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# CaEch1-mediated mitophagy regulates vegetative growth, conidiation, appressorium formation, and pathogenicity in *Colletotrichum camelliae*

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## Abstract

Anthracnose, caused by *Colletotrichum camelliae*, poses a significant threat to the yield and quality of *Camellia oleifera*. Mitophagy, a selective form of autophagy, is crucial in maintaining mitochondrial quality and intracellular homeostasis. To date, an optimized experimental system for studying mitophagy in *C. camelliae* has yet to be established, and the role of mitophagy in the pathogenesis of *C. camelliae* remains unclear. Here, we characterized the function of CaEch1, a homolog of *Magnaporthe oryza* MoEch1, in the anthracnose fungus *C. camelliae*. Our findings indicate that mitochondria-localized CaEch1, plays a vital role for mitophagy in *C. camelliae*. Also, the CaEch1$_{1\text{-}29}$-GFP could serve as a reliable marker for monitoring mitophagy in *C. camelliae*. Additionally, the knockout of *CaECH1* resulted in defects in fungal growth, conidiation, and appressorium formation. Pathogenicity assays further revealed that knockout of *CaECH1* significantly reduced the virulence of *C. camelliae*. In summary, our research underscores the importance of CaEch1 in growth, conidiation, appressorium formation, and virulence in *C. camelliae*. This suggests its potential as a marker for mitophagy and provides valuable insights for developing new fungicides targeting anthracnose.

**Keywords** Anthracnose, *Colletotrichum camelliae*, CaEch1, Pathogenicity, Mitophagy

## Background

*Camellia oleifera*, a distinctive woody oil crop in China, serves both as an ecological tea fruit and as a valuable resource for poverty alleviation (Guo et al. 2022). It plays a crucial role in ensuring energy security, preserving the environment, and improving oil quality (Wang et al. 2017; Zhang et al. 2022). However, *Camellia oleifera* is vulnerable to various pathogens, leading to a significant reduction in oil tea quality and hindering the healthy and sustainable development of the oil tea industry (Lu et al. 2020; Gong et al. 2020). Anthracnose, caused by *Colletotrichum camelliae*, poses a substantial threat to tea oil production (Lu et al. 2018; Lv et al. 2024). Controlling this disease remains challenging due to the lack of resistant genes or immune varieties against anthracnose.

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Therefore, it is increasingly important to deepen the understanding of the pathogenic mechanism of *C. camelliae* to develop more effective strategies for disease control.

Mitochondria are double membrane-bound organelles that play a critical role in various cellular functions by producing most of the required adenosine triphosphate (ATP) (Chinopoulos and Adam-Vizi 2010). Mitophagy, a selective form of autophagy, is essential for maintaining the appropriate quantity and quality of mitochondria, thereby reducing ROS production during cellular exposure to starvation stress in respiratory growth (Lennicke and Cochemé 2021; Song et al. 2020; Youle and Narendra 2010). In filamentous fungi, mitophagy is pivotal for differentiation in sexual and asexual development, as well as pathogenesis (Kanki et al. 2015; He et al. 2014; Patkar et al. 2012; Kou et al. 2016; Shen et al. 2022). For instance, the enoyl-CoA hydratase MoEch1 and sorting nexin protein MoAtg24 in *Magnaporthe oryzae* are crucial for mitochondrial morphology/mitophagy and infectious growth (Xiao et al. 2020). In *Ustilaginoidea virens*, UvSnx4 plays a role in development, stress adaptation, and pathogenicity through its participation in mitophagy (Shi et al. 2023). The leucine zipper-EF-hand-containing transmembrane protein FgLetm1 of *Fusarium graminearum* also influences mitochondrial morphology and plant infection (Tang et al. 2018). A recent study indicates that prohibitin proteins ChPhb1 and ChPhb2 of *Colletotrichum higginsianum* regulate the translocation of ChATG24, impacting mitophagy and virulence (Yan et al. 2022). These findings highlight the importance of mitophagy in conidial differentiation and pathogenicity of filamentous fungi. However, the molecular mechanism of mitophagy is not extensively clear in *C. camelliae*.

CaEch1 is a homolog of the rice blast fungus enoyl-CoA hydratase MoEch1, a mitochondrial β-oxidation enzyme, which contains a consensus mitochondrial targeting peptide sequence at its N-terminus (1–29 aa). In *M. oryzae*, knockout of *MoECH1* affects the hypha growth, conidiation, and virulence (Patkar et al. 2012). Meanwhile, MoEch1 locates in mitochondria. Similarly, the Ech1 homolog catalyzes by the *Aspergillus nidulans* the second step in the breakdown of fatty acids, which is essential for fatty acid utilization (Hynes et al. 2008). Localization studies revealed that AnEch1$_{1\text{-}29}$ (MTS) was present in tubular and punctate mitochondria. However, the role of the mitochondrial β-oxidation pathway in pathogenesis of *C. camelliae* and themitophagy remains unexplored.

In summary, we demonstrated that Ech1 plays an important role in vegetative growth, conidiation, and pathogenicity in *C. camelliae*. We also found that the mitochondrial β-oxidation enzyme is essential for mitochondrial morphology/mitophagy. Additionally, our study indicates that CaEch1 could be a suitable marker for monitoring mitophagy in *C. camelliae*.

## Results

### Identification of CaEch1 in *C. camelliae*

To identify the homolog of Ech1 in *C. camelliae*, we used a BLASTp search with the *Magnaporthe oryzae* MoEch1 protein sequence as a query. The analysis revealed that CaEch1 is homologous to MoEch1, encoding a polypeptide of 288 amino acids. Protein sequence alignment showed a high similarity of CaEch1 with Ech1 homologs in other fungi, including *Metarhizium robertsii* (33% identity), *Ustilaginoidea virens* (81% identity), *Beauveria bassiana* (76% identity), *Fusarium graminearum* (83% identity), *Colletotrichum fructicola* (99% identity), *Magnaporthe oryzae* (77% identity), *Aspergillus niger* (62% identity), and *Saccharomyces cerevisiae* (33% identity) (Additional file 1: Figure S1). All these Ech1 homologs contain an Enoyl-CoA hydratase domain (ECH_2) and conserved N-terminal residues (Fig. 1a). The evolutionary analysis indicated that CaEch1 is most closely related to the CfEch1 (Fig. 1b). These findings suggested that Ech1 is highly conserved across various fungi and may play an important role in *C. camelliae*.

