Mitochondrial prohibitin complex regulates fungal virulence via ATG24-assisted mitophagy

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Prohibitins are highly conserved eukaryotic proteins in mitochondria that function in various cellular processes. The roles of prohibitins in fungal virulence and their regulatory mechanisms are still unknown. Here, we identified the prohibitins ChPhb1 and ChPhb2 in a plant pathogenic fungus Colletotrichum higginsianum and investigated their roles in the virulence of this anthracnose fungus attacking crucifers. We demonstrate that ChPhb1 and ChPhb2 are required for the proper functioning of mitochondria, mitophagy and virulence. ChPhb1 and ChPhb2 interact with the autophagy-related protein ChATG24 in mitochondria, and ChATG24 shares similar functions with these proteins in mitophagy and virulence, suggesting that ChATG24 is involved in prohibitin-dependent mitophagy. ChPhb1 and ChPhb2 modulate the translocation of ChATG24 into mitochondria during mitophagy. The role of ChATG24 in mitophagy is further confirmed to be conserved in plant pathogenic fungi. Our study presents that prohibitins regulate fungal virulence by mediating ATG24-assisted mitophagy.

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Mitochondria are semi-autonomous double membrane-bound organelles that generate most of the adenosine triphosphate (ATP) required for diverse cellular functions and are crucial to various physiological processes, including lipid metabolism, redox signaling, calcium and iron homeostasis, and programmed cell death\(^1\). Prohibitin proteins, which belong to the SPFH (stomatin/prohibitin/flotillin/HIPK) family, are ubiquitously expressed and highly conserved in eukaryotes\(^2\). The prohibitin complex contains two subunits, Phb1 and Phb2, which physically associate with each other to form a high-molecular-weight complex in the inner mitochondrial membrane\(^3\).

The prohibitin complex plays a variety of roles in mitochondria, including mitochondrial DNA (mtDNA) maintenance, protein synthesis and degradation, assembly of the oxidative phosphorylation system, maintenance of cristae structure, and apoptosis\(^4\). Deletion of Phb1 and Phb2 did not cause any observable aberrant growth phenotype in Saccharomyces cerevisiae, but these proteins play a critical role in mitochondrial turnover and the lifespan of this yeast\(^5\). Prohibitins are required for embryonic development in Caenorhabditis elegans\(^6\) and mouse\(^7\). Prohibitins also act as pleiotropic regulators of plant development. In Arabidopsis thaliana, Phb3 plays an essential role in ethylene signaling, nitric oxide accumulation, plant defense, induction of salicylic acid biosynthesis, cell division, senescence, and root meristem cell proliferation\(^8\). However, the roles of prohibitins in fungal pathogens are unknown.

Phb2 is a cellular process responsible for selectively removing damaged or unwanted mitochondria\(^9\). The prohibitin proteins Phb2 is essential for Parkin-mediated mitophagy by functioning as a mitochondrial receptor in mammalian cells, thereby affecting the recruitment of the autophagy protein LC3 to initiate mitochondrial clearance\(^10\). In yeast, the mitochondrial outer membrane protein Atg32 functions as a mitochondrion specific receptor that interacts with the core autophagic machinery Atg8 and Atg11 on the surfaces of mitochondria to induce mitophagy\(^11,12\). Although mitophagy is an evolutionarily conserved process, Atg32 homologs have only been identified in yeast. Therefore, it appears that ATG proteins that function in selective autophagic pathways, such as mitophagy, are not conserved like those in nonselective autophagic pathways in filamentous fungi\(^13\). In the filamentous fungus, Magnaporthe oryzae, mitophagy mediated by the sorting nexin MoAtg24 is essential for proper biotrophic development and invasive growth in rice (Oryza sativa)\(^14,15\). In Fusarium graminearum, FgSnx4 (also known as ATG24) is ubiquitously expressed at all developmental stages and localizes to early endosomes, and the FgSnx4 deletion mutant shows defects in polarized growth and virulence\(^16\). However, the regulatory mechanisms of mitophagy in fungal pathogenesis are not well understood.

Colletotrichum higginsianum, a hemibiotrophic fungal pathogen, is the causal agent of anthracnose disease in various cruciferous plants including the model plant Arabidopsis thaliana\(^17\). At the start of the hemibiotrophic lifecycle of C. higginsianum on A. thaliana, conidia contact the leaf surface and differentiate into melanized appressoria to facilitate penetration, followed by in planta growth, and formation of primary and secondary hyphae\(^18\). The C. higginsianum–A. thaliana pathosystem is an attractive model to examine hemibiotrophic fungal–plant interactions\(^19\).

In the current study, we identified the prohibitin proteins ChPhb1 and ChPhb2 from C. higginsianum and demonstrated that these proteins can interact with each other in mitochondria and are involved in proper mitochondrial functioning, mitophagy and virulence. Moreover, we showed that the mitophagic protein, ChATG24, interacts with both ChPhb1 and ChPhb2 in the mitochondria and is also essential for mitophagy and virulence. Deletion of either ChPhb1 or ChPhb2 perturbs the translocation of ChATG24 into the mitochondria. Collectively, these findings...
reveal that prohibitins recruit ChATG24 into the mitochondria to modulate mitophagy, thereby affecting fungal virulence.

**Results**

**Characterization of ChPhb1 and ChPhb2.** To characterize prohibitin genes in a phytopathogenic fungus, we used the amino acid sequences of prohibitins (Phb1 and Phb2) from *S. cerevisiae* as queries for a BLASTP search against the *C. higginsianum* genome. Two proteins, Ch063_12292 and Ch063_08657, were identified as the *C. higginsianum* homologs of *S. cerevisiae* Phb1 and Phb2, respectively. Both ChPhb1 and ChPhb2 contain a conserved PHB domain (Fig. 1a).

