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A Novel Light-Responsive Gene *BcCfaS* Regulates the Photomorphogenesis and Virulence of *Botrytis cinerea* via Lipid Metabolism

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ABSTRACT

Light is a fundamental environmental factor for living organisms on earth—not only as a primary energy source but also as an informational signal. In fungi, light can be used as an indicator for both time and space to control important physiological and morphological responses. *Botrytis cinerea* (*B. cinerea*) is a devastating phytopathogenic fungus that exploits light cues to optimize virulence and the balance between conidiation and sclerotia development, thereby improving its dispersal and survival in ecosystems. However, the components and mechanisms underlying these processes remain obscure. Here, we identify a novel light-signaling component in *B. cinerea*, *BcCfaS,* which encodes a putative cyclopropane fatty-acyl-phospholipid synthase. *BcCfaS* is strongly induced by light at the transcriptional level and plays a crucial role in regulating photomorphogenesis. Deletion of *BcCfaS* results in reduced vegetative growth, altered colony morphology, impaired sclerotial development, and enhanced conidiation in a light-dependent manner. Moreover, the mutant exhibits serious defects in stress response and virulence on the host. Based on a lipidomics analysis, a number of previously unknown fungal lipids and many *BcCfaS*-regulated lipids are identified in *B. cinerea*, including several novel phospholipids and fatty acids. Importantly, we find that *BcCfaS* controls conidiation and sclerotial development by positively regulating methyl jasmonate (MeJA) synthesis to activate the transcription of light-signaling components, revealing for the first time the metabolic base of photomorphogenesis in fungi. Thus, we propose that *BcCfaS* serves as an integration node for light and lipid metabolism, thereby providing a regulatory mechanism by which fungi adapt their development to a changing light environment. These new findings provide an important target for antifungal design to prevent and control fungal disease.

1. Introduction

Light is a prerequisite for life, as it not only provides a source of energy but also acts as a crucial environmental signal that informs most living organisms—ranging from plants and animals to microbes—about their surroundings and drives adaptive behavior [1,2]. As sessile organisms, fungal species can utilize surrounding light cues to measure the time of day and obtain information about whether they are in soil, inside a host, or exposed to air and other stresses [3]. This information then directs the transcriptional reprogramming and regulates many physiological and morphological processes that enable the fungi to optimize their growth and development according to the prevailing conditions [4]. In fungi, light plays an essential role in regulating the balance between asexual and sexual development, which can promote the survival and dispersal of fungal species in ecosystems [5]. Moreover, several fungal pathogens can use light, perhaps in combination with a circadian clock, to adjust their pathogenic capacity and achieve maximal infection success at a specific time of day [6–8]. These processes have detrimental effects on human life, as the uncontrolled dissemination and optimal virulence of fungi can cause destructive plant and human diseases. On the other hand, fungal photobiology has useful aspects for humans, since many fungal metabolites (e.g., pigments and antiviral compounds) that

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accumulate after exposure to light are used in the food and medicine industries [9,10]. Therefore, elucidating the molecular mechanisms underlying fungal photobiology would help to improve their application to biotechnology and further our understanding of their pathogenic life cycle, which can be targeted for developing antifungal strategies.

Botrytis cinerea (*B. cinerea*) is a destructive phytopathogenic fungus that causes gray mold disease on over 1400 host plants, including virtually all vegetables and fruits [11,12]. Due to its scientific and economic importance, *B. cinerea* ranks second among the world's top 10 fungal plant pathogens [13]. This fungus initiates its pathogenic life cycle typically by means of conidia, which are the key determinant of the spread and severity of gray mold disease, as they can be easily dispersed by air. Sclerotia can also serve as a primary inoculum, as they can survive in soil or on plant debris for years and germinate to form mycelia and conidia when conditions become favorable. *B. cinerea* can infect all plant parts including stems, leaves, flowers, fruits, and seeds at both preharvest and postharvest stages [12,14]. Our previous studies found that *B. cinerea* can produce reactive oxygen species via different mechanisms to regulate development and pathogenesis, providing an important target for antifungal design [15–17]. In addition to its broad host range and sophisticated pathogenic strategies, the ability of *Botrytis* to adapt to a dynamically changing environment makes *Botrytis* disease management more challenging. Like most filamentous fungi, *B. cinerea* has evolved the capacity to simultaneously perceive and respond to dynamic changes in light. In this mold, light plays an important role in determining whether it will produce asexual spores for dispersal or form sclerotia for survival. Furthermore, light can regulate the vegetative growth, spore germination, secondary metabolism, stress response, and virulence that enable *B. cinerea* to achieve an optimal pathogenic life cycle [18]. Given its visualized physiological responses to light and the availability of genome sequences, *B. cinerea* may provide an ideal model system to investigate the photobiology of fungal pathogens.