We then employed a qRT-PCR assay to monitor the expression level of *CaECH1* at different infection stages, including 12, 24, 48, 72, and 96 h post-inoculation (hpi). The results showed that *CaECH1* is significantly upregulated during the infection process, with its expression levels increasing 6–40 folds compared to the 0 hpi (Fig. 1c). These findings suggest that *CaECH1* plays an essential role during the infection stage in *C. camelliae*.

### Knockout and complementation of *CaECH1*

To investigate the biological function of *CaECH1* in *C. camelliae*, we knocked out the *CaECH1* using a homologous recombination strategy with *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Fig. 2a). The Southern blot assay confirmed the gene replacement event, with the 3.6 kb band in the Wild-type (WT) strain shifting to a 5.3 kb band in Δ*Caech1* mutants (Fig. 2b). The three resulting mutant strains displayed smaller colonies compared to the WT. The Δ*Caech1*-6 and Δ*Caech1*-7 transformants were selected for further experiments.

To confirm that the altered phenotypes in the Δ*Caech1* mutants were solely linked to the gene replacement event, a vector (p3300neo-CaEch1) carrying a complete *CaECH1* gene copy with its native promoter was transformed into Δ*Caech1*-6. The complementation strains (Δ*Caech1*-C) were confirmed by qRT-PCR (Fig. 2c, d). The expression level and colony morphology

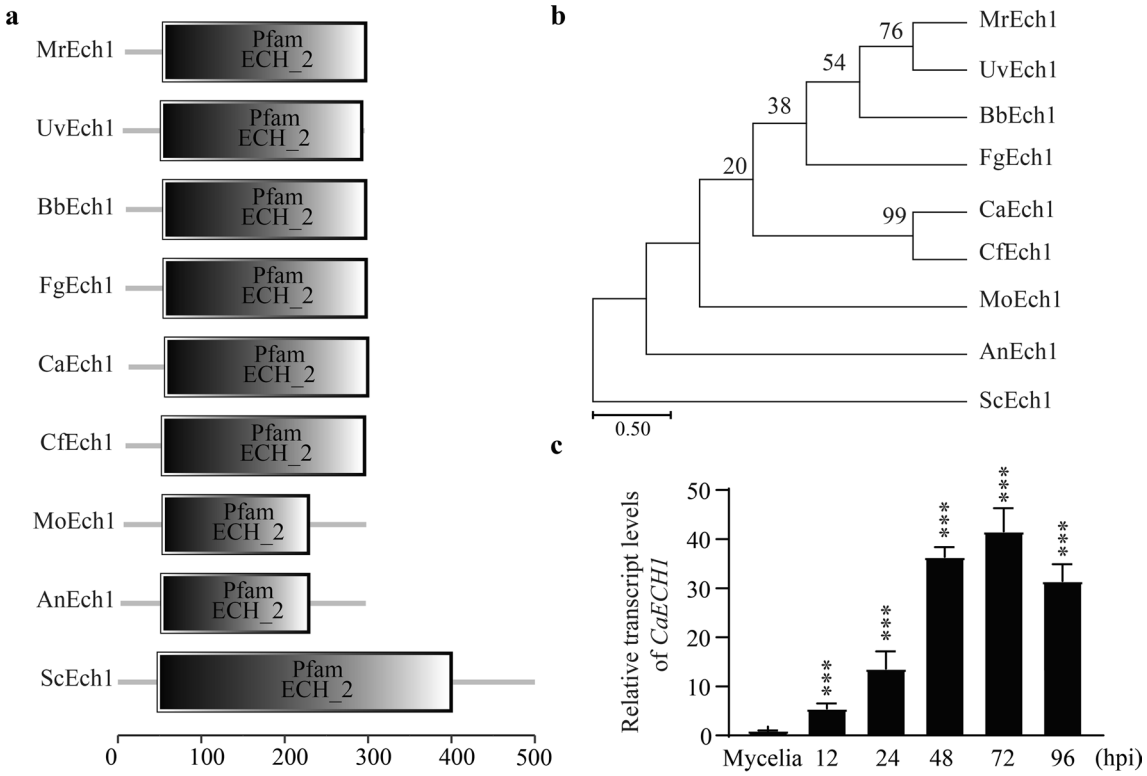


**Fig. 1** Identification of CaEch1 in *C. camelliae*. **a** Analysis of functional domains in Ech1 homologs. The Ech1 homologs analyzed included *Metarhizium robertsii* (XP_007822149.1), *Ustilaginoidea virens* (XP_042996294.1), *Beauveria bassiana* (XP_008594428.1), *Fusarium graminearum* (XP_011327030.1), *Colletotrichum camelliae* (KAH0421588.1), *Colletotrichum fructicola* (XP_031886528.1), *Magnaporthe oryzae* (XP_003717318.1), *Aspergillus niger* (XP_001399422.1), and *Saccharomyces cerevisiae* (NP_010321.1). **b** Phylogenetic analysis of Ech1 homologs from different organisms. **c** The relative expression level of the *CaECH1* gene in C. *camelliae* was analyzed. The expression level of *CaECH1* during infection stages was normalized using the *ACTIN* gene as an internal control. Data were shown as Mean ± SD (n = 3). *** indicates the significant difference at the 0.001 level

of Δ*Caech1*-C strains were comparable to those of WT strains, indicating that Δ*Caech1*-C functionally complemented the phenotype of Δ*Caech1*.

### CaEch1 is involved in vegetative growth, conidiation, and conidial morphology

Given that the colonies of Δ*Caech1* mutants appeared smaller than those of WT, we assessed hyphal growth by inoculating mycelial plugs of the WT, Δ*Caech1*, and Δ*Caech1*-C strains on PDA plates for 7 days. The results showed that the colony diameters of the Δ*Caech1*-6 and Δ*Caech1*-7 mutants were considerably reduced compared to WT during the cultured process. However, the vegetative growth defect of Δ*Caech1* was restored in the Δ*Caech1*-C strain (Fig. 3a, c). These results indicated that CaEch1 is required for fungal growth in *C. camelliae*.