Phylogenetic analysis of prohibitins from various organisms showed that ChPhb1 and ChPhb2 are divided into two distinct clades (Fig. 1b), suggesting that ChPhb1 and ChPhb2 are paralogous and that Phb1 and Phb2 are highly conserved in different species. We examined the expression profiles of ChPhb1 and ChPhb2 during each growth phase including vegetative hyphae, conidia, and the infection process on *A. thaliana* using qRT-PCR. ChPhb1 and ChPhb2 were highly expressed at the necrotrophic stage at 72 h post-inoculation (hpi) (Fig. 1c). These results suggested that prohibitin genes might play important roles in the formation of secondary hyphae during infection.

**ChPhb1 and ChPhb2 are required for full virulence.** To examine the functions of prohibitins in *C. higginsianum*, we generated both single and double deletion mutants of ChPhb1 and ChPhb2, and their complemented strains (Supplementary Fig. 1). We analyzed the phenotypic changes in vegetative growth, conidia formation, biomass, and appressorial formation in the deletion mutants, wild-type strain Ch-1 and complemented strains. The ΔChPhb1 deletion mutant displayed significantly attenuated growth and showed obviously reduced conidiation compared to the wild-type strain, while complementation of ΔChPhb1 in ΔChPhb2 deletion mutant could restore growth and conidiation (Fig. 2a–d). However, the ΔChPhb2 deletion mutant showed no obvious defects in conidial germination or appressorial formation (Fig. 2e). No phenotypic changes were detected in the ΔChPhb1 deletion mutant (Fig. 2a–d). Moreover, the ΔChPhb1/ΔChPhb2 double deletion mutant showed significantly attenuated vegetative growth and conidiation, which was similar to the phenotype of the ΔChPhb2 deletion mutant (Fig. 2a–e). These results indicated that ChPhb2, but not ChPhb1, is involved in vegetative growth and conidiation.

To further explore the roles of ChPhb1 and ChPhb2 in host colonization and virulence, we conducted virulence and plant infection assays using the ΔChPhb1 and ΔChPhb2 single deletion mutants and the ΔChPhb1/ΔChPhb2 double deletion mutant on *A. thaliana* seedlings. At 7 dpi, typical water-soaked and collapsed lesions appeared on *Arabidopsis* leaves following inoculation with the wild-type and all complemented strains. In contrast, the ΔChPhb1, ΔChPhb2, and ΔChPhb1/ΔChPhb2 deletion mutants caused few and small necrotic lesions to form on infected leaves (Fig. 2f). We also assayed appressorial penetration and infectious growth in *Arabidopsis* leaves by microscopy. Compared to wild-type and complemented strains, the formation of primary hyphae was not significantly affected in ΔChPhb1 or ΔChPhb2, but only 52% of the primary hyphae of ΔChPhb1 and 30% of the primary hyphae of ΔChPhb2 developed into secondary hyphae at 4 dpi (Fig. 2g, h). The ΔChPhb1/ΔChPhb2 double deletion mutant exhibited even less secondary hyphae formation than the single mutants, but there were no significant differences between ΔChPhb2 and ΔChPhb1/ΔChPhb2. These results indicated that the ChPhb1 and ChPhb2 deletion mutants are defective in the switch to necrotrophic growth and that ChPhb2 might be more directly responsible for the reduced virulence.

**ChPhb1 interacts with ChPhb2 in mitochondria.** In budding yeast and mammals, Phb1 and Phb2 form large, multimeric ring complexes at the inner membranes of mitochondria. In a yeast two-hybrid (Y2H) assay, blue colonies of Y2HGold with pGBKKT7-ChPhb1 and pGAK17-ChPhb2 were observed on SD-Leu-Trp-His/X-a-gal plates, demonstrating that ChPhb1 interacts with ChPhb2 (Fig. 3a). Previous studies in mammals showed that Phb1 and Phb2 can interact as a heterodimeric complex. To further confirm the role of ChPhb1 and ChPhb2, yeast two-hybrid assays of ChPhb1 or ChPhb2 self-interaction were assessed and no self-interactions were found for ChPhb1 or ChPhb2 (Fig. 3a). These results indicated that ChPhb1 and ChPhb2 might form as a heterodimeric complex. This interaction between ChPhb1 and ChPhb2 was further validated by Co-IP assays in vivo. We generated ChPhb1–3×FLAG and ChPhb2–GFP fusion constructs and used them to co-transform the wild-type strain. Both ChPhb1–3×FLAG (46 kDa) and ChPhb2–GFP (61 kDa) fusion proteins were detected in the immunoprecipitate containing anti-FLAG M2 beads (Fig. 3b). The strain that only carried ChPhb1–3×FLAG was used as a control, and it was not detected in the immunoprecipitate (Fig. 3b). These results indicated that ChPhb1 interacts with ChPhb2 in vivo.

To examine the subcellular localizations of ChPhb1 and ChPhb2, we expressed ChPhb1 and ChPhb2 under the control of their native promoters fused with enhanced green fluorescent protein (eGFP) tags at their C termini and expressed them in the ΔChPhb1 and ΔChPhb2 mutants, respectively. The complementation strains which complement the phenotypes of deletion mutants were obtained (Fig. 2 and Supplementary Fig. 1). Punctate and tubular GFP fluorescent signals were observed in vegetative hyphae of the ChPhb1-GFP and ChPhb2-GFP tagged mutants. Upon staining with the red fluorescent mitochondrial dye MitoTracker, punctate or tubular GFP signals were seen to completely co-localized with the mitochondrial structures labeled by red fluorescence (Fig. 3c). These results indicated that both ChPhb1 and ChPhb2 localize to mitochondria.

To further enable direct visualization of interactions between ChPhb1 with ChPhb2, we performed bimolecular fluorescence complementation (BiFC) assays using ChPhb1 fused to the C-terminal fragment of YFP (YFP-CF) and ChPhb2 fused to the N-terminal fragment of YFP (YFP-NTF). The two constructs expressing ChPhb1-YFP-CF and YFP-NTF-ChPhb2 were co-transformed into *C. higginsianum* strain Ch-1 while the constructs YFP-CF and YFP-NTF were co-transformed into strain Ch-1 as a negative control. GFP signals were only detected in transformants co-expressing ChPhb1-YFP-CF and YFP-NTF-ChPhb2 but not in the negative control, suggesting that ChPhb1 interacts with ChPhb2 in mitochondria (Fig. 3d).

