. lunata*.

To determine the roles of ROS in *C. lunata*, we first examined ROS generation using 2',7'-dichlorofluorescein diacetate (H₂DCFDA). This cell-permeable indicator remains non-fluorescent until it is hydrolyzed and oxidized within the cell to yield dichlorofluorescein (DCF). DCF launches a green fluorescence when it is excited by a laser beam of 504 nm (LeBel et al. 1992). Microscopic observation showed that ROS were produced within conidia, germ tubes, and appressoria but large amounts of ROS were not observed in incipient appressoria (Supplementary Fig. S1). Ascorbic acid (AA), an oxygen radical scavenger (Smirnoff 2000), was further used to explore the role of ROS in *C. lunata* development. The *C. lunata* wild-type (WT) strain CX-3 was cultured on complete medium (CM) supplemented with 10 and 100 mM AA. The CX-3 strain incubated on CM was used as control. AA inhibited hyphal growth, and the effects were AA concentration dependent (Fig. 1A). The growth rate of CX-3 cultured on CM containing 10 and 100 mM AA was 85 and 24%, respectively, that of the control (Fig. 1B). Accordingly, conidial yield with 10 and 100 mM AA supplementation was 53 and 9%, respectively, that of the control (Fig. 1C). Moreover, AA addition delayed conidial germination. At 2, 4, 8, and 24 h postinoculation (hpi), the germination rate of CX-3 incubated in 10 mM AA was 88, 85, 93, and 100%, respectively, that of the control (Fig. 1D). When exposed to 100 mM AA, CX-3 colonies exhibited reduced pigmentation (Fig. 1A). The germination rate of CX-3 incubated in 100 mM AA was 70, 69, 83, and 94% that of the control at 2, 4, 8, and 24 hpi, respectively (Fig.

1D). Germ tubes displayed abnormal morphology at all concentrations of AA used (Fig. 1E). At 2, 4, and 8 hpi, the germ tube length of CX-3 in 10 mM AA was 66, 53, and 33%, respectively, that of the control, while the germ tube length in 100 mM AA was 44, 34, and 15%, respectively, that of the control (Fig. 1F). More remarkable than the delayed germination caused by AA treatment was its inhibition on appressorial formation. The number of conidia able to form appressoria with 10 mM AA supplementation was 58% that of the control whereas, with 100 mM AA supplementation, appressoria were almost absent (Fig. 1G).

Diphenylene iodonium (DPI) inhibits NOX activity, thereby blocking superoxide anion (O_2^-) generation (O'Donnell et al. 1993). At 2 hpi, the germination rate of CX-3 exposed to 25 and 250 μ M DPI was 93 and 87%, respectively, that of the control (Fig. 1H). Accordingly, germ tube length with 25 and 250 μ M DPI treatment was 70 and 54%, respectively, that of the control (Fig. 1I and J). At 4 hpi, the number of conidia able to form appressoria in the presence of 25 and 250 μ M DPI was approximately 88 and 75%, respectively, that of the control (Fig. 1K). Taken together, the results showed that ROS are generated in hyphae, conidia, and appressoria of *C. lunata* during germination and appressorial formation. ROS are required for melanin biosynthesis, conidiation, germination, hyphal development, and appressorial formation in *C. lunata*.

Identification of *CINOX2* and phylogenetic analysis.

The protein sequence of NADPH oxidase B (GenBank accession number OWY44306) of *Alternaria alternata* was BLASTed against the *C. lunata* strain m118 database (JGI MycoCosm fungal genome resource) for ortholog identification. The sequence of the putative *NOX2* gene of the *C. lunatus* m118 strain was then BLASTed against the *C. lunata* strain CX-3 genome provided by the NCBI (Gao et al. 2014). A single ortholog was identified and designated as *CINOX2*. CX-3 genomic DNA and RNA were used as templates to amplify the *CINOX2* gene and its cDNA, respectively, before sequencing and submission to NCBI. The *CINOX2* gene (GenBank accession number MN205394) contains 1,686 bp and four introns. The *CINOX2* protein (GenBank accession number QIE06315) consists of 561 amino acids (aa) and exhibits significant homology with members of the fungal NOX2 clade (Supplementary Fig. S2). *C. lunata* *BRN1* was designated as *CIBRN1*. Its cDNA sequence (GenBank accession number DQ358052) and protein sequence (GenBank accession number ABC86900) were obtained from NCBI (Lanisnik Rižner and Wheeler 2003; Liu et al. 2011). *CIBRN1* is 912 bp long and encodes a 267-aa product. *CINOX2* protein sequence alignments were generated with ClustalW. MEGA6 software was used to construct a phylogenetic tree (Tamura et al. 2013). To evaluate the biological functions of *CINOX2* and *CIBRN1*, the genes were deleted in the hemibiotrophic *C. lunata* strain CX-3, and the resultant mutant strains ($\Delta Clnox2$ and $\Delta Clbrn1$) were complemented by respectively integrating a single copy of *CINOX2* or *CIBRN1* (Supplementary Figs. S3 and S4).

CINOX2 and *CIBRN1* regulate *C. lunata* hyphal growth and biomass.