Ech1 homologs are known to related to the asexual development in *M. oryzae* (Patkar et al. 2012). To investigate the function of *CaECH1* in conidiation, we evaluated conidial production by the WT, Δ*Caech1*, and complemented transformant Δ*Caech1*-C. After 7 days of induction on PDA plates, conidia were counted and measured by microscope. The results showed that Δ*Caech1* mutants exhibited a marked reduction in conidiation compared with WT (Fig. 3e). Interestingly, the conidial length of Δ*Caech1* was slightly reduced compared to WT and Δ*Caech1*-C (Fig. 3b, d). These results suggest that CaEch1 plays a crucial role in asexual development in *C. camelliae*.

### CaEch1 is involved in pathogenicity in *C. camelliae*

To determine whether CaEch1 plays a role in plant infection, the WT, Δ*Caech1*, and Δ*Caech1*-C strains were inoculated onto the leaves of susceptible cultivar *Camellia oleifera* under high humidity (90%) and 12-h light/dark conditions. After 7 days of incubation, the Δ*Caech1* strains developed lesions at a considerably slower rate, resulting in smaller lesion areas than those of WT and Δ*Caech1*-C strains (Fig. 4a, b). These results indicate that CaEch1 is important for fungal virulence in *C. camelliae*.

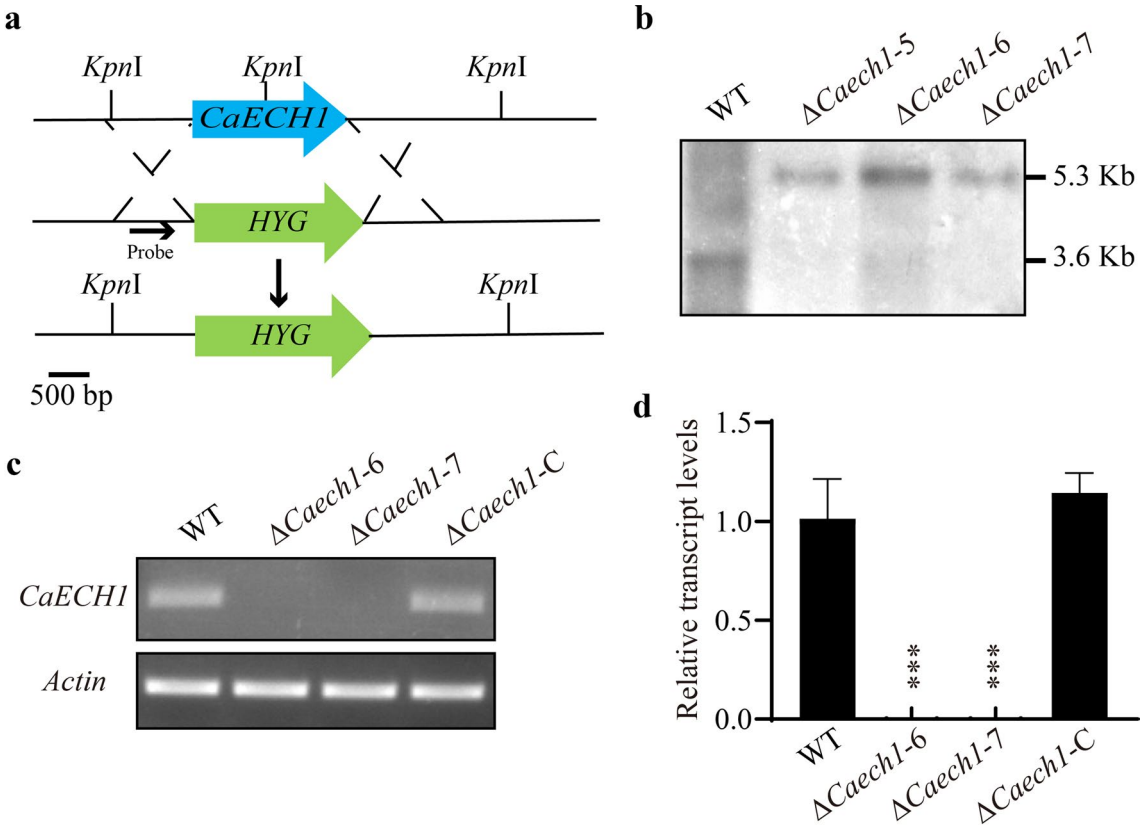


**Fig. 2** Disruption of *CaECH1* and complementation analysis. **a** Strategy for disrupting the *CaECH1* scheme. *HYG*, *hygromycin resistance gene cassette*. **b** Southern blot analysis of WT and Δ*Caech1* strains. The genomic DNA of the specified strains was digested using *KpnI* and analyzed by Southern blotting with a probe situated upstream of the *CaECH1* coding region. In the confirmed *CaECH1* knockout mutants, a 5.3 Kb band in WT shifted to 3.6 Kb. **c** Verification of *CaECH1* deletion mutants by RT-PCR analysis. *CaECH1* expression was evident in WT and Δ*Caech1*-C, no detectable band was found in the Δ*Caech1*-6 and Δ*Caech1*-7 mutants. **d** The expression levels of *CaECH1* were determined by qRT-PCR assay. The results were calculated based on three biological replicates. Asterisks denoted significant differences at a *P*-value < 0.001 level

### CaEch1 is required for conidial germination and appressorium formation

*Colletotrichum camelliae* employs a specialized structure, the appressorium, to invade host cells. The reduced pathogenicity of the Δ*Caech1* strain implies that conidial germination and appressorium formation may be defective. To verify this hypothesis, we observed the conidial germination and appressorium formation on hydrophobic coverslips. The results showed that the conidia of the WT and complemented transformants could germinate normally, but the Δ*Caech1* strain barely germinated conidia (Fig. 5a). Furthermore, the appressorium morphogenesis differed markedly between the WT and Δ*Caech1* mutants (Fig. 5b, c). In the WT, the appressorium morphogenesis is usually formed normally, while more than half of the appressoria in the Δ*Caech1* strain was misshapen (Fig. 5d). These findings suggest that CaEch1 is important for conidial germination and appressorium development in *C. camelliae*.