**ChPhb1 and ChPhb2 are important for mitochondrial structure and transmembrane potential.** Since ChPhb1 interacts with ChPhb2 in mitochondria, we explored whether the deletion of the two prohibitin proteins would result in defects in mitochondrial function. After staining with MitoTracker red, the mitochondria in hyphae showed elongated tubules in the wild-type and complemented mutant strains, whereas the red fluorescent signals were diffuse in the ΔChPhb1 and ΔChPhb2 deletion mutants as well as the ΔChPhb1/ΔChPhb2 double deletion mutant (Fig. 4a). We then expressed the mitochondria-localized fission protein ChFis1-GFP in the ΔChPhb1 and ΔChPhb2 deletion mutants to examine mitochondrial fission. Analysis of green fluorescent signals in mitochondria revealed elongated tubules in the hyphae, with no
difference detected among the strains (Supplementary Fig. 2). However, detailed transmission electron microscopy (TEM) analysis revealed defective morphogenesis of cristae in the absence of either prohibitin (Fig. 4b). These results suggest that ChPhb1 and ChPhb2 are crucial for maintaining mitochondrial structure but are not required for mitochondrial fission.

The intensity of the MitoTracker deep red changed along with mitochondrial transmembrane potential ($\Delta \Psi$). Thus, we tested whether mitochondrial $\Delta \Psi$ was disturbed in the $\Delta$ChPhb1, $\Delta$ChPhb2, or $\Delta$ChPhb1/Phb2 deletion mutants using JC-1 dye. When treated with dye, an increase in red fluorescence was observed in the wild type, with a mean green/red fluorescence ratio of the probe reaching 3.57 ± 0.62, which is indicative of an increase in mitochondrial $\Delta \Psi$ (Fig. 4c and Supplementary Fig. 3). Under similar exposure conditions, monomeric JC-1–associated green fluorescence in the $\Delta$ChPhb1, $\Delta$ChPhb2, and $\Delta$ChPhb1/ChPhb2

Fig. 2 ChPhb1 and ChPhb2 are required for the full virulence of C. higginsianum. a Morphology of colonies of each strain grown on PDA at 25 °C for 7 days. b Statistical analysis of colony growth. Colony diameter was measured after culturing at 25 °C for 7 days. c Statistical analysis of mycelial biomass. The biomass of each strain was measured after shake-cultured in PDB for 7 days. d Statistical analysis of conidial production. Conidiation was measured by collecting conidia from PDA plates after 7 days. e Appressorial formation rates of each strain on a hydrophobic surface. f Pathogenicity assay on Arabidopsis plants. Conidial suspensions of each strain were sprayed onto the plants and incubated at 25 °C for 4 days. g Infection structures of each mutant. Ap appressoria, Ph primary hyphae, Sh secondary hyphae. Scale bar, 20 μm. h Percentage of infection structure formation on Arabidopsis leaves. For bar graphs, error bars represent the standard deviation, and absence of lowercase letters in common indicate significant differences using LSD at $P = 0.05$. 
Fig. 3 ChPhb1 interacts with ChPhb2 in mitochondria. a The interaction of ChPhb1 and ChPhb2 was detected using a Y2H assay. Yeast transformants expressing plasmids harboring the indicated constructs were assayed for growth on nonselective plates (SD/-Leu/-Trp) and selective plates (SD/-Leu/-Trp/-His) with 1 mM X-gal for 3 days. b Co-IP assay of the interaction between ChPhb1 and ChPhb2. c Laser-scanning confocal microscope (LSCM) observation of the subcellular localizations of ChPhb1:GFP and ChPhb2:GFP in mitochondria of hyphae. DIC differential interference contrast, GFP green fluorescent protein. Scale bar, 5 μm. d BiFC assay of the interaction between ChPhb1 and ChPhb2. Hyphae were stained with Mito-tracker and then observed by confocal fluorescence microscope. Scale bar, 5 μm.
deletion mutants was more pronounced compared to the wild type. Furthermore, the two single deletion mutants exhibited significantly higher green/red fluorescence ratio than the ΔChPhb1/ChPhb2 double deletion mutant. These results indicated that both ChPhb1 and ChPhb2 are involved in generating mitochondrial ΔΨ.

The loss of ΔΨ is thought to be directly associated with the accumulation of reactive oxygen species (ROS)\(^\text{26}\). To test this hypothesis, we examined the sensitivity of the ΔChPhb1 and ΔChPhb2 mutants to increased ROS levels. The ΔChPhb1, ΔChPhb2, and ΔChPhb1/ChPhb2 mutants showed increased sensitivity to menadione compared with the wild type (Fig. 4d and Supplementary Fig. 4). No significant changes were observed between ΔChPhb1, ΔChPhb2 or ΔChPhb1/ChPhb2 mutant and the wild type on the plates amended with H\(_2\)O\(_2\) (Supplementary Fig. 4b). Menadione generates ROS specifically in mitochondria. These results indicated that ChPhb1 and ChPhb2 were required for responses to mitochondrial ROS, and overall suggested that these prohibitins are crucial for maintaining mitochondrial structure and function.

ChPhb1 and ChPhb2 are involved in mitophagy. Eukaryotes have evolved several quality control mechanisms that preserve mitochondrial homeostasis and prevent cellular damage. Mitophagy