To determine the roles of *CINOX2* and *CIBRN1* in *C. lunata* hyphal growth, we cultured the WT strain CX-3, $\Delta Clnox2$ and $\Delta Clbrn1$ mutant strains, and $\Delta Clnox2$ -C and $\Delta Clbrn1$ -C complemented strains on potato dextrose agar (PDA), CM, and minimal medium (MM) plates. Compared with the WT CX-3, $\Delta Clnox2$ and $\Delta Clbrn1$ showed very similar phenotypes (Fig. 2A). Both mutants produced less melanin, and melanin was hardly observed in $\Delta Clnox2$ during the first 2 days (Supplementary Fig. S5). Moreover, the hyphal growth of the $\Delta Clnox2$ and $\Delta Clbrn1$ strains was slightly faster than that of CX-3 grown

Fig. 1. Exposure to antioxidants affects hyphal growth, melanin biosynthesis, and development in *Curvularia lunata*. Ascorbic acid (AA) inhibits hyphal growth and melanin biosynthesis in *C. lunata*. The CX-3 strain was cultured on complete medium (CM) containing 10 and 100 mM AA for 5 days. Then, the growth was **A**, photographically documented and **B**, quantitatively analyzed. **C**, Conidiation of strains shown in **A** was assessed. AA inhibits conidial germination in *C. lunata*. Conidia of CX-3 were incubated in 10 and 100 mM AA for the indicated hours before **D**, the germination rate was calculated. **E**, Germinated conidia were photographed and **F**, the germ tube length was measured. **G**, AA abolishes appressorial formation. Conidia able to form appressoria were examined at 4 h postinoculation (hpi). Diphenyleneiodonium chloride (DPI) delays conidial germination. Conidia of CX-3 were incubated in 25 and 250 μM DPI before **H**, the germination rate was calculated at 2 hpi. **I**, Germinated conidia were imaged and **J**, the germ tube length was measured at 4 hpi. **K**, DPI affects the development of infection-related structures in *C. lunata*. ND = not detected. Bar = 20 μm. Data represent means ± standard error of the mean from three independent experiments. Asterisks *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

Fig. 2. *CINOX2* and *CIBRN1* play roles in *Curvularia lunata* growth and asexual development. Loss of *CINOX2* or *CIBRN1* promotes fungal growth and biomass accumulation. CX-3, $\Delta Clnox2$, $\Delta Clnbrn1$, $\Delta Clnox2-C$, and $\Delta Clnbrn1-C$ were incubated on solid potato-dextrose agar (PDA), complete medium (CM), and minimal medium (MM) for 5 days before **A**, colony morphologies were imaged and **B**, hyphal growth was quantified. **C**, Tested strains were cultured in liquid CM for 5 days before culture fluids (top panel), culture filtrates (middle panel), and freeze-dried mycelia (bottom panel) were photographed. **D**, Biomass of the tested strains. Disruption of *CINOX2* or *CIBRN1* **E**, promotes conidiation; **F**, reduces 1,8-dihydroxynaphthalene melanin accumulation on conidia and appressorial tips; and **G**, alters conidial size. *CINOX2* and *CIBRN1* deletion mutants have **H**, increased germination rates but **I**, impaired appressorial formation. Bar = 15 μm . Data represent means $\pm$ standard error of the mean from three independent experiments. Asterisks *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

on different media. (Fig. 2B). To investigate the roles of *CINOX2* and *CIBRN1* in the determination of fungal biomass, the WT, mutants, and complemented strains were incubated in liquid CM. Culture filtrates of the CX-3 and complemented strains were transparent and colorless, whereas the filtrates of $\Delta Clnox2$ and $\Delta Clbrn1$ were turbid and pigmented (Fig. 2C), meaning a greater amount of pigment was released into the media by the $\Delta Clnox2$ and $\Delta Clbrn1$ mutants. In addition, the biomass of $\Delta Clnox2$ and $\Delta Clbrn1$ was increased compared with that of the WT strain (Fig. 2D). These results showed that deletion of *CINOX2* and *CIBRN1* facilitates *C. lunata* hyphal growth and biomass accumulation.

***CINOX2* and *CIBRN1* are redundant for conidiation and conidial germination but are required for normal conidia and appressorial formation in *C. lunata*.**

To test whether *CINOX2* and *CIBRN1* affect *C. lunata* conidial development, conidia of the tested strains were collected from PDA plates at 5 days postinoculation (dpi). Analysis of conidiation revealed that the conidial yield of $\Delta Clnox2$ and $\Delta Clbrn1$ was significantly increased compared with the WT (Fig. 2E). Both mutants produced smaller conidia, and the conidia and appressorial tips of the mutants were coated with less DHN melanin (Fig. 2F). The length and width of the conidia in $\Delta Clnox2$ were approximately 89 and 88%, respectively, of those of CX-3, and $\Delta Clbrn1$ produced narrower conidia with normal length (Fig. 2G). At 2, 4, and 6 hpi, the relative germination rate was 137, 113, and 105%, respectively, in $\Delta Clnox2$ and 141, 117, and 105%, respectively, in $\Delta Clbrn1$ compared with the WT (Fig. 2H). The appressorial formation of the tested strains was further quantitatively analyzed. Appressorial formation of $\Delta Clnox2$ was similar to that of $\Delta Clbrn1$, and they all were approximately 70% that of the WT strain (Fig. 2I). Our data showed that *CINOX2* and *CIBRN1* are redundant for conidiation and conidial germination but are required for conidial development, appressorial formation, and melanin biosynthesis in *C. lunata*.