Cyclopropane–fatty–acyl-phospholipid (CFA) synthases are highly conserved proteins found in a variety of organisms. They are enzymes that catalyze the transfer of the methyl group from S-adenosyl methionine to unsaturated lipids [19–21]. CFA synthases have been extensively studied in bacteria, in which they catalyze the cyclopropanation of membrane phospholipids and increase bacterial adaption to environments with changing conditions such as temperature, salinity, oxidation, and pH [22–24]. In several human bacterial pathogens, such as *Helicobacter pylori* (*H. pylori*) and *Mycobacterium tuberculosis*, CFA synthase is required for resistance to antibacterial agents and virulence [25–27]. In plants, CFA synthases play important roles in lipid metabolism, and the deletion of CFA synthases has been shown to cause compositional changes in fatty acid species [20]. Fungal CFA synthase has only been sporadically studied in the model mushroom *Coprinopsis cinerea*, where it was found to be associated with fruiting body formation [28]. However, to the best of our knowledge, CFA synthase has not been previously reported in pathogenic fungi.

In this study, we identified a CFA synthase coding gene *BcCfaS* as a novel light-responsive signaling component in *B. cinerea*. *BcCfaS* plays an indispensable role in light-regulated physiological and morphological processes. With lipid profiling and chemical complementation analyses, we show that *BcCfaS* acts as an important light-dependent regulator in repressing conidiation and promoting sclerotial development by regulating methyl jasmonate (MeJA) synthesis. Thus, we propose that *BcCfaS* coordinates light signaling, development, and lipid metabolism in *B. cinerea*, thereby improving its dissemination and adaptation in nature.

2. Materials and methods

2.1. Strains and growth conditions

This study used *B. cinerea* strain B05.10 as the wild type (WT) and as the recipient for mutant strain construction in this study. Protoplast transformation and regeneration were performed on Schenk and Hildebrandt (SH) medium [29]. For phenotypic analysis, all strains were routinely grown at 22 °C on potato dextrose agar (PDA) medium (200 g of potato, 20 g of dextrose, 15 g of agar, and 1000 mL of distilled water) under white light conditions for conidia production or under dark conditions for sclerotia formation. White light (7000 lx) was provided by white fluorescent tubes in the incubator. For conidial suspension preparation, spores were collected from 10-day-old sporulating cultures, filtered through two layers of Miracloth and diluted to a concentration as required.

2.2. Sequence analysis of the BcCfaS gene

The genome sequence of *BcCfaS* was downloaded from the *B. cinerea* database,[†] and *BcCfaS* homology sequences from different fungi were collected from GenBank,[‡] utilizing the *BcCfaS* sequence as a query for the Basic Local Alignment Search Tool (BLAST) search program. A multiple sequence alignment analysis was conducted using the EMBOSS Needle webtool. The phylogenetic tree was created with MEGA 5.0 software, as described in our previous report [30].

2.3. Plasmid constructs and fungal transformants

The deletion mutants of *BcCfaS* were obtained by means of the homologous recombination method reported previously [29]. In brief, two fragments with approximately 1.0 kb of sequences flanking the *BcCfaS* gene were amplified with specific primers and introduced into pLOB7 in the upstream and downstream of the hygromycin phosphotransferase gene (HPH) cassette using the ClonExpress II One Step Cloning Kit (Vazyme, China). The 4.4-kb fragment, including the flanking sequences and the HPH cassette, was amplified and transformed into the protoplasts via the polyethylene glycol (PEG)-mediated transformation method. The transformants were selected on SH medium containing 50 $\mu g \cdot mL^{-1}$ hygromycin B (Roche, Germany). To obtain homokaryotic mutants, single spore isolation was performed using a selective medium supplemented with 150 $\mu g \cdot mL^{-1}$ hygromycin B. Southern blot analysis was conducted to identify single-copy genomic integration according to a previously reported method [31].