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Fig. 4 ChPhb1 and ChPhb2 are important for mitochondrial structure and function. a Mitochondrial morphology in hyphal tips stained by Mito-tracker. Scale bar, 10 μm. b TEM observation of mitochondria in hyphae. Scale bar, 1 μm. c The mitochondrial membrane potential was evaluated by calculating the ratio of red to green fluorescence intensity for at least 10 fields under microscope. For each sample, box plot indicates median, lower and upper quartiles, and minimum and maximum values of ratio. For each box plot, the bottom and top horizontal lines indicate the first and third quartiles of the data, respectively. The horizontal line inside each box plot shows the median value. d Inhibition ratio calculated based on colony diameter of strains subjected to menadione stress for 7 days. Error bars represent the standard deviation. For bar graphs, error bars represent the standard deviation, and absence of lowercase letters in common indicate significant differences using LSD at P = 0.05.
is a cellular process that selectively removes mitochondria through autophagy; this process is triggered by the presence of severely damaged or superfluous mitochondria. We generated ΔChPhb1 and ΔChPhb2 deletion mutants carrying Mito-GFP and used them to monitor mitophagy. The two Mito-GFP strains were cultured in liquid CM for 2 days, then shifted to BG for 48 h to induce mitochondrial biogenesis, and shifted to MN for 12 h to induce mitophagy. In glycerol medium, GFP signals were randomly distributed in the cytoplasm of the mycelia, except for the vacuoles. When the mycelia were transferred from glycerol medium to nitrogen-starvation medium, GFP signals were clearly observed in mycelial vacuoles of the wild type but not of the ΔChPhb1, ΔChPhb2 or ΔChPhb1/ΔChPhb2 mutants (Fig. 5). These results indicated that mitophagy was blocked upon the loss of ChPhb1 or ChPhb2.

To determine whether ChPhb1 and ChPhb2 are involved in non-specific autophagy, we expressed GFP-ATG8 in the wild type and deletion mutants to monitor non-specific autophagy. In the ΔChPhb1 and ΔChPhb2 deletion mutants, a vacuolar GFP signal was detected after starvation in MN medium for 5 h, just as with the wild-type strain (Supplementary Fig. 5), suggesting that ChPhb1 and ChPhb2 are not essential for non-specific autophagy. Since previous studies reported that ATG8 is essential for all types of autophagy, we generated a ΔChATG8 deletion mutant carrying Mito-GFP. The results showed that no vacuolar GFP signal was observed in the ΔChATG8 mutant (Fig. 5), suggesting that delivery of Mito-GFP in C. higginsianum to the vacuole indeed required ChATG8. Overall, these results suggested that ChPhb1 and ChPhb2 are specifically involved in mitophagy in C. higginsianum.

ChATG24 interacts with ChPhb1 and ChPhb2 in the mitochondria. To further explore how ChPhb1 and ChPhb2 participate in mitophagy, we performed Y2H assays to examine their interactions with the ATG proteins, ATG7, ATG8, ATG11, and ATG24, which might be associated with mitophagy in pathogenic fungi. We co-transformed yeast cells with various combinations of AD and BD constructs and selected the cells on SD-Leu-Trp-His. Only ChATG24 was found to interact with ChPhb1 and ChPhb2 (Fig. 6a).

To validate the interactions between ChATG24 and prohibitin proteins in vivo, we performed Co-IP assays. We generated the ChPhb1–3×FLAG and ChATG24-GFP fusion constructs and introduced them into wild-type strain Ch-1 via co-transformation. Immunoblot analysis with anti-FLAG and anti-GFP antibodies detected the presence of a 30-kDa or a 73-kDa band, respectively. In proteins eluted from anti-FLAG M2 beads, the 73-kDa ChATG24-GFP band was detected in the transformant expressing the ChPhb1–3×FLAG and the ChATG24-GFP constructs, but not in the transformant expressing the ChPhb1–3×FLAG construct only (Fig. 6b). Similar methods were used to test the transformant expressing ChATG24-3×FLAG with ChPhb2-GFP fusion constructs (Fig. 6c). These results suggest that both ChPhb1 and ChPhb2 interact with ChATG24 in vivo.

To further confirm the interactions between ChPhb1 and ChPhb2, ChPhb1 and ChAtg24, or ChPhb2 and ChAtg24 in the absence of ChATG24, ChPhb1 or ChPhb2, three fusion constructs of ChPhb1–3×FLAG and ChATG24-GFP, ChPhb1–3×FLAG and ChATG24-3×FLAG, or ChATG24-3×FLAG and ChPhb2-GFP were separately transformed into the ΔChATG24, ΔChPhb2 or ΔChPhb1 mutant. Results of Co-IP assays showed that ChPhb1 interacted with ChPhb2 in the ΔChATG24 mutant, ChPhb1 interacted with ChAtg24 in the ΔChPhb2 mutant, and ChPhb2 interacted with ChAtg24 in the ΔChPhb1 mutant (Fig. 6d), suggesting that the absence of any one of these three proteins did not affect the interactions between the other two.

We further confirmed the interactions between ChATG24 and the two prohibitin proteins using BiFC assays. Constructs expressing ChPhb1-YFPCTF and YFPNTF, ChATG24 were co-transformed into wild-type C. higginsianum to test the ChATG24-ChPhb1 interaction, and constructs expressing ChATG24-YFPCTF and YFPNTF, ChPhb2 were co-transformed into wild-type C. higginsianum to test the ChATG24-ChPhb2 interaction. YFP signals were only detected in transformants co-expressing ChPhb1-YFPCTF and YFPNTF, ChATG24 or ChATG24-YFPCTF and YFPNTF, ChPhb2, but not in the negative control (Fig. 6d), suggesting that ChATG24 interacts with both ChPhb1 and ChPhb2. After staining with the mitochondrial dye MitoTracker, YFP fluorescence was observed as punctate or tubular patterns that completely co-localized with the mitochondrial structures showing red fluorescence. These results indicated

![Fig. 5 ChPhb1 and ChPhb2 are involved in mitophagy. Mycelia from each strain were cultured in CM for 2 days, shifted to BM-G for 30 h and then starved in MM-N for 12 h. The strains subjected to MN starvation were co-stained with 10 μM CMAC. Confocal microscopy with Mito-GFP and CMAC-stained vacuoles was performed to detect mitophagy in each strain. Scale bar, 5 μm.](image-url)
that ChATG24 interacts with the two prohibitin proteins, ChPhb1 and ChPhb2, in mitochondria.

**ChATG24 is also important for fungal virulence and mitophagy.** Since the role of ChATG24 in *C. higginsianum* has not been fully elucidated, we further characterized it using a targeted gene replacement strategy with the \( \Delta \text{ChATG24} \) deletion mutant (Supplementary Fig. 6). Fungal growth and conidiation were significantly reduced in this mutant compared to the wild type (Fig. 7a–c). However, compared to the wild type, the \( \Delta \text{ChATG24} \) deletion mutant showed no obvious defects in conidial germination or appressorial formation. In pathogenicity assays, the \( \Delta \text{ChATG24} \) deletion mutant showed reduced virulence on *A. thaliana* compared to the wild type (Fig. 7d). In addition, the \( \Delta \text{ChATG24} \) deletion mutant was defective in the switch to necrotrophic growth (Fig. 7e–f).