***CINOX2* and *CIBRN1* negatively regulate sexual reproduction.**

To explore the functions of *CINOX2* and *CIBRN1* in sexual reproduction, three outcrosses were set up: CX-3 $\times$ ZD958 (control), $\Delta Clnox2 \times$ ZD958, and $\Delta Clbrn1 \times$ ZD958. As a result, melanin-pigmented pseudothecia and asci were produced on the surface of maize leaf substrates in all cross matings (Fig. 3A). Quantitative analysis indicated that the pseudothecium number of the $\Delta Clbrn1 \times$ ZD958 and $\Delta Clnox2 \times$ ZD958 crosses was approximately 160 and 140%, respectively, that of the CX-3 $\times$ ZD958 cross (Fig. 3B). The corresponding ascus number was approximately 128 and 120%, respectively, that of CX-3 $\times$ ZD958 (Fig. 3C). Our data demonstrated that the *CINOX2* and *CIBRN1* genes play a role in *C. lunata* sexual reproduction and negatively regulate the production of pseudothecia and asci.

***CINOX2* and *CIBRN1* regulate *C. lunata* CWI, oxidative-stress adaptation, and sensitivity to intracellular superoxide radicals.**

To test whether *CINOX2* and *CIBRN1* affect fungal stress adaptation, we measured the sensitivity of the WT CX-3, mutants, and complemented strains to various stress-mimicking agents, including the intracellular O_2^- -producing mimetic agent menadione (Kreiner et al. 2002), cell-wall-perturbing reagents sodium dodecyl sulfate (SDS) and Congo red (CR), oxidative stress agent hydrogen peroxide (H_2O_2), and osmotic stress agent NaCl. Compared with the WT, the stress-adaptive phenotypes of the $\Delta Clnox2$ and $\Delta Clbrn1$ strains were markedly similar, except for pigmentation (Fig. 4A). For example, the

$\Delta Clnox2$ and $\Delta Clbrn1$ mutants grown on CM plates amended with CR did not have transparent halos, suggesting that cellulase was absent in both mutants. $\Delta Clnox2$ grown on CM plates with added H_2O_2 had sharply reduced pigmentation, whereas pigmentation in the $\Delta Clbrn1$ strain under H_2O_2 showed minimal change. In comparison with the WT, $\Delta Clnox2$ and $\Delta Clbrn1$ displayed decreased sensitivity to CR, SDS, and menadione (Fig. 4B and C) but not to H_2O_2 and NaCl (Fig. 4D and E). The relative inhibition rate of the $\Delta Clnox2$ and $\Delta Clbrn1$ mutants was 33 and 30%, respectively, under CR at $60 \mu g\ ml^{-1}$ and was 89 and 87%, respectively, under 0.01% SDS compared with the WT (Fig. 4B). The relative inhibition rate of the $\Delta Clnox2$ and $\Delta Clbrn1$ mutants was 61 and 60%, respectively, compared with the WT when the tested strains were treated by $60 \mu M$ menadione (Fig. 4C). These findings showed that *CINOX2* and *CIBRN1* are involved in CWI and cellulase synthesis, and confer *C. lunata* sensitivity to intracellular superoxide radicals.

***CINOX2* and *CIBRN1* are required for *C. lunata* virulence.**

The $\Delta Clnox2$ and $\Delta Clbrn1$ mutants induced fewer necrotic lesions compared with the WT and complemented strains at 6 dpi (Fig. 4F). The disease index related to $\Delta Clnox2$ and $\Delta Clbrn1$ was 62 and 70%, respectively, that of the WT strain (Fig. 4G). The lesion number caused by $\Delta Clnox2$ and $\Delta Clbrn1$ was 45 and 49%, respectively, that of CX-3 (Fig. 4H). The length and width of lesions induced by $\Delta Clnox2$ were 130 and 126%, respectively, of those of CX-3. The lesion length and width caused by $\Delta Clbrn1$ were 131 and 110%, respectively, of those of CX-3 (Fig. 4I). The above data showed that *CINOX2* and *CIBRN1* play vital roles during *C. lunata* early infection.

***CINOX2* localizes to the plasma membrane.**

NOX protein localization in fungi has been well characterized (Schürmann et al. 2013; Siegmund et al. 2015; C. Zhang et al. 2016). In *C. lunata*, *CINOX2* was predicted to localize to the plasma membrane. We further constructed the H3Pro:: *CINOX2-mCherry* vector that can constitutively express *CINOX2-mCherry* fusion protein (Fig. 5A). We introduced H3Pro::H2B-*mCherry* and H3Pro:: *CINOX2-mCherry* vectors into the CX-3 strain. H2B is a nuclear-localized protein from *M. grisea*. Analysis of the nuclear localization of H2B showed that *C. lunata* is a unique hyphomycete containing multiple cell nuclei. In *C. lunata* conidia, *CINOX2* localized to the plasma membrane and was absent within conidial septa. In germinating conidia, *CINOX2* localized to the plasma membrane as well as to some intracellular organelles in conidia and hyphae (Fig. 5B). In *P. anserina*, the NOX complex was found to be assembled in the endoplasmic reticulum (ER) and the vacuolar system (Lacaze et al. 2015). Therefore, we speculated that these intracellular organelles might be ER and vesicles originating from the ER.

Disruption of *CINOX2* impairs O_2^- and H_2O_2 production.