For gene complementation vector construction, the *BcCfaS* expression cassette, including the full coding sequence of *BcCfaS* with its upstream fragment and downstream fragments, was amplified and introduced into Spe I/EcoR I-digested pNAN-OGG [32]. The complementation of the *BcCfaS* deletion mutant was accomplished via the targeted integration of *BcCfaS* at the *bcniiA* gene locus, resulting in the replacement of the nourseothricin-resistance cassette and the expression cassette. The primers used for generating the deletion and complementation strains are presented in Table S1 in Appendix A.

To construct the *BcCfaS*-Green fluorescent protein (GFP) fusion cassette, the open reading frame of *BcCfaS* was amplified and assembled with the NotI-digested pNAN-OGG. The 7.4-kb fragment containing the flanking sequences of *bcniiA*, the *BcCfaS*-GFP fusion cassette, and the nourseothricin resistance cassette was amplified with specific primers and then transformed into the pro-

[†] https://fungi.ensembl.org/Botrytis_cinerea/Info/Index.
[‡] https://www.ncbi.nlm.nih.gov/BLAST.

toplasts of the WT strain. The transformants were selected on SH medium supplemented with 50 μg·mL^{-1} nourseothricin. For subcellular localization analysis, the mycelia were cultured in liquid potato dextrose broth (PDB) on a shaker (22 °C, 200 r·min^{-1}, 4 h), collected, and incubated in darkness or light for an additional 2 h. Endoplasmic reticulum localization was performed using ER-tracker red (Thermo Fisher Scientific, USA) staining. The fluorescence of the fusion protein was observed by means of a confocal microscope (ZEISS LSM 980, Germany).

2.4. Fungal growth and development assays

For the mycelial growth rate, conidial production, and sclerotia formation assays, mycelial plugs (2 mm in diameter) taken from the 3-day-old fungal culture were inoculated on PDA medium and incubated under continuous white light or dark conditions. The colony diameters of different strains were measured using the crossing method and recorded at the times indicated in the figure legends. Conidiation was determined by counting the number of conidia using an automated cell counter (Countstar, China), and sclerotial formation was observed and recorded by counting the number of sclerotia. To assess the colony characteristics, cultures were grown on PDA medium with sterile cellophane and detected at 3 days post inoculation (dpi) with a fluorescence microscope (Leica DM 2500, Germany).

2.5. Pathogenicity assay

Apple fruits (*Malus pumila* Mill cv. Fuji), strawberry fruits (*Fragaria ananassa* L.), and tomato leaves without physiological or pathological damages were used for the pathogenicity assay. Before inoculation, the fruits were sterilized with 2% sodium hypochlorite, washed with running water, and wounded with a sterilized needle. For infection, the conidial suspension was diluted to 2 × 10^5 spores·mL^{-1} in sterilized distilled water with 1/4 volume of PDB. A 5 μL aliquot of a conidial suspension of each strain was added to the wound of the fruits or dropped onto the upper surface of the tomato leaves. Inoculated tissues were incubated in a humidity chamber at 22 °C without light. Lesion formation was observed and recorded at the times indicated in the figure legend. The experiment was conducted in triplicate, with each repetition including at least 20 fruits per strain.

2.6. Stress adaptation assay

To evaluate stress tolerance, 5 μL of conidial suspension (1 × 10^6 conidia·mL^{-1}) of each strain was pipetted onto PDA medium amended with diverse stress agents, including the osmotic agents NaCl and KCl, oxidative stress generator H_2O_2, hypoxia-induced factor $CoCl_2$, and cell-wall-disturbing agent Congo Red. The adaptive capacity of each strain to the stress agents was assessed by measuring culture diameters and relative mycelial growth inhibition at 3 dpi [33]. The experiment was conducted in triplicate, with each repetition including at least three colonies per strain.