### CaEch1 localizes on mitochondria and is important for maintaining mitochondria morphology

To investigate the subcellular localization of CaEch1, we fused CaEch1 with a GFP tag at the N-terminus and introduced it into the Δ*Caech1* mutant. The GFP signal exhibited a vesicular, punctate, or tubular pattern, similar to mitochondria in the hyphae of *C. camelliae*. When CaEch1-GFP-expressing hyphae were co-incubated with the mitochondria-specific marker Mitotracker Red CMXRos, the GFP fluorescence co-localized with mitochondria structures, suggesting the CaEch1 localization in mitochondria in *C. camelliae* (Fig. 6a, b). In addition, the results of transmission electron microscopy (TEM) revealed that mitochondria in the Δ*Caech1* strain exhibited markedly vesicular mitochondria compared to the spherical mitochondria with normal cristae (Additional file 1: Figure S2). Taken together, these findings indicate that CaEch1 plays a crucial role in regulating mitochondrial morphology.

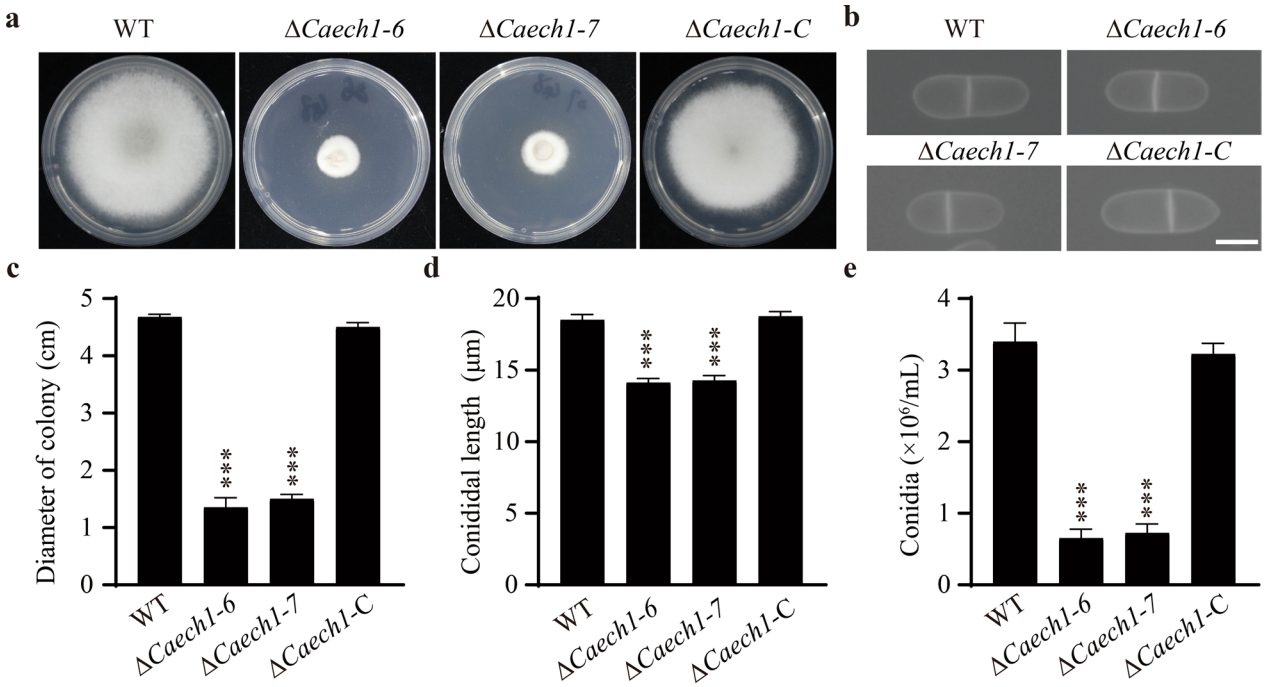


**Fig. 3** CaEch1 is required for mycelial growth, conidiation, and conidial morphology. **a** Disruption of *CaECH1* decreased mycelial growth in *C. camelliae*. Each strain was grown on PDA plates for 7 days prior to being photographed. **b** Conidial morphology of WT, Δ*Caech1*, and Δ*Caech1*-C strains were measured. Scale bar, 5 μm. **c** The diameter of 7-day-old colonies were measured and presented with mean ± SD. **d** The colony diameters of the indicated strains. **e** Conidiation capacity of WT, Δ*Caech1*, and Δ*Caech1*-C strains were incubated on PDA medium for 7 days. Columns represent mean, error bars represent SD, and *P* values, ***, $P < 0.001$. The experiment in this figure was repeated three times