The O_2^- and H_2O_2 levels in the WT CX-3, $\Delta Clnox2$ mutant, and $\Delta Clnox2$ -C complemented strain were examined using nitro blue tetrazolium chloride (NBT) and 3,3'-diaminobenzidine (DAB) staining approaches. NBT forms a dark-blue formazan precipitate when reduced by O_2^- (Malagnac et al. 2004), while DAB can react with H_2O_2 to generate deep-brown polymer (Begum et al. 2011). The conidia and elongating germ tubes of the WT and complemented strain were more heavily stained by NBT and DAB than those of the $\Delta Clnox2$ mutant (Fig. 6A). The gray value produced by NBT and DAB staining in $\Delta Clnox2$ hyphae was 73 and 32%, respectively, that of CX-3 (Fig. 6B), suggesting that disruption of *CINOX2* severely

impairs O_2^- and H_2O_2 production in *C. lunata* hyphae. ROS production in conidia and appressoria of the tested strains was further inspected using H_2DCFDA . The results showed that the conidia and appressoria of $\Delta Clnox2$ emitted a weaker fluorescent signal than the WT and complemented strains

(Fig. 6C). The relative gray value of the conidia and appressoria in the $\Delta Clnox2$ mutant was 75 and 78%, respectively, that of CX-3 (Fig. 6D), suggesting that loss of *CINOX2* results in less intracellular ROS production in conidia and appressoria.

Fig. 3. *CINOX2* and *CIBRN1* negatively regulate pseudothecium and ascus production in *Curvularia lunata*. **A**, Morphological characteristics of pseudothecia and asci generated on the surface of maize leaf substrates in cross matings of CX-3 (*MAT1-2*) × ZD958 (*MAT1-1*), $\Delta Clnox2$ (*MAT1-2*) × ZD958 (*MAT1-1*), and $\Delta Clbrn1$ (*MAT1-2*) × ZD958 (*MAT1-1*). **B**, Pseudothecium number per square centimeter of maize leaves and **C**, ascus number per pseudothecium. At least 25 pseudothecia were opened and the ascus number in each pseudothecium was recorded. Data represent means $\pm$ standard error of the mean from three independent experiments. Asterisk (*) indicates $P < 0.05$.

Fig. 4. Stress sensitivity and virulence assay of *ClNOX2* and *ClBRN1* deletion mutants. In a sensitivity assay of $\Delta Clnox2$ and $\Delta Clbrn1$ to stress-mimicking agents, CX-3, $\Delta Clnox2$, $\Delta Clbrn1$, and the complemented strains were inoculated on complete medium (CM) amended with sodium dodecyl sulfate (SDS), Congo red (CR), NaCl, hydrogen peroxide (H_2O_2), and menadione. **A**, Colony morphologies were photographed at 5 days postinoculation. Red arrows indicate the transparent halos. Loss of *ClNOX2* or *ClBRN1* reduced the sensitivity to **B**, SDS and CR and **C**, menadione but did not affect stress adaption to **D**, H_2O_2 and **E**, NaCl. $\Delta Clnox2$ and $\Delta Clbrn1$ displayed lower virulence than CX-3 and complemented strains. **F**, Disease symptoms caused by CX-3, $\Delta Clnox2$, $\Delta Clbrn1$, $\Delta Clnox2-C$, and $\Delta Clbrn1-C$. Conidial suspensions (1×10^6 conidia ml^{-1}) of the tested strains were sprayed on the fourth leaf of maize plants. Tween 20 (0.02%) was used as a control (mock). Photographs were obtained at 6 days postinoculation. Pathogenicity shown in **F** was inspected. **G**, Disease index and **H**, lesion number but not **I**, lesion size caused by $\Delta Clnox2$ and $\Delta Clbrn1$ were decreased. Data represent means $\pm$ standard error of the mean from three independent experiments. Asterisks ** and *** indicate $P < 0.01$ and 0.001 , respectively.

***CINOX2* and *CIBRN1* are involved in melanin biosynthesis.**

To confirm the roles of *CINOX2* and *CIBRN1* in melanin production, intracellular DHN and extracellular DOPA melanin were extracted using the acidity-alkali sedimentation method (Gao et al. 2015). Melanin detection showed that intracellular melanin produced by the $\Delta Clnox2$ mutant was approximately 44% that of CX-3, and intracellular melanin in $\Delta Cibrn1$ was hardly detected (Fig. 6E). In contrast, extracellular melanin produced by $\Delta Clnox2$ and $\Delta Cibrn1$ was much more than that of CX-3 (Fig. 6F). To explore the regulatory roles of *CINOX2* in DHN melanin biosynthesis, mRNA levels of the DHN melanin biosynthesis genes *CISCD1* (GenBank accession number BAJ83952) and *CIBRN1* were examined in CX-3 and $\Delta Clnox2$ mutant. The expression level of *CISCD1* in the $\Delta Clnox2$ mutant was decreased by approximately fourfold compared with WT, while the expression level of *CIBRN1* remained unchanged (Fig. 6G). These results suggest that *CINOX2* regulates the transcription of *CISCD1* but not *CIBRN1* in *C. lunata*.

***CIM1* regulates the development and virulence of *C. lunata*.**

The MAPKs (*Clm1*, *Clh1*, and *Clk1*) identified in *C. lunata* are reported to positively regulate the transcription of DHN melanin biosynthesis genes and generation of acetyl-CoA (Gao et al. 2013; Ni et al. 2018; Wang and Chen 2011). Among the $\Delta Clm1$, $\Delta Clh1$, and $\Delta Clk1$ mutants, $\Delta Clm1$ has a visible discoloration phenotype (Wang and Chen 2011). Compared with the WT and $\Delta Clm1$ -C, $\Delta Clm1$ displayed faster growth (Fig. 7A and B) and increased germination rates (Fig. 7E). Conidia of $\Delta Clm1$ are smaller and coated with less DHN melanin (Fig. 7C). The *CIM1* deletion mutant was less sensitive to CR in comparison with the WT and $\Delta Clm1$ -C (Fig. 7A and D). Additionally, conidiation (Fig. 7F), cellulase synthesis (Fig. 7A),

and appressorial formation (Fig. 7G) were inhibited in the $\Delta Clm1$ mutant, which leads to virulence reduction (Fig. 7H to J).