To determine whether ChATG24 is required for mitophagy, we generated \( \Delta \text{ChATG24} \) deletion mutants carrying Mito-GFP. In
mitophagy monitoring assays, GFP signals were randomly distributed in the cytoplasm (except for vacuoles) of mycelia in glycerol medium. Following transfer to MN medium, green fluorescent signals were observed in mycelial vacuoles of the wild type but not the ΔChATG24 deletion mutant (Fig. 7g), indicating that mitophagy was defective in the mutant. These results indicated that ChATG24 is essential for hyphal growth, conidiation, virulence, and mitophagy.

Deletion of ChPhb1 or ChPhb2 perturbs the translocation of ChATG24 into mitochondria. To examine the subcellular localization of ChATG24, we generated a C. higginsianum strain expressing ChATG24-GFP under the control of its native promoter. ChATG24-GFP was undetectable in mycelia cultured in liquid CM. In a previous study, ATG24 was not expressed in CM, but it was expressed and localized to mitochondria under nitrogen starvation conditions [17]. Therefore, we cultured the ΔChATG24 deletion mutant introduced harboring ChATG24-GFP under nitrogen starvation conditions and examined the subcellular localization of the fusion protein. Upon induction by nitrogen starvation, the ChATG24-GFP signal was diffusely distributed in the cytoplasm where it aggregated as punctate structures. After labeling mitochondria with MitoTracker, ChATG24-GFP partially co-localized with MitoTracker-stained mitochondria (Fig. 8a). After labeling lipid droplets with Nile Red, ChATG24-GFP co-localized with the stained lipid droplets (Fig. 8a). Overall, these results indicate that ChATG24 primarily localizes to the cytoplasm in lipid droplets and mitochondria under nitrogen starvation conditions.

To further confirm the notion that prohibitin is a mitophagy cargo of ChATG24, we examined the subcellular localization of GFP-ChATG24 in the ΔChPhb1 and ΔChPhb2 deletion mutants. The ChATG24-GFP signal in both the ΔChPhb1 and ΔChPhb2 deletion mutants was diffusely distributed in the cytoplasm and aggregated as punctate structures, but the punctate structures in the mutants were significantly larger than those of the wild type (Fig. 8b). A co-localization assay was carried out with ChATG24-GFP and Mito-tracker. Fluorescence observation and fluorescence intensity analysis showed that most ChATG24-GFP appeared in mitochondria in Ch-1 while only a small part of ChATG24-GFP appeared in mitochondria in ChPhb1 or ChPhb2 deletion mutants (Fig. 8c). These results suggested that the deletion of ChPhb1 or ChPhb2 perturbs the translocation of ChATG24 into the mitochondria.
ChATG24 rescues the defects of the mitophagy-deficient mutant ΔMoATG24. MoATG24 is a mitophagy-specific receptor that localizes to the mitochondria and is required for virulence in Magnaporthe oryzae17. Mitophagy mediated by MoATG24 is required for the proper invasive growth of M. oryzae18. Thus, to examine whether the function of ChATG24 in mitophagy is conserved in plant pathogenic fungi, we expressed ChATG24 under the control of the constitutive promoter RP27 in ΔMoATG24. The growth defect was completely rescued in ΔMoATG24 expressing ChATG24 (Fig. 9a, b).

To investigate whether ChATG24 could rescue the pathogenicity of ΔMoATG24, we inoculated susceptible rice seedlings (Oryza sativa cv. LTH) with the complementation mutant at the fifth leaf stage by spraying with a conidial suspension. Compared to the ΔMoATG24 mutant, which only occasionally produced small lesions, the complementation strains com ChATG24-2 and com ChATG24-11 gave numerous typical necrotic lesions (Fig. 9c).

To examine the differences between plant infection by ΔMoATG24 and the complementation strains, we observed invasive hyphae under the microscope at 24 and 48 hpi (Fig. 9d, e). At 24 hpi, less than 1% of ΔMoATG24 appressoria were capable of invading the rice sheath. In contrast, nearly 76% of appressoria of the complementation strains successfully penetrated the rice sheath. At 48 hpi, the penetration rates of appressoria of the wild type (P131) and complemented mutants com ChATG24-2 and com ChATG24-11 were comparable: 71% of wild-type invasive hyphae and 67% and 66% of invasive hyphae in com ChATG24-2 and com ChATG24-11, respectively, formed branches. By contrast, only 1% of invasive hyphae in ΔMoATG24 formed branches. Thus, ChATG24 restored the pathogenicity of ΔMoATG24, suggesting that ChATG24 shares overlapping functions with MoATG24.

Discussion
Prohibitins are eukaryotic proteins with highly conserved functions, playing key roles in transcriptional regulation during cell proliferation, differentiation, and cellular aging; regulation of sister chromatid cohesion; cellular signaling apoptosis; and mitochondrial biogenesis8. The prohibitin complex has a variety
of putative functions within mitochondria. In this study, we characterized the prohibitin genes \textit{ChPhb1} and \textit{ChPhb2} in \textit{C. higginsianum} and found a pathogenic mechanism linking prohibitin and mitophagy in this fungal pathogen.

Phb1 and Phb2 share more than 50% identical amino acid residues. Conserved domains shared within a gene family usually exhibit common functions. In the present study, we found that \textit{C. higginsianum} Phb1 and Phb2 both contain a PHB domain, which is characteristic of the SPFH family of membrane proteins. Phb2, a subunit of the prohibitin complex, exerts many relevant functions via interactions with Phb1.