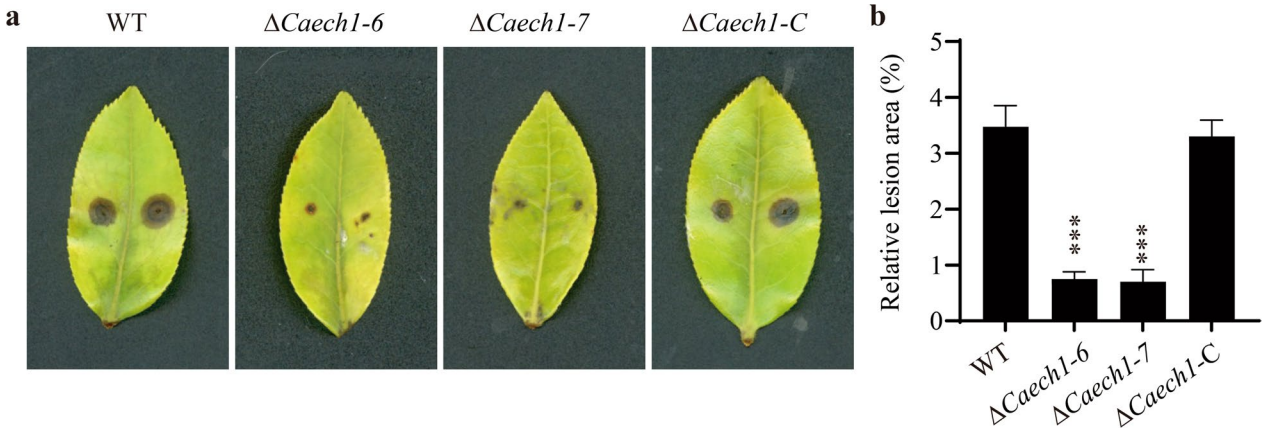


**Fig. 4** CaEch1 is required for virulence in *C. camelliae*. **a** Less lesions were formed on the leaves of *Camellia oleifera* inoculated with Δ*Bduth1* strains. Conidial suspensions of the WT, Δ*Bduth1*, and complemented strains were inoculated onto susceptible cultivar leaves of *Camellia oleifera* in high humidity (90%) under 12-h light/dark conditions. **b** Quantification analysis of lesion area on inoculated leaves. The values were obtained from three biological replicates and repeated three times. The asterisk denotes a significant difference by Student's *t*-test at $P < 0.001$

### CaEch1 could serve as a marker for mitophagy in *C. camelliae*

Mitophagy is a cellular process that selectively removes mitochondria to the vacuole. However, due to the lack of a mitochondrial marker to monitor mitophagy, the molecular mechanism underlying mitophagy remains unclear in *C. camelliae*. To determine whether CaEch1 could serve as a potential marker for monitoring mitophagy, we generated a construct encoding N-terminal 29 residues of CaEch1 fused to the N-terminus

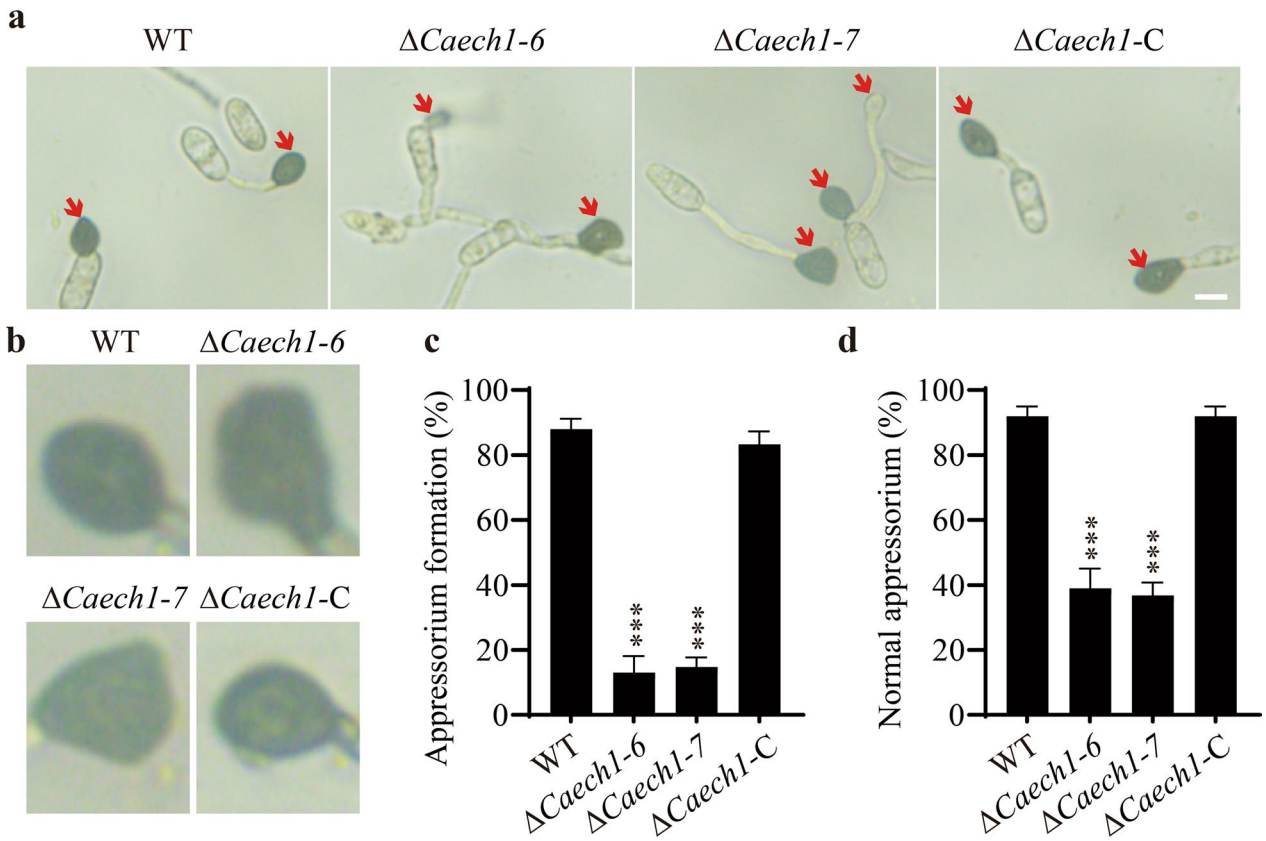


**Fig. 5** CaEch1 regulate conidial germinates and appressorium formation in *C. camelliae*. **a** The conidial suspensions of the WT, Δ*Caech1*, and complemented strains Δ*Caech1*-C, were inoculated onto hydrophobic coverslips. At 30 h post-inoculation (hpi), conidia germinate to form appressorium. **b** The morphology of appressorium in each strain. **c** The percentage of conidia that form appressoria in the WT and derivative strains. **d** The percentage of normal appressoria in WT, Δ*Caech1*, and complemented strains Δ*Caech1*-C. Mean and SD were calculated from three biological replicates. Significant at ***$P < 0.001$. Scale bar, 10 μm

of GFP (CaMts-GFP) and introduced it into the WT strain. The localization of CaMts-GFP was similar to CaEch1-GFP (Fig. 6 and Additional file 1: Figure S3). The CaMts-GFP strain was cultured in a nutrient-rich liquid PDB medium for 2 days and then shifted to the SD-N (nitrogen starvation) medium for 12 h. Simultaneously, the mycelia were stained with CMAC (7-amino-4-chloromethylcoumarin) dye to visualize vacuoles and scanned using a confocal microscope. As shown in Fig. 7a, GFP fluorescence noticeably increased in the CMAC-stained vacuole. In addition, a western blot assay confirmed the degradation of the CaMts-GFP fusion protein under deficient conditions, indicating an increased degradation of CaMts-GFP over time (Fig. 7b). The mitochondrial outer membrane protein Porin was employed as a marker to detect the occurrence of mitophagy (Additional file 1: Figure S4). These findings suggest that CaEch1 is essential for mitophagy and that CaMts-GFP could serve as an appropriate marker for monitoring mitophagy in *C. camelliae*.