***CINOX2*- and *CIM1*-mediated signaling pathways regulate *C. lunata* melanin biosynthesis.**

The above results suggest that the morphology, development, and pathogenicity of $\Delta Clm1$ are associated with reduced levels of DHN melanin. Quantitative reverse-transcription PCR (RT-qPCR) analysis showed that *CIM1* negatively regulated *CINOX2* at the transcriptional level, while deletion of *CINOX2* had little effect on the transcript-level expression of *CIM1* (Fig. 8A). In the vascular wilt fungus *F. oxysporum*, NOX-mediated H_2O_2 production, as a signal, is located upstream of the Ste2-dependent phosphorylation of Mpk1 (Nordzieke et al. 2019). In the mycoparasite *Coniothyrium minitans*, NOX-derived ROS affect CmSlr2 localization (Wei et al. 2016). Therefore, we speculated that *CINOX2* might posttranscriptionally regulate *CIM1*. Interestingly, *CINOX2* regulated the expression level of *CISCD1*, while *CIM1* regulated that of *CIBRN1* (Fig. 8B), suggesting that both *CINOX2* and *CIM1* regulate DHN melanin biosynthesis.

DISCUSSION

DHN melanin has diverse functions in the fungal kingdom (Cordero and Casadevall 2017). Hemibiotrophic fungus *M. grisea* forms appressoria and ruptures the host surface by exerting physical force. Melanin produced in *M. grisea* serves to generate turgor pressure and provides structural resilience and rigidity to the rapidly expanding appressoria (Dean et al. 2005; de Jong et al. 1997). Necrotrophic fungus *B. cinerea* cannot generate melanized appressoria. It uses chemical methods instead of mechanical pressure to invade host plants (Choquer et al. 2007). In *B. cinerea*, loss of the DHN melanin biosynthesis genes *bcbn1* and *bcpks13* enhanced hyphal growth, extracellular enzymatic secretion, and virulence (Zhang et al. 2015). Appressoria of hemibiotrophic *Curvularia lunata* are dome-shaped, melanin-pigmented structures. They swell at the tips of extended germ tubes from both ends of the four-celled conidia (Supplementary Video S1). Loss of the DHN melanin biosynthesis gene *CIBRN1* in *C. lunata* inhibited appressorial formation and cellulase synthesis, indicating that DHN melanin is involved in the biotrophic and necrotrophic phase in *C. lunata*. In addition, *CIBRN1* elimination altered conidial morphology and CWI, enhanced asexual development and sexual reproduction, and increased accumulation of extracellular melanin. Because DHN melanin is an important cell wall component in fungi (Chatterjee et al. 2015; Wang et al. 1996), we speculate that DHN melanin plays an important role in *C. lunata* development and virulence through cell wall assembly (Fig. 8C).

Fungal melanin deposition is linked with cell wall chitin (Carzaniga et al. 2002; Chatterjee et al. 2015; Wang et al. 1996). Once chitin metabolism is disrupted, the melanin deposited in the cell wall will be released into the extracellular environment, leading to a “leaky melanin” phenotype (Tsirilakis et al. 2012). In this study, the levels of melanin released into the extracellular space were significantly increased in $\Delta Clnox2$ and $\Delta Cibrn1$, which is consistent with the turbid and pigmented culture filtrates of both mutants, suggesting that differently colored DHN melanin intermediates cannot be fixed in the cell wall by chitin but, instead, are released into the extracellular milieu.

NOXs play essential roles in a variety of developmental and pathogenic processes such as cellular differentiation, polarity establishment, fruit body formation, invasive hyphal growth,

Fig. 5. Subcellular localization of the *CINOX2* protein in *Curvularia lunata*. **A**, Schematic diagram of the H3Pro::MoH2B-mCherry and H3Pro::CINOX2-mCherry vectors. **B**, MoH2B-mCherry (control) and CINOX2-mCherry fusion proteins were expressed in the CX-3 strain. White arrows indicate intracellular organelles in conidia and hyphae. Images were obtained using laser-scanning confocal microscopy. Bar = 15 μ m.

and appressorial formation (Irani 2000; Tamura et al. 2000). In *M. oryzae*, regulated synthesis of ROS by NOX directly controls septin and F-actin dynamics at the appressorium pore to affect appressoria-mediated cuticle rupture and plant cell invasion (Ryder et al. 2013). Furthermore, septins and F-actin are

mislocalized in *M. oryzae* melanin-deficient mutants (Ryder et al. 2019). The $\Delta Clnox2$ strain was melanin deficient, and the phenotypes of the $\Delta Clnox2$ and $\Delta Clbrn1$ were remarkably similar, suggesting that *CINOX2* may control the septin and cortical F-actin network by regulating DHN melanin