2.7. RNA isolation and real time quantitative polymerase chain reaction (RT-qPCR)

The total RNA was isolated from the harvested samples using an RNA extraction reagent (Omega, USA), and 1 μg was used to generate complementary DNA (cDNA) using the PrimeScript RT reagent kit (TaKaRa, Japan). SYBR Premix Ex Taq (TaKaRa) was used for RT-qPCR analysis on the Step One Plus Real-Time PCR System (Applied Biosystems, USA). Three technical replicates were performed, and the *BcactA* gene was used as the internal control, as previously reported [15]. Gene-specific primers are presented in Table S1.

2.8. Lipidomic profiling

For lipid extraction, the WT and the Δ*BcCfaS* mutant were cultured for 48 h on PDA medium with sterile cellophane; half of the cultures were then transferred to white light conditions. After another 12 h of incubation, all cultures were harvested and ground into uniform powder using liquid nitrogen. Approximately 100 mg of powder was dissolved in 750 μL of methanol and 2.5 mL of methyl tertiary butyl ether, and then incubated for 1 h on a shaker, as previously reported [34]. Next, 625 μL of water was added and the mixture was incubated for 10 min, followed by centrifugation at 1000*g* for 5 min. The upper phase was isolated, and the lower layer was extracted again with 1 mL of methyl tertiary butyl ether/methanol/water (10:3:2.5 (v/v/v)). The combined organics were dried in nitrogen (N_2) and re-dissolved in 100 μL of isopropanol. The lipid extracts were analyzed using a Thermo Vanquish UHPLC liquid chromatography system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher, Germany). The conditions for lipid separation were as previously described [35]. Mass spectrometry analyses were carried out using a mass spectrometer operating in positive or negative ionization mode (with a spray voltage of 3 kV, a capillary temperature of 350 °C, sheath gas at 20 psi (1 psi = 6.895 kPa), an auxiliary gas rate of 5 L·min^{-1}, and sweep gas at 1 L·min^{-1}) using a full-scan analysis over *m*/*z* 114–1700. To obtain accurate qualitative and relative quantitative results, raw mass data were normalized and analyzed using Compound Discoverer 3.01 software (Thermo Fischer). Data processing and statistical analyses were performed as previously described [35]. The differential lipid metabolites were screened with a variable importance in projection (VIP) score > 1, a *P* value < 0.05, and a fold change ≥ 1.5 or ≤ 0.67. For clustering heat maps, the intensity areas of differential metabolites were log2-transformed and then normalized using the min–max approach. Rows were clustered using the Euclidean distance and complete linkage.

2.9. Evaluation of MeJA accumulation by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

For LC-MS/MS analysis, the samples were prepared as described in Section 2.8. MeJA extraction, purification, and analysis were performed as previously reported [36], with slight modifications. In brief, mycelial powder was dissolved in three volumes of cold 80% ethanol solution. After incubation at 4 °C overnight with gentle rotation, the supernatant was collected via centrifugation at 6000*g* at 4 °C for 20 min and passed through a Waters Sep-pak C18 cartridge. The analytical conditions used for LC-MS/MS were as follows: column, ACQUITY UPLC HSS T3 column (2.1 mm × 100.0 mm, 1.8 μm; Waters, USA); solvent system, water (0.1% formic acid) and acetonitrile (0.1% formic acid); gradient program, 5:95 (v/v) from 0 to 4 min, 90:10 (v/v) from 4 to 8 min, and 5:95 (v/v) from 8 to 10 min; flow rate, 0.38 mL·min^{-1}; temperature, 45 °C; and injection volume, 10 μL.

2.10. Statistical analysis

Statistical analyses were performed with SPSS statistical software (SPSS Inc., USA) via one-way analysis of variance. Mean separations were carried out using Student's *t*-test or Duncan's multiple range test. Values of $P \leq 0.05$ were considered to be statistically significant.