## Discussion

Anthracnose, caused by *Colletotrichum camelliae*, has posed a serious threat to the yield and quality of tea-oil trees in recent years, making it important to study the pathogenesis of *C. camelliae* (Liu et al. 2023; Lin et al. 2020). Mitophagy, an evolutionarily conserved cellular process, selectively degrades superfluous, damaged, and dysfunctional mitochondria (Novak 2012; Shen et al. 2022). However, no study has been performed to target the function of mitophagy in *C. camelliae* virulence. Here, we identified enoyl-CoA hydratase Ech1 and confirmed that it is required for mitophagy, implying that CaEch1 could serve as a marker for mitophagy in *C. camelliae*. Moreover, CaEch1 plays crucial roles in hyphal growth, conidiation, appressorium formation, and pathogenicity in *C. camelliae*.

Mitochondria, double-membrane-enclosed organelles, conduct the majority of cellular oxidative processes and generate most of the cell's adenosine triphosphate (ATP), serving as the main energy source

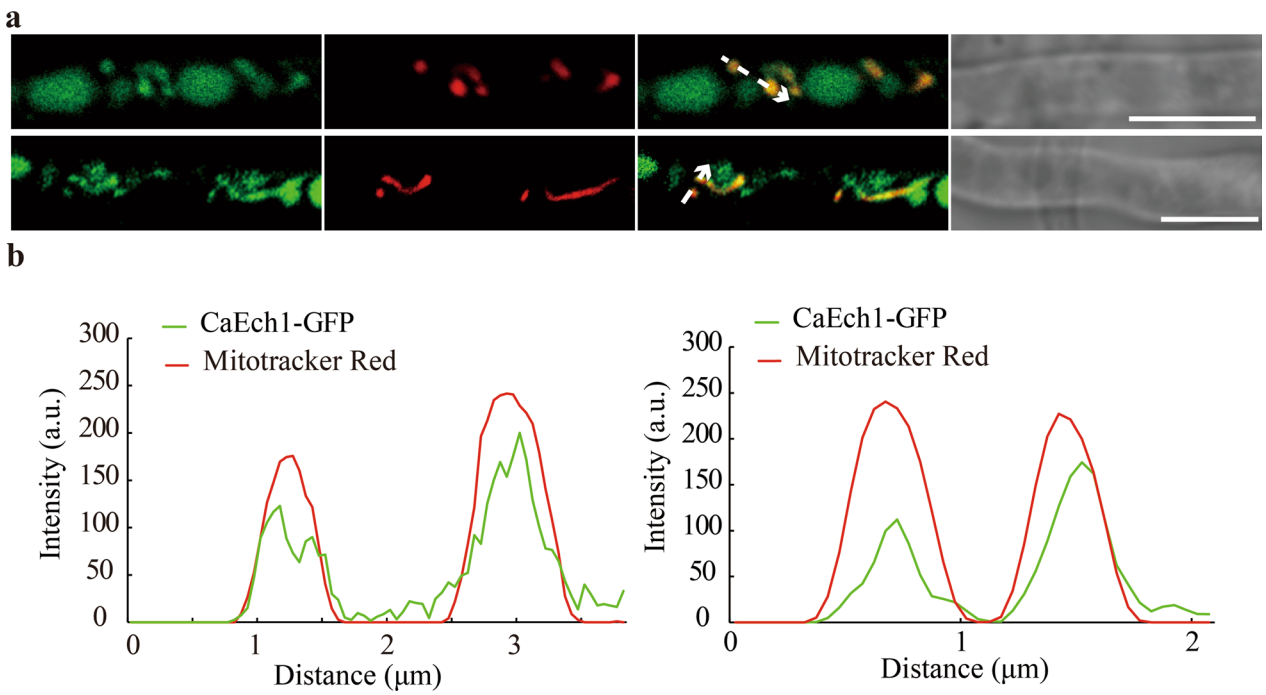


**Fig. 6** CaEch1 is located on mitochondria. **a** CaEch1 is colocalized with the mitochondrial specific marker Mitotracker Red and were observed by confocal microscopy. Scale bar, 5 μm. **b** Fluorescence densities of CaEch1 and Mitotracker Red were analyzed using Image J software