Fig. 6. *CINOX2* regulates reactive oxygen species (ROS) and melanin production in *Curvularia lunata*. *CINOX2* elimination affects ROS generation. **A**, Germinated conidia of CX-3, $\Delta Clnox2$, and $\Delta Clnox2-C$ were stained with nitro blue tetrazolium chloride (NBT) or 3,3'-diaminobenzidine (DAB) and viewed by light microscopy. **B**, The relative production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by gray value in hyphae shown in **A**. **C**, CX-3, $\Delta Clnox2$, and $\Delta Clnox2-C$ were stained with 2',7'-dichlorofluorescein diacetate and viewed by laser-scanning confocal microscopy. **D**, Relative ROS production by gray value in conidia and appressoria shown in **C**. *CINOX2* and *CIBRN1* are involved in intracellular and extracellular melanin biosynthesis. **E**, Quantification of intracellular melanin production. The melanin extracted from CX-3, $\Delta Clnox2$, and $\Delta Clbrn1$ was dissolved in NaOH solution (upper panel). **F**, Quantification of extracellular melanin. **G**, *CINOX2* does not affect the transcript-level expression of *CIBRN1* but regulates the mRNA level of *CISCD1*. The gels (lower panel) show representative quantitative reverse-transcription PCR data for *CIBRN1* and *CISCD1* compared with *ACTIN*. ND = not detected. Bar = 15 μ m. Data represent means $\pm$ standard error of the mean from three independent experiments. Asterisks ** and *** indicate $P < 0.01$ and 0.001, respectively.

biosynthesis during plant infection. In *P. anserina*, a weakened cell wall resulting from pigment deficiency or absence facilitates ascospore germination (Lambou et al. 2008). *CINOX2* negatively regulates hyphal growth, asexual development, and sexual reproduction in *C. lunata*. We speculate that the accelerated development of $\Delta Clnox2$ is related to a weakened cell wall and the increased production of secondary metabolites resulting from DHN melanin deficiency.

The regulatory mechanisms underlying *C. lunata* DHN melanin biosynthesis remain obscure. In this study, the CX-3 strain produced less melanin when treated with AA, suggesting that ROS are essential for DHN melanin production. *CINOX2* partly controlled intracellular H_2O_2 and ROS production. Furthermore, *CINOX2* and *CIM1* regulated the expression level of *CISC1* and *CIBRN1* genes, respectively. *CIM1* negatively regulated the expression level of *CINOX2*. Taken together, the

Fig. 7. *CIM1* is involved in *Curvularia lunata* development and virulence. **A**, Colony morphologies of CX-3, $\Delta CIm1$, and $\Delta CIm1-C$. Tested strains were cultured on potato dextrose agar (PDA), complete medium (CM), minimal medium (MM), and CM supplemented with Congo red (CR) at 60 $\mu g mL^{-1}$ or 0.01% sodium dodecyl sulfate (SDS). Red arrows indicate the transparent halos. **B**, *CIm1* disruption enhanced hyphal growth. **C**, *CIm1* elimination altered the conidial morphology. Conidia and appressorial tips of the $\Delta CIm1$ mutant were coated with less melanin. **D**, Relative inhibition rate of hyphal growth by SDS and CR. **E**, Relative germination rate of the tested strains. **F**, The *CIM1* deletion mutant has decreased conidial number and **G**, impaired appressorial formation. $\Delta CIm1$ displayed lower virulence than CX-3 and the complemented strain. **H**, Disease symptoms caused by CX-3, $\Delta CIm1$, and $\Delta CIm1-C$. Tween 20 (0.02%) was used as a control (mock). Pathogenicity shown in H was inspected. **I**, Disease index and **J**, lesion number caused by $\Delta CIm1$ were significantly decreased. Bar = 15 μm . Data represent means $\pm$ standard error of the mean from three independent experiments. Asterisks ** and *** indicate $P < 0.01$ and 0.001, respectively.

Fig. 8. Regulatory roles of *CINOX2* and *CIM1* in 1,8-dihydroxynaphthalene (DHN) melanin biosynthesis and multiple functions of DHN melanin in *Curvularia lunata*. **A**, *CIM1* negatively regulates *CINOX2* transcriptionally, whereas deletion of *CINOX2* has little effect on the transcript-level expression of *CIM1*. Gels show representative quantitative reverse-transcription PCR (RT-qPCR) data for *CINOX2* and *CIM1* compared with *ACTIN*. ND = not detected. **B**, *CIM1* regulates *CIBRN1* transcriptionally. Gels show representative RT-qPCR data for *CIBRN1* and *CISCD1* compared with *ACTIN*. **C**, A model describing the regulatory mechanism of *CINOX2* in *C. lunata* development and virulence. *CINOX2* generates reactive oxygen species (ROS), which have a role in DHN melanin biosynthesis and may posttranscriptionally regulate *CIM1*. As feedback, *CIM1* negatively regulates *CINOX2* expression. *CINOX2* and *CIM1* are involved in DHN melanin biosynthesis by regulating the transcription of *CISCD1* and *CIBRN1*, respectively. DHN melanin has various functions beyond maintaining osmotic pressure in appressoria. Melanin deficiency in *C. lunata* may result in cytoskeleton remodeling, which then affects cellulase synthesis, appressorial (AP) formation, and conidial morphology, thereby affecting *C. lunata* virulence. DHN melanin is also involved in extracellular melanin production, cell wall integrity, and fungal development.

results support the idea that the *CINOX2*-derived ROS signaling pathway and the *CLM1*-mediated CWI signaling pathway are involved in DHN melanin biosynthesis as a cross-linked manner (Fig. 8C). To further understand how *CINOX2* regulates *CLM1*, it is important to examine posttranscriptional modifications and the localization of *CLM1* in a $\Delta Clnox2$ background. The $\Delta Clnox2$ strain presented almost no pigmentation when grown on CM with added H_2O_2 , indicating that *CINOX2* functions by regulating melanin biosynthesis in the process of *C. lunata* infection and colonization. Therefore, it is important to measure the swelling pressure of appressoria and evaluate the transcript-level expression of *CINOX2*, *CIBRN1*, and *CISCD1* at different infection times.

MATERIALS AND METHODS

Strains cultures.