However, our results suggested that \textit{ChPhb2}, but not \textit{ChPhb1}, is involved in vegetative growth and conidiation. The PHB domain of \textit{ChPhb2} is essential for maintaining the structure and function of mitochondria. The mitochondrial inner membrane is anchored via its N-terminal hydrophobic regions. The N-terminal hydrophobic regions of \textit{ChPhb1} and \textit{ChPhb2} differ, perhaps explaining why \textit{ChPhb2} showed some distinct functions from \textit{ChPhb1} in \textit{C. higginsianum}.

In this study, we found that there were no significant differences between \textit{ChPhb2} and \textit{ChPhb1}/\textit{ChPhb2} mutants in vegetative growth, conidiation or pathogenicity. However, the \textit{ChPhb1}/\textit{ChPhb2} deletion mutant showed significant damage in mitochondrial transmembrane potential and reduced menadione sensitivity compared to \textit{ChPhb2} mutant. The degree of mitochondrial damage caused by deletion of \textit{ChPhb1}, \textit{ChPhb2}, or \textit{ChPhb1}/\textit{ChPhb2} did not correspond to their respective vegetative growth or conidiation. These indicated that \textit{ChPhb1} and \textit{ChPhb2} functioned not only individually but also collaboratively in mitochondria.

Previous studies showed that prohibitin1 and prohibitin2 function as a heterodimeric complex. Our Y2H results indicated that \textit{ChPhb1} and \textit{ChPhb2} function presumably as a heterodimeric complex in \textit{C. higginsianum}, which was consistent with the findings of most studies in human tissues and yeasts. This could explain why \textit{ChPhb1} and \textit{ChPhb2} function independently.

Phylogenetic analysis indicated that Phb1 and Phb2 in \textit{C. higginsianum} share high homology with prohibitins of human, mouse, and other fungi, suggesting that the regulatory functions of prohibitins in various species might also be similarly. However, defects in prohibitins do not cause any observable growth phenotypes in \textit{S. cerevisiae}. Prohibitins are required for
embryonic development in *C. elegans* and mouse. In contrast, ChPhb2 is required for vegetative growth and conidiation in *C. higginsianum*, indicating that the roles of prohibitin in cellular differentiation may differ markedly among animals, yeasts, and phytopathogenic fungi.

Prohibitins are highly conserved mitochondrial proteins that have a variety of putative functions within mitochondria. The prohibitin complex has been associated with mtDNA maintenance, mitochondrial biogenesis and degradation, biogenesis and assembly of the oxidative phosphorylation system, maintenance of the cristae structure, and membrane lipid homeostasis. In the current study, ChPhb1 and ChPhb2 were shown to localize to the mitochondria. This is in line with the finding that Phb1 and Phb2 localize to the mitochondrial inner membranes in the cells of various eukaryotic organisms.

We provided several lines of evidence that deletion of ChPhb1 or ChPhb2 causes mitochondrial dysfunction, which results in increased ROS synthesis. First, ChPhb1 and ChPhb2 were verified to be located in the mitochondria; however, in a MitoTracker-staining experiment, differential fluorescent signals in the cytoplasm were detected in the ΔChPhb1 and ΔChPhb2 mutants. In a previous study, upon the depletion of prohibitin, mitochondria appeared fragmented and disorganized. These findings suggested that the deletion of ChPhb1 and ChPhb2 causes mitochondrial dysfunction. Second, a detailed TEM analysis revealed defective morphogenesis of cristae in the absence of either prohibitin in *C. higginsianum*. Similarly, defective morphogenesis of cristae has been reported in plant and yeast mitochondria depleted of prohibitin. Third, the mitochondrial ΔΨ was disordered in the ΔChPhb1 and ΔChPhb2 mutants. This observation is consistent with the finding that in yeast and human cells, the loss of the prohibitin complex results in a defect in mitochondrial ΔΨ. We attempted many times to measure mitochondrial ROS levels levels in these strains using DCFH-DA and mitoSOX-red. However, we were unable to obtain consistent data either when attempting to directly detect in situ or when using isolated mitochondria of *C. higginsianum*. Future work is needed to develop a more efficient method of ROS detection in mitochondria of *C. higginsianum*.

The loss of ΔΨ is directly associated with the accumulation of ROS. Exogenously added menadione specifically generates ROS in mitochondria. The ChPhb1 and ChPhb2 deletion mutants showed strongly increased sensitivity to menadione compared to the wild type. These results suggest that the deletion of ChPhb1 and ChPhb2 significantly increased ROS levels in mitochondria. This is in line with the previous finding that a lack of prohibitin results in increased ROS production. Thus, the role of prohibitin in maintaining proper mitochondrial function is highly conserved in eukaryotes.

The disruption of mitophagy results in distorted mitochondrial function, mitochondrial membrane depolarization, decreased ATP levels, increased oxygen utilization, and elevated ROS levels. Phb2 is involved in mitochondrial homeostasis and plays a critical role in mitophagic responses in evolutionarily distant models, such as human cancer cells and *C. elegans*. The current study provided evidence that mitophagy was delayed in ΔChPhb1 and ΔChPhb2. Cell biology analyses proved that mitophagy is significantly induced under nitrogen starvation condition. Mitophagy is quite different from nonspecific macroautophagy, which typically engulfs cellular components including mitochondria. This is consistent with the finding that Phb2 is a crucial mitophagy receptor involved in targeting mitochondria for autophagic degradation in mammalian cells. Furthermore, exogenously added menadione specifically induces ROS formation in mitochondria. In the current study, ChPhb1 and ChPhb2 deletion mutants showed conspicuously increased sensitivity to mitochondrial ROS, but not to exogenous H₂O₂. This observation confirms the roles of ChPhb1 and ChPhb2 in mitophagy.

Mitophagy plays essential roles in cellular differentiation and pathogenesis in filamentous fungi. For example, mitophagy is essential for conidial differentiation and fungal pathogenesis in *M. oryzae*. Similarly, in *A. oryzae*, the mitophagy-related protein ATG26 is involved in conidiation, growth of aerial hyphae, and invasive growth. Otherwise, mitophagy is also required for the development of blastospores and starvation stress in *Beauveria bassiana*. However, the relationship between mitophagy and fungal pathogenesis remains elusive and needs to be further explored.