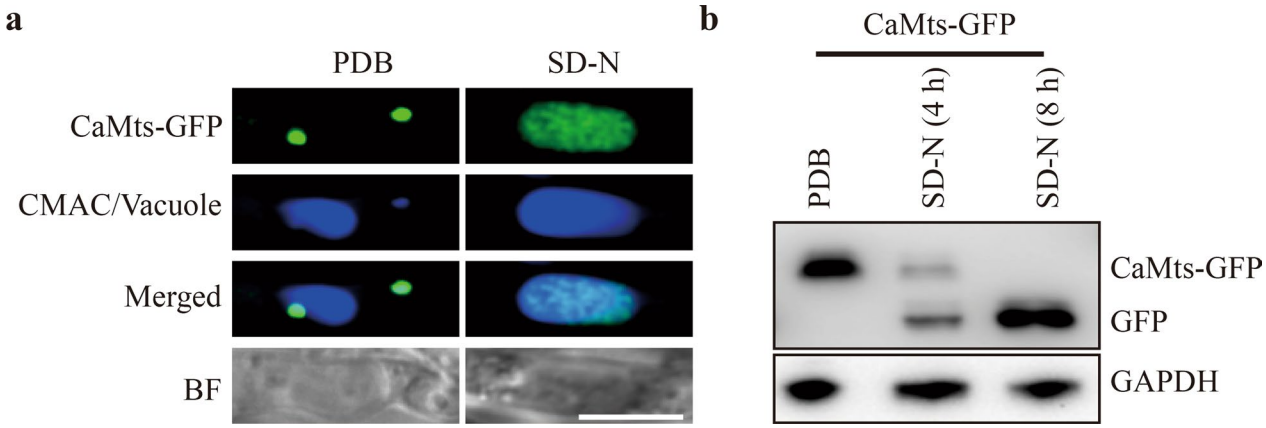


**Fig. 7** Monitoring of mitophagy using CaMts-GFP. **a** The localization of CaMts-GFP was observed under nutrient-rich (PDB) and nitrogen-starved (SD-N) conditions.s The specified strain was grown in the PDB medium for 2 days, followed by transfer to an SD-N medium for 12 h. The vacuoles were stained with CAMC (7-amino-4-chloromethylcoumarin) prior to observation using a confocal laser scanning microscope. Scale bar, 5 μm. **b** CaMts-GFP degradation was observed under nitrogen starvation conditions using Western blot assay with an anti-GFP antibody. GAPDH served as an internal reference

(Chinopoulos and Adam-Vizi 2010; Kou et al. 2019). Mitophagy plays a crucial role in eliminating damaged mitochondria to maintain cellular stability (Kurihara et al. 2012). Mitochondria dysfunction results in the accumulation of defective mitochondria within the cell, leading to various physiological and metabolic disturbances. In mammals, dysfunctional mitochondria are linked to a range of major diseases, including neurodegenerative disorders, diabetes, and heart diseases (Luan et al. 2023; Mary et al. 2022; Eldeeb et al. 2022). Similarly, alterations in mitochondrial morphology are observed in certain plant pathogens with deficiencies in infectious growth. In *Beauveria bassiana*, disruption of mitophagy-related gene *ATG11* resulted in serious

defects in mitochondrial morphology and virulence (Ding et al. 2018). In *Aspergillus oryzae*, the *ATG26* knockout strain which is a mitophagy-deficient mutant, strongly reduced conidial and hyphae growth, as well as invasive growth (Kikuma et al. 2017). Mutation of mitochondrial fission machinery genes in *Magnaporthe oryzae*, such as *DNM1*, *MDV1*, and *FIS1*, impacted both mitophagy and mitochondrial morphology, leading to a decrease in virulence (Zhong et al. 2016). These findings suggest that healthy mitochondrial function is essential for successful infection by plant pathogens.

Ech1 has an Enoyl-CoA hydratase domain and a conserved N-terminate peptide, which may serve as a consensus mitochondrial targeting peptide sequence (1–29 aa) (Patkar et al. 2012). To uncover the characterization of the most conserved Ech1 family proteins, we investigated the homologous gene in other fungi. In *A. nidulans*, Ech1 was localized in the mitochondria (Maggio-Hall and Keller 2004). In *M. oryzae*, $Ech1_{1\text{-}29}$ (MTS) was found on the tubular and punctate mitochondria and played a role in vegetative growth, conidiation, and pathogenicity (Patkar et al. 2012). However, the molecular mechanism of Ech1 remains unclear in *C. camelliae*. Therefore, we conducted BLASTp analyses using the MoEch1 protein sequence as a query to identify the homologous protein in *C. camelliae*, CaEch1. Our findings suggest that CaEch1-GFP (CaMts-GFP) can be used as a reliable marker for mitophagy in *C. camelliae*. First, we observed that CaEch1 is required for mitophagy, as the Δ*Caech1* mutant strain exhibited vesicular mitochondria (Additional file 1: Figure S2). Second, localization studies revealed that the CaEch1-GFP (CaMts-GFP) strain was located in mitochondria (Additional file 1: Figure S3), and the vegetative growth of CaEch1-GFP strain was similar to that of the WT strain (Fig. 3). Additionally, the GFP signal of CaMts-GFP was predominantly found in vacuoles under SD-N conditions. Furthermore, the degradation of the CaMts-GFP fusion protein and the occurrence of mitophagy was detected by immunoblotting (Fig. 7b). Overall, CaEch1-GFP could act as a specific marker for monitoring mitophagy in *C. camelliae*.

## Conclusions

In summary, our study found that CaEch1 plays a crucial role in the fungal growth, conidiation, and pathogenicity of *C. camelliae*. In addition, CaMts-GFP proved to be a reliable marker for monitoring the mitophagy in *C. camelliae*. These results shed light on the molecular mechanism by which CaEch1 regulates virulence in *C. camelliae* and provided insight into potential targets for the development of new fungicides to control anthracnose.

## Methods

### Sequence analysis

The genetic and protein sequences of Ech1 homologs utilized in this research were acquired from the National Center for Biotechnology Information database. The CaEch1 motif and analogous sequences were examined using the SMART software tool. The phylogenetic tree of Ech1 homologs was established through MEGA 10.0 employing a neighbor-joining algorithm (Meng et al. 2022).

### Generation of the mutant and complemented strains

To obtain the mutants of *CaECH1*, a gene knockout strategy was based on homologous recombination using *Agrobacterium tumefaciens-mediated transformation* (ATMT). The knockout vector was designed using pGKO, as described previously (Chen et al. 2020). Briefly, upstream and downstream fragments of *CaECH1*, each approximately 1 Kb, were amplified from the genomic DNA of the WT strain. These fragments were sequentially ligated to flank the *hygromycin* resistance gene cassette in the pGKO plasmids using Recombination exnase® multis (Vazyme Biotech Co., Ltd, C155-02). The recombination vector pGKO-CaEch1 was introduced into the *HBMC-171* strain via the ATMT method (Meng et al. 2020), and positive transformations were selected on PDA medium with 250 μg/mL *hygromycin* B and 200 μMcefatoxime. Transformants were isolated and subsequently screened by PCR, qRT-PCR, and southern blot assay.