In this study, the *C. lunata* CX-3 strain (a laboratory strain) was used as the WT for transformation and as a control. Conidial suspensions of all the tested strains were stored in an ultralow-temperature freezer and inoculated on PDA plates at 28°C for 5 days before experiments.

AA and DPI inhibition assays.

To examine the inhibition of AA on *C. lunata* hyphal growth and conidiation, CX-3 was inoculated on CM with added 10 or 100 mM AA, and cultured at 28°C in the dark for 5 days. Then colony diameters were measured. Conidia from the above plates were suspended in 100 ml of sterile water and quantified with a hemocytometer. To test the effect of AA and DPI on conidial germination, conidia were suspended in AA and DPI aqueous solutions and incubated on glass slides at 28°C for indicated times. The germinated conidia from 100 conidia were counted under a microscope. The germinated conidia were photographed and the germ tube length was measured. To assess the effect of AA and DPI on appressorial formation, conidia suspended in AA and DPI were incubated on coverslips at 28°C for 4 h. Then, appressoria-generating conidia from 100 conidia were counted.

Transformation of *C. lunata*.

To generate the pPZP100HCINOX2 construct for *CINOX2* deletion, a 1,663-bp fragment containing the targeted *CINOX2* domain, 5'-flanking region, and 3'-flanking region was amplified using CX-3 genomic DNA as the template. The fragment was digested with the *EcoRI/XbaI* restriction enzymes and ligated into the pPZP100 vector. The targeted *CINOX2* domain of the resulting plasmid was replaced with a hygromycin-resistance cassette predigested with *KpnI* (Supplementary Fig. S3A). To generate the pPZP100HCIBRN1 construct for *CIBRN1* deletion, the pPZP100H vector containing the *HPH* gene was integrated with the 5'- and 3'-flanking regions of *CIBRN1* (Supplementary Fig. S3B). Candidate transformants were obtained using *Agrobacterium tumefaciens*-mediated transformation (ATMT) (L. Zhang et al. 2016). Transformants were selected with hygromycin B at 200 $\mu\text{g ml}^{-1}$ and further confirmed by PCR (Supplementary Fig. S4A and B). Primers are shown in Supplementary Table S1.

Vectors for the complementation of the *CINOX2* and *CIBRN1* deletion mutants were constructed by one-step cloning. To generate the *CINOX2* complemented vector, pPZP100 plasmid was linearized using *HindIII* and *KpnI*. Four fragments, including the 5'-flanking region with part of the *CINOX2* gene, neomycin phosphotransferase (*NPTII*) resistance gene from plasmid pII99, the 3'-flanking region with part of the *CINOX2* gene, and the linearized pPZP100 were recombined using MonClone Hi-Fusion Cloning Mix (Vazyme, Nanjing, China) (Supplementary Fig. S3C). To generate the vector for *CIBRN1*

complementation, pPZP100 was digested using *HindIII* and *KpnI*. Four fragments, including the 5'-flanking region with part of the *CIBRN1* gene, *NPTII*, green fluorescent protein, and 3'-flanking region with part of the *CIBRN1* gene were cloned into linearized pPZP100 with MonClone Hi-Fusion Cloning Mix (Vazyme) (Supplementary Fig. S3D). The resulting recombinant plasmids were sequenced and transformed via ATMT into the *CINOX2* and *CIBRN1* deletion mutants. PDA containing G418 at 200 $\mu\text{g ml}^{-1}$ was used for transformant selection. Transformants with *NPTII* resistance were validated by PCR assays (Supplementary Fig. S4C and D). Primers are shown in Supplementary Table S2. CE Design and Primer Premier 5 software were used to design all primers.

Fungal growth and biomass assay.

For the fungal growth assay, the tested strains were cultured on PDA, CM, and MM plates at 28°C for 5 days. Then, the colony diameters were measured. For the biomass assay, four mycelial plugs (5 mm in diameter) of each strain were transferred into 100 ml of liquid CM and incubated at 28°C and 100 rpm for 5 days. The mycelia were separated from the filtrate using filter paper. Then, the mycelia were dried and weighed.

Asexual development assay.

The tested strains were incubated on solid CM for 5 days; then, conidiation was determined using a hemocytometer. Conidia were observed and photographed using a laser-scanning confocal microscope (LSCM). The length and width of 100 conidia were measured using cellSens software provided by the LSCM system (OLYMPUS FV3000). For the conidial germination assay, conidia suspended in sterile water were cultured on glass slides at 28°C for 2 h. For the appressorial formation assay, conidia suspended in liquid CM were incubated on coverslips at 28°C for 4 h. In all, 100 conidia/replicate were examined under a microscope.

Sexual reproduction experiments.

For the sexual reproduction assay, the WT CX-3 (*MAT1-2*), $\Delta CINOX2$ (*MAT1-2*), and $\Delta CIBRN1$ (*MAT1-2*) were crossed to the *C. lunata* ZD958 (*MAT1-1*) strain. The cross-mating strains were cultured on Sach's medium ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ at 1.0 g liter⁻¹, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ at 0.25 g liter⁻¹, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at 0.25 g liter⁻¹, and CaCO_3 at 0.85 g liter⁻¹) under full light at 24°C for 21 days (Lu et al. 2017). To determine fertility potential, the number of pseudothecia on maize leaf substrates was checked, and asci were quantified in at least 25 pseudothecia.

Stress sensitivity assay.