The conserved nature of ATG24 proteins in yeast and other filamentous fungi demonstrates a deep evolutionary heritage in eukaryotes. In *M. oryzae*, MoATG24-GFP co-localizes with mitochondria under nitrogen starvation conditions. In *F. graminearum*, FgAtG24-GFP is ubiquitously expressed during all stages of development and localizes to early endosomes. Nevertheless, in the current study, ChATG24-GFP localized to the cytoplasm with lipid bodies under nitrogen starvation conditions. This difference in the intracellular localization of ATG24 among *C. higginsianum*, *M. oryzae*, and *F. graminearum* could account for its specialized function in each organism. The fungal growth defect and virulence caused by deletion of ATG24 appears to be consistent among *C. higginsianum*, *M. oryzae*, and *F. graminearum*. MoATG24-mediated mitophagy is required for the proper invasive growth of *M. oryzae*. To explore the conservation of ATG24 in mitophagy, we expressed GFP-ChATG24 under the control of the strong constitutive promoter RP27 in the ΔMoatG24 mutant. The growth defects and virulence were completely rescued in ΔMoatg24 expressing ChATG24-GFP, indicating that ATG24 plays a relatively conserved role in mediating mitophagy in *C. higginsianum* and *M. oryzae*.

During selective autophagy, the recognition of a specific cargo depends on a series of receptor proteins (e.g., ATG19p in cytoplasm-to-vacuole targeting and ATG32p in mitophagy). Such receptors sense stimuli that induce mitophagy and couple mitochondrial dynamics to the quality control machinery. In the current study, we uncovered a direct relationship between prohibitin and ChATG24. Co-IP and BiFC assays provided evidence that both ChPhb1 and ChPhb2 interact with ChATG24 in the mitochondria. Moreover, ΔChATG24 is defective in mitophagy. Our findings suggest that prohibitin regulates fungal virulence by mediating ChATG24-assisted mitophagy.

This study provides insight into the relationship between prohibitin and mitophagy in a fungal pathogen, and reveals a new mitophagy regulation mechanism: prohibitin acts as a ChATG24-assisted mitophagy regulator. To the best of our knowledge, this is the first functional analysis of prohibitins in a phytopathogenic fungus. These findings expand our understanding of the mitophagy mechanism in fungi.

### Methods

**Fungal strains, media and growth conditions.** Colletotrichum higginsianum strain IMI349061 (Ch-1) is fully virulent on *A. thaliana* ecotype Col-0 and was used as the reference wild-type strain in all experiments. All strains derived from the reference strain were maintained and cultured on potato dextrose agar (PDA) at 25 °C in the dark. *All C. higginsianum* strains used in this study are listed in Supplementary Data 2. For fungal DNA or RNA isolation, five-day-old mycelia collected from liquid cultures grown in potato dextrose broth (PDB) under shaken conditions (180 rpm) were used. Comidia washed from the culture were used for *A. tumefaciens*-mediated transformation (ATMT). To generate the ATMT constructs and complementation mutants, the transformants were isolated and screened on PDA supplemented with 50 μg/ml hygromycin B (Roche, Mannheim, Germany) or 150 μg/ml nymocin (Amresco, OH, USA), respectively.
Deletion and complementation of target genes. To replace the ChPhb1 gene, ~1.1 kb of flanking sequences of upstream and downstream of ChPhb1 were amplified with specific primers (Supplementary Data 2). The PCR product of the upstream and downstream flanking sequence were ligated into pMD18T-HYG vector to generate the initial vector pMD18T-Fl-HYG-F2. This vector was digested with HindIII and KpnI and ligated with pNeo3300III. The resulting constructs were transformed into the wild-type strain Ch-1 via ATMT47. The hygromycin resistant transformants were further confirmed by reverse-transcription PCR (RT-PCR) analysis (Supplementary Data 2) and Southern blotting according to the manufacturer’s instructions (Amersham Gene Images Alkphos Direct Labelling and Detection System, GE Healthcare, UK). ChPhb2 and ChATG24 deletion mutants were generated using the same strategy. To generate ChPhb1/ChPhb2 double knockout mutants, the F1-HYG-F2 sequence of ChPhb2 was ligated with the pNeo3300III-bar vector using a similar strategy. The resulting constructs were transformed into the ΔChPhb1 mutant. The putative knockout mutants with hygromycin B resistance and bialaphos resistance (bar) were further confirmed by RT-PCR analysis (Supplementary Data 2).

To construct the complementation vector, the ChPhb1 complement fragment, containing its native promoter region and the entire gene coding region without the termination codon was cloned into pNeo3300-GFP vector. The complementation vector was transformed using ATMT co-culture with the ΔChPhb1 deletion mutant. Putative complementation mutants with G418 resistance were selected for RT-PCR (Supplementary Fig. 1). Similar methods were used to complement the ΔChPhb2 and ΔChATG24 deletion mutants.

Phenotypic analyses. Mycelial plugs (9 mm in diameter) of the wild-type strain and mutants were transferred to PDA plates and incubated in the dark at 25 °C. The assays for vegetative growth and conidial production were performed as described28. Mycelial plugs were collected by scraping from liquid cultures that had been grown in 250 ml PDB and shaken at 180 rpm at 25 °C for 7 days; the mycelia were dried at 50 °C and weighed. For conidial germination and appressorial formation, droplets of 10 μl of conidial suspensions (1 × 106 conidia/ml) were dropped onto plastic coverslips and incubated at 25 °C for 24 h. Conidial germination and appressorial formation were measured. The percentages of conidial germination and appressorial formation were determined by microscopic examination of at least 100 conidia or appressoria. ROS stress response assays were carried out in PDA plates supplemented with 2 mM menadione and 3 mM H2O2, separately, and incubated in the dark at 25 °C for 7 days. Each test was repeated three times.