3. Results

3.1. Identification of BcCfaS as a novel light-responsive gene in B. cinerea

To identify the key regulatory elements in the light-signaling pathway, we conducted a high-throughput RNA sequencing analysis of *B. cinerea* in response to 60 min of light exposure. In this way, a large number of light-responsive genes were screened (data not shown), among which *Bcin03g06110* was significantly induced by light at the transcriptional level (Fig. 1(a)). *Bcin03g06110* was annotated as encoding a putative CFA synthase family protein in the NCBI database.[†] We thus named this gene *BcCfaS*. To further confirm the light-responsive expression pattern of *BcCfaS*, the WT strain was first grown in constant darkness for 60 h, and then transferred to white light conditions for the indicated times ranging from 15 min to 240 min. Next, it was harvested and analyzed via RT-qPCR. The data revealed that the expression level of *BcCfaS* was rapidly induced after 15 min of light exposure and reached its maximum at 60 min with a 14.2-fold increase, displaying a fast and strong light response (Fig. 1(b)).

The *BcCfaS* gene is composed of six introns and seven exons located on chromosome III, with an open reading frame of 1026 bp that encodes an endoplasmic reticulum-localized protein of 342 amino acids (Fig. 1(c)). Protein sequence alignment analysis indicated that BcCfaS shares a high similarity with orthologs from other filamentous fungi, such as *Monilinia fructicola* (*M. fructicola*) (99.1%), *Sclerotinia sclerotiorum (S. sclerotiorum)* (98%), *Magnaporthe oryzae* (*M. oryzae*) (87.2%), *Colletotrichum gloeosporioides* (*C. gloeosporioides*) (91.8%), *Verticillium dahliae* (*V. dahliae*) (91.2%), *Lasiodiplodia theobromae* (*L. theobromae*) (84.2%), *Botryosphaeria dothidea* (*B. dothidea*) (83.4%), *Aspergillus nanangensis* (*A. nanangensis*) (80.2%), *Aspergillus affinis* (*A. affinis*) (75.9%), *Ustilago hordei* (*U. hordei*) (62.0%), and *Ustilago maydis* (*U. maydis)* (64.7%). To investigate the phylogenetic relationship of these proteins, we performed a phylogenetic analysis. This analysis revealed that BcCfaS is highly conserved in filamentous fungi, indicating that it is relatively closely related to the leotiomycetes fungi, including *M. fructicola* and *S. sclerotiorum* (Fig. 1(d)).

3.2. BcCfaS is an important regulator of photomorphogenesis

To explore the biological function of *BcCfaS* in *B. cinerea*, we generated a targeted deletion mutant of *BcCfaS* by means of homologous recombination. The entire open reading frame of *BcCfaS* in WT strain B05.10 was replaced by a hygromycin resistance cassette. Two independent gene-replacement mutants, Δ*BcCfaS*-1 and Δ*BcCfaS*-2, were selected from the hygromycin-resistant transformants for single spore isolation and then identified by polymerase chain reaction (PCR) verification, southern blot analysis, and expression profile analysis (Fig. S1 in Appendix A). Moreover, to confirm the phenotype of *BcCfaS*, a complementary strain was also constructed via an ectopic integration of its full-length nucleotide sequence into the Δ*BcCfaS*.

We first examined the effect of *BcCfaS* disruption on vegetative growth under either light or dark conditions on PDA medium. As shown in Figs. 2(a) and (b), the growth rate of Δ*BcCfaS* was significantly reduced under these conditions; however, the degree of the growth defect was dependent on whether light was present or not. After 2 days of incubation, the colony diameter of Δ*BcCfaS* exhibited a 42% reduction under dark conditions but only a 25% reduction under continuous light conditions, as compared with the WT and complementary strains. Interestingly, the light-regulated growth pattern was different between the WT and the deletion mutant. When exposed to continuous light, the WT strain showed a slight decrease in growth rate. However, the Δ*BcCfaS* strain grew more rapidly in light than in darkness, indicating that *BcCfaS* acts as a positive regulator of the light-dependent inhibition of vegetative growth. In addition, microscopic examination revealed that Δ*BcCfaS* displayed an abnormal colony morphology with densely packed clumps of hyphae under dark but not under light conditions (Fig. 2(a)). These results suggested that *BcCfaS* regulates vegetative growth in a light-dependent manner in *B. cinerea*.

The rapid responsiveness of *BcCfaS* to light at the transcriptional level indicated that *BcCfaS* may be an important light-signaling component of *B. cinerea*. Since light plays a dominant role in regulating fungal development, we were interested in whether *BcCfaS* is involved in light-regulated developmental processes. After 7 days of growth in dark conditions, the WT strain started to form sclerotia, but the Δ*BcCfaS* mutants could hardly develop any sclerotia (Fig. 2(c)). When observations were made after 14 days of incubation, Δ*BcCfaS* could only form fewer and bigger sclerotia (Figs. 2(d) and (e)) and instead produced abundant conidia (Fig. 2(f)), pointing to an important role for *BcCfaS* in repressing conidiation and activating sclerotial development in dark conditions. When grown under light, Δ*BcCfaS* exhibited earlier and more excessive conidial formation, although the growth rates were reduced compared with those of the WT. The number of conidia of Δ*BcCfaS* was up to threefold higher than that of the WT strain at 7 dpi (Fig. 2(g)), indicating that *BcCfaS* also acts as a repressor of light-induced conidiation. All the developmental defects were fully restored by reintroducing the *BcCfaS* gene into the deletion strain Δ*BcCfaS*. These findings suggest that *BcCfaS* is a key regulator of light-dependent development in *B. cinerea*.

To determine the molecular mechanisms of *BcCfaS* in light response and light-dependent development, we examined the expression of 11 predicted photoreceptor-encoding genes and five light-responsive genes, including *BcLTF1*, *BcLTF2*, *BcLTF3*, *BcVEL1*, and *BcFRQ1*, which have been shown to regulate the photomorphogenesis of *B. cinerea*. RT-qPCR showed that five photoreceptor-encoding genes (*BcLOV3*, *BcWCL2*, *BcPHY1*, *BcPHY2*, and *BcBOP1*) and three light-responsive genes (*BcLTF1*, *BcLTF2*, and *BcFRQ1*) were down-regulated in Δ*BcCfaS* under both dark and light conditions (Fig. 2(h)). Furthermore, the expressions of *BcCRY1*, *BOP2*, and *BcLTF3* were significantly decreased in Δ*BcCfaS* only under dark conditions, while the transcription of other genes was not significantly changed (Fig. S2 in Appendix A). These results demonstrate that *BcCfaS* is necessary for the proper expression of photoreceptor-encoding genes and light-responsive genes.

3.3. BcCfaS is required for full virulence

To test whether *BcCfaS* is involved in the virulence of *B. cinerea*, we inoculated the conidial suspensions of the WT, Δ*BcCfaS*, and complementary strain on tomato leaves and on apple and strawberry fruits. As shown in Fig. 3, loss of *BcCfaS* resulted in dramatically decreased virulence on various plant hosts. At 24 h after infection, disease symptoms had fully emerged on the tomato leaves inoculated with the WT and complementary strains, while no lesions were observed on Δ*BcCfaS*-inoculated tomato leaves. When inoculated leaves were incubated for 48 h, the Δ*BcCfaS* mutant only caused very small disease lesions that were 48.6% of those of the control (Figs. 3(a) and (b)). Similarly, the lesion diameters of the mutant exhibited a 60.3% reduction on wounded apple fruit (Figs. 3(c) and (d)) and a 37.8% reduction on strawberry fruit (Figs. 3(e) and (f)) at 72 h after infection. The reduction of virulence in the deletion mutants was completely recovered in the complementary strain. These results indicate that *BcCfaS* is required for the full virulence of *B. cinerea*.

[†] https://www.ncbi.nlm.nih.gov/.

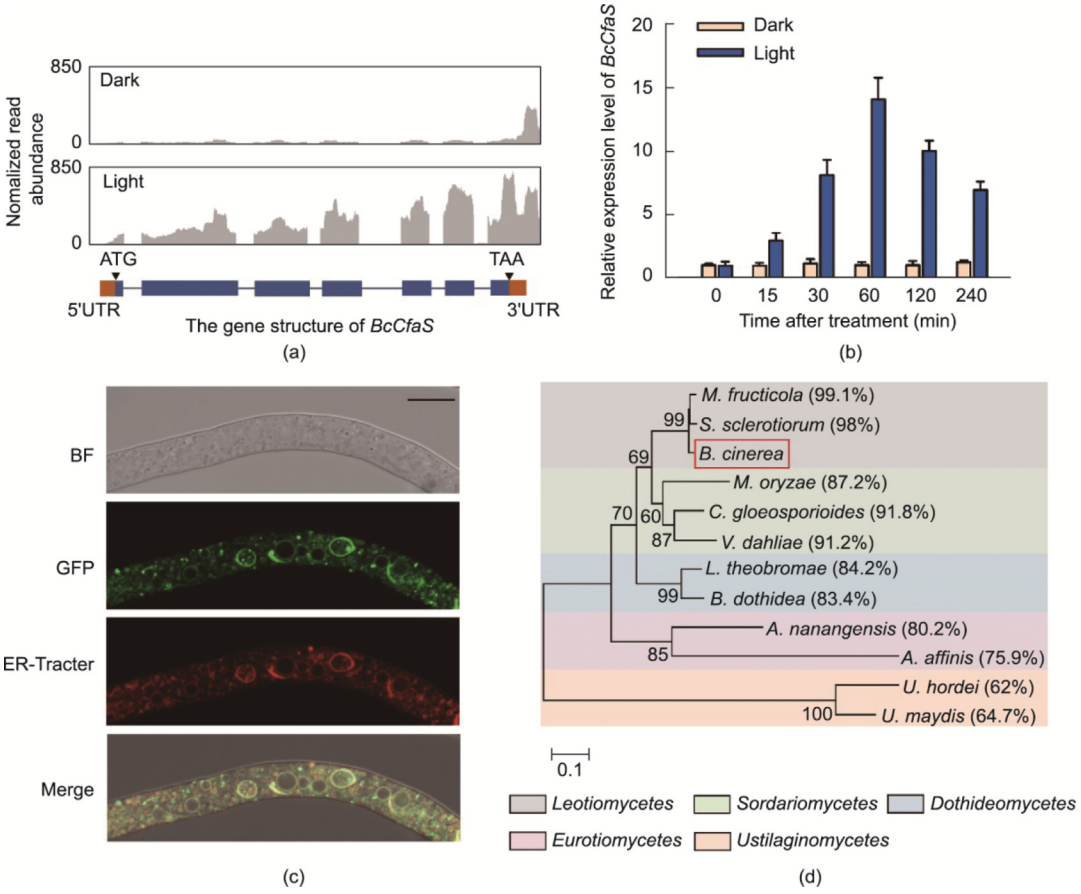

Fig. 1. *BcCfaS* is a novel light-responsive gene in *B. cinerea*. (a) The Integrative Genomics Viewer showing the RNA sequencing reads abundance at *BcCfaS* locus in WT strain B05.10 grown in dark or after light treatment for 60 min. The gene structure of *BcCfaS* indicates untranslated regions (orange boxes), exons (blue boxes), and introns (blue lines). (b) Relative expression of *BcCfaS* after light exposure was determined by RT-qPCR analysis. The mycelia of the WT strain were grown on PDA medium in dark for 60 h, and then transferred to white light (treatment group) or continued to grow in dark (negative control) for 15, 30, 60, 120, 240 min. (c) The subcellular localization of BcCfaS. Bar = 10 μm. (d) Phylogenetic analysis of BcCfaS orthologs from different fungal species. The phylogenetic tree was constructed using MEGA 5.0 with a maximum likelihood method. The horizontal bar indicates the relative distance in the phylogenetic tree. GenBank accession numbers are as follows: *Monilinia fructicola* (*M. fructicola*) (KAA8572885.1), *Sclerotinia sclerotiorum* (*S. sclerotiorum*) (APA08127.1), *B. cinerea* (ATZ48386.1), *Magnaporthe oryzae* (*M. oryzae*) (EHA57053.1), *Colletotrichum gloeosporioides* (*C. gloeosporioides*) (ELA27655.1), *Verticillium dahliae* (*V. dahliae*) (RXG42730.1), *Lasiodiplodia theobromae* (*L. theobromae*) (KAB2573584.1), *Botryosphaeria dothidea* (*B. dothidea*) (KAF4302948.1), *Aspergillus nanangensis* (*A. nanangensis