Complemented strains were generated using the p3300neo-GFP plasmid vector with a geneticin resistance gene (G418). The full-length genomic copy, composed of 1.7 kb of the promotor and coding region of *CaECH1*, was cloned using primers ech1-cF/cR and then inserted into p3300neo-GFP. The sequenced construct was transformed into Δ*Caech1* by ATMT. The positive transformants were verified by PCR and RT-PCR. All primers used in this study are listed in Additional file 2: Table S1.

### Fungal strains and culture conditions

*Colletotrichum camelliae* Wild-type strain *HBMC-171* was provided by Professor Junbin Huang from Huazhong

Agriculture University (Hubei, China). PDA (Potato sucrose agar; potato 200 g/L, glucose 20 g/L, and agar 15 g/L) medium was used for standard culture maintenance and conidiation. To evaluate vegetative growth, PDA plates inoculated with mycelial plugs were incubated in darkness for 2 days and then transferred to continuous light for 5 days at 25°C. For harvesting conidia, 5 ml of sterile water were introduced to the colonies and gently scraped with an inoculating loop. The suspension was filtered through three layers of lens paper to hinder mycelial debris, and the conidia were collected by centrifugation. For conidial germination and appressorium formation assays, conidia were dropped onto glass slides and artificial hydrophobic films, respectively. Following a 24-h incubation at 25°C, the rates of germination and appressorium formation were scanned and recorded using a microscope.

#### qRT-PCR analysis

Total RNA from mycelia cultured in PDB was extracted using the fungal RNA kit 200 (OMEGA). The resulting total RNA was reverse transcribed, and qPCRs were performed using a Primescript™ RT reagent kit with gDNA Eraser and SYBR Green Premix Ex Taq™ (Tli RnaseH Plus) following the manufacturer's instructions. The Actin gene was used as an endogenous reference gene, and the results were calculated using $2^{-\Delta\Delta Ct}$method (Meng et al. 2021). These experiments were repeated three times independently. The primers used for this experiment are listed in Additional file 2: Table S1.

#### Inoculation assay

The detached *Camellia oleifera* leaf infection assay was performed to assess pathogenicity in the mutant strains. The WT, Δ*Caech1*, and Δ*Caech1*-C complemented strains were cultured on PDA medium for 7 days. Leaves of Changlin 40 were inoculated with conidial suspensions ($1\times10^6$ spores/mL) of each strain. The inoculated leaves were incubated in a 25°C humidified chamber with a 14-h light and 10-h dark cycle for 7 days. The disease symptoms were recorded, and the relative lesion area was measured using the Image J software. All experiments were repeated three times with three biological replicates each time.

#### Generation of the CaEch1-GFP strain and Western blot analysis

A short stretch encoding the N-terminal 29 residues of CaEch1 was fused to the N-terminus of GFP (MTS-GFP). Briefly, approximately 1.5 kb of upstream flanking sequences and 87 bp of the coding region of CaEch1 were PCR amplified and ligated to p3300neo-GFP. The recombinant plasmid was then transferred to the WT strain by ATMT. Positive transformants were verified by western blot and fluorescence microscopy.

For the induction of mitophagy, the CaMts-GFP strain was inoculated in the PDB (potato sucrose agar, potato 200 g/L, glucose 20 g/L) medium for 2 days, and then transferred to SD-N (Yeast nitrogen base without amino acids 1.7 g/L, glucose 20 g/ L) medium for 5 and 10 h, respectively. Total proteins from mycelia were collected and extracted with lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, and 1 × proteinase inhibitor). Equal amounts of proteins were loaded and separated on a 12% SDS-PAGE and transferred to a PVDF membrane. The anti-GFP antibody (Huabio, ET1607-31) and an anti-rabbit IgG were used for the blotting processes. GAPDH (Cat. No. R1208–3, HuaBio, China) was used as an internal reference. Results were detected using the ChemiDoc XRS + system (Bio-Rad, Hercules, USA).

#### Fluorescence Microscopy

The GFP signal in fluorescent strains was observed and recorded by confocal fluorescent LSM 880 microscope (Zeiss, Oberkochen, Germany). For mitochondria staining, hyphae were incubated with the mitochondria-specific marker Mitotracker Red CMXRos for 30 min in the dark at room temperature, then washed with deionized water before observation. To visualize the vacuole, mycelia were incubated with 10 μM CellTracker™ Blue CMAC Dye (7-amino-4-Chloromethylcoumarin, Molecular Probes, C2110) for 20 min at 25°C, then washed with deionized water before microscopic observation. GFP and CMAC were imaged with 488 nm and 405 nm laser excitation, respectively.

#### Transmission electron microscopy observation

Fresh mycelia were carefully incubated with 2.5% glutaraldehyde in phosphate buffer for at least 4 h. The samples were then promptly dispatched to Zhejiang University for processing and subsequent observation under transmission electron microscopy (Hitachi H-7650) (Zhong et al. 2016).

#### Statistical analysis

All experiments were conducted in triplicate. Final data analysis was performed using SPSS via single-factor ANOVA, with statistically significant results indicated by

asterisks. Error bars represent the standard deviation of the three replicates.

**Abbreviations**

| | |
|---|---|
| ATP | Adenosine triphosphate |
| BF | Bright field |
| CFW | Calcofluor white |
| CMAC | 7-Amino-4-chloromethylcoumarin |
| d | Day(s) |
| GFP | Green fluorescent protein |
| h | Hour(s) |
| hpi | Hours post inoculation |
| Kb | Kilobase |
| PDB | Potato sucrose agar |
| ROS | Reactive oxygen species |
| WT | Wild-type |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00299-w.

**Additional file 1.**: **Figure S1.** The alignment of the SUN family members from different organisms. **Figure S2.** CaEch1 is important for mitochondrial morphology. **Figure S3.** CaMts-GFP is partly located on mitochondria. **Figure S4.** Immunoblotting detection of the mitochondria outer membrane protein Porin.

**Additional file 2. Table S1.** Primers used in this study.

**Author contributions**

SC and CL planned and designed the research. LC, LW, and YC performed the experiments and analyzed the data. SM and MX wrote the manuscript. All authors have read and agreed to the latest version of the manuscript for publication.

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## Declarations

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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