Pathogenicity and plant infection assays. To test virulence, 5 ml conidial suspension from 4-week-old seedlings of susceptible A. thaliana ecotype Col-0 with a sprayer as described previously48. Inoculated plants were incubated in a chamber at 25 °C under dark and saturated humidity conditions for 24 h, and then transferred to a growth chamber at 25 °C, with a light/dark photoperiod of 12/12 h. Lesion formation was examined at 7 days post-inoculation (dpi). Each test was repeated three times. Trypan blue staining was used to assess the extent of fungal colonization, inoculated leaf tissues were fixed and destained in a methanol:chloroform:glacial acetic acid (6:3:1) solution, rehydrated in water, stained with trypan blue49, and viewed by light microscopy.

Mitophagy monitoring assays. For mitophagy monitoring assays, the MTS-GFP expressing strain was first grown in complete medium (CM) (1 g/L yeast extract, 3 g/L peptone, 0.5 g/L sucrose, and 0.5 g/L yeast nitrogen base) and then incubated in basal medium with glycerol (BG; 1.6 g/L yeast nitrogen base, 2 g/L asparagine, 1 g/L NH4NO3, and 1.5% v/v glycerol) for 30 h. The mycelia were then starved by 6 h incubation in nitrogen-starvation minimal medium (MN) containing 2 mM PMSF (0.5 g/L KCl, 0.5 g/L MgSO4, 1.5 g/L KH2PO4, 0.1% v/v trace elements, 10 g/L glucose, pH 6.3)43. The samples were stained with 10 μM Cell Tracker Blue CMAC-Dye (Molecular Probes, C2110) for 30 min at 25 °C before observation. For autophagy monitoring assays, CM-cultured mycelia were washed and incubated in MM-N for 6 h to induce nonselective autophagy.

Yeast two-hybrid assays. Yeast two-hybrid (Y2H) analysis was carried out using the Matchmaker Gold Yeast Two Hybrid System (Takara Clontech). Details of the primers for the constructs used in Y2H are listed in Supplementary Data 2. Yeast strains carrying pGBK7-T53-pGaDIT7-T or pGBD/pGAD were used as positive and negative controls, respectively. Yeast transformants expressing plasmids which harbor the indicated constructs were assayed for growth on nonselective plates (SD/-Leu/-Trp) and selective plates (SD/-Leu/-Trp/-His) with 1 mM X-gal for 3 days.

Co-immunoprecipitation assays. Co-immunoprecipitation (Co-IP) assays were used to confirm the protein interactions in vivo. ChPhb1 cDNA was cloned into pNeo3300-FLAG, while ChPhb2 cDNA was cloned into pNeo3300-GFP. The resulting vectors (ChPhb1–3×FLAG and ChPhb2–GFP) were co-transformed into the wild-type strain. All proteins were isolated from transformants expressing both ChPhb1–3×FLAG and ChPhb2–GFP, and incubated with anti-Flag M2 affinity resins (Sigma Aldrich). Proteins that bound to the M2 resins were eluted after a series of washing steps following the manufacturer’s instructions. Immunoblot analysis was performed with anti-FLAG (1:2000 dilution; Sigma Aldrich, USA) and anti-GFP (1:10000 dilution; TransGen Biotech, China) primary antibodies. Immunoblot signals were detected using the ChemiDoc XRS + system (Bio-Rad). Interactions of ChPhb1–3×FLAG/ChPhb2–GFP, ChPhb1–3×FLAG/ChATG24-GFP and ChATG24-GFP/ChPhb2–GFP were also measured based on the above methods. Similar methods were used to test the deletion strain ΔChPhb1 expressing ChATG24-GFP/ChPhb2–GFP fusion constructs, and deletion strain ΔChPhb2 expressing ChPhb1–3×FLAG with ChATG24-GFP fusion construct.

Protein extraction and immunoblot analysis. For immunoblot analysis, total proteins were isolated from vegetative hyphae as described22, and then were separated by 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Ontario, Canada) with a Bio-Rad electroblotting apparatus. Immunoblot signals were measured using the ChemiDoc XRS + system (Bio-Rad).

Bimolecular fluorescence complementation assays. BifC assays were conducted to confirm protein interactions in vivo. The ChPhb1–1×YFP and ChATG24–2×CFP fusion constructs were generated by cloning the ChPhb1 and ChATG24 fragments, respectively, into pNeo3300-CYFP. The ChPhb2–2×NTPF and ChATG24–2×NCPF fusion constructs were generated respectively by cloning the ChPhb2 or ChATG24 fragment, into pNeo3300-NYPF. The construct pairs ChPhb1–1×YFP/ChPhb2–2×NTPF, ChPhb2–2×NTPF/ChATG24–2×CFP, ChPhb1–1×YFP/ChATG24–2×CFP were co-transformed into wild-type Ch-1 using ATMT, with NYPF- and CFP-expressing strains used as negative controls. Transforms that were resistant to hygromycin
and neomycin were isolated and confirmed by PCR. YFP signals were examined under a Leica SP8 LSCM.

**Bioinformatic**s. The full-length sequences of ChPhb1 and ChPhb2 were downloaded from the genomic database (http://fungi.ensembl.org/Colletotrichum_higginsianum/Info/Index) of C. higginsianum isolate IMI349063. Protein domain and motif predictions were performed with DOG (Domain Graph, version 1.0) software. Homologs to the ChPhb1 and ChPhb2 protein sequences from different organisms were identified from the GenBank database using the BLASTP algorithm. A phylogenetic tree was generated with MEGA software (version 7.0, http://www.megasoftware.net/index.php) using the neighbor-joining (NJ) algorithm.

**Statistics and reproducibility.** All values and error bars in graphs are means ± standard error of the mean (SEM); respective n values are indicated in Figure legends. Statistical analysis was calculated by two-tailed Student’s t tests using the Graphpad Prism 8 software.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**. The source data underlying the graphs and real-time PCR analysis within the paper can be found in Supplementary Data 1. Unprocessed blots can be found in Supplementary Figs. 7–11. All other data, vectors and plasmids are available from the corresponding authors on reasonable request.

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**Author contributions**

YY conducted most of the experiments and wrote the manuscript. TJ, YQ, LC, CX and LH conducted parts of the research. HJ and BC gave critical suggestions for structure and writing of the manuscript. HT revised the manuscript. ZL supervised all aspects of the study, reviewed critically, and revised the manuscript. All authors read and approved the contents of this manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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