For the stress sensitivity assay, the tested strains were inoculated onto CM supplemented with the following elements: 0.01% SDS, CR at 60 $\mu\text{g ml}^{-1}$, 0.7 M NaCl, 0.05 and 0.1% H_2O_2 , and 60 μM menadione. The strains were cultured at 28°C for 5 days. Then, colony morphologies were observed and photographed and colony diameters were measured. The percentage of inhibition of mycelial growth (PIMG) was calculated using the formula $\text{PIMG} = [(C - N)/(C - 5)] \times 100$, where *C* is the colony diameter of the untreated control, *N* is that of stress treatment, and 5 is the colony diameter of mycelial plugs (Wang et al. 2015).

Subcellular localization.

The Wolf PSORT tool predicted plasma membrane targeting of *CINOX2* (score: 25). To test plasma membrane targeting, plasmid pKD9-mCherry driven by the *H3* promoter (*M. grisea*) was linearized with *BamHI* and *SmaI* restriction enzymes and

ligated with *CINOX2* cDNA to generate vector H3Pro:: *CINOX2-mCherry*. To determine whether the H3 promoter from *M. grisea* functions in *C. lunata*, the vector H3Pro::H2B-*mCherry* containing *M. grisea* nuclear-localized H2B protein was used as control. Vectors H3Pro::H2B-*mCherry* and H3Pro:: *CINOX2-mCherry* were transformed into the WT CX-3 via ATMT. Transformants that had hygromycin resistance and shared the same morphology with CX-3 were randomly selected. Fluorescent images were obtained using LSCM at the wavelength of 580 nm excitation and 610 nm emission. Primer sequences were shown in Supplementary Table S3.

Virulence assay.

Conidia (1×10^6 conidia ml^{-1}) were sprayed on the fourth leaf of 1-month-old maize plants. Conidial suspension was used at 2 ml/plant. The inoculated plants were transferred into a chamber with high humidity and a temperature of 25°C. After 48 h, we returned them to the greenhouse where the temperature remained 25°C under a cycle of 16 h of light and 8 h of darkness. At 6 dpi, disease severity was evaluated and the maize leaves were photographed (Zhang et al. 2019). The length and width of at least 50 lesions were measured. For each tested strain, at least four maize plants were inoculated for each experiment.

Detection of ROS localization and production.

O_2^- anion and H_2O_2 in CX-3, ΔClnox2 , and complemented strains were detected using NBT and DAB staining. Conidia were inoculated on coverslips in a moist chamber at 28°C for 4 h. Then, sterile water was replaced with 100 μl of 0.3 mM NBT or DAB aqueous solution at 1 mg ml^{-1} . After 20 min, formazan precipitates by NBT or brown precipitates by DAB were observed and photographed with a light microscope (Axio Scope A1; Zeiss). ROS in conidia and appressoria of the tested strains were examined using H_2DCFDA . Conidia were incubated at 28°C for 0 and 4 h before they were treated with 10 μM H_2DCFDA for 20 min. The H_2DCFDA samples were photographed with an LSCM (OLYMPUS FV3000) under an excitation wavelength of 504 nm and an emission wavelength of 529 nm. The NBT, DAB staining, and fluorescent signal were quantitatively analyzed based on gray value with ImageJ software.

Melanin extraction and measurement.

Melanin was extracted as previously described (Gao et al. 2015). The tested strains were cultured in 100 ml of potato dextrose broth at 28°C and 150 rpm for 6 days. Mycelia and filtrates were separated by using four layers of gauze. The filtrates were adjusted to pH 2 with 7 M HCl and centrifuged at 1,000 rpm for 10 min to obtain the precipitates of extracellular melanin. The mycelia were lyophilized and boiled in 15 ml of deionized water for 5 min. Then, the mycelia were dissolved in 1 M NaOH and autoclaved at 121°C for 20 min. The supernatant obtained by centrifugation was acidified with high-concentrated HCl and precipitated overnight. After being dried in a 20°C desiccator for 12 h, the precipitates of intracellular melanin were obtained. The precipitates of intracellular and extracellular melanin were transferred into 5 ml of HCl (7 M) and boiled for 2 h. Then, they were centrifuged and dissolved in 1 M KOH. After repeating three times, the precipitates were washed with 0.01 M HCl and deionized water. The precipitates were dried in a 20°C desiccator for 24 h before they were weighed.

RT-qPCR.

Conidial suspensions (1×10^6 conidia ml^{-1}) of CX-3, ΔClnox2 , and ΔClnml were transferred into 100 ml of liquid CM and cultured at 28°C and 150 rpm for 5 days. Then, mycelia

were lyophilized, and RNA was extracted using Invitrogen TRIzol. A PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) was used to remove DNA contaminations. We used cDNA, primer pairs (Supplementary Table S4), and SYBR green dye (Invitrogen) to perform RT-qPCR on a Bio-Rad iCycler. The Bio-Rad iCycler was run with the following settings: 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s. The relative expression level was calculated with the $2^{-\Delta\Delta\text{Ct}}$ method. A CX-3 strain cultured in liquid CM was used as a control group for all analyses. We used the internal reference gene *ACTIN* (GenBank accession number AGK07294) to normalize gene expression.

Statistical analysis.

All data were derived from at least three independent experiments in which triplicate treatments were examined. The quantitative data were normalized as 100% to make different treatment experiments comparable. Significance was assessed using SPSS Statistics 22 and the Student's *t* test. Differences were considered significant at $P < 0.05$.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

CE Design: <https://crm.vazyme.com/cetool/en-us/simple.html>
ClustalW software: <https://www.ebi.ac.uk/Tools/msa/clustalw2>
JGI MycoCosm database: <https://mycocosm.jgi.doe.gov/Coclul2/Coclul2.home.html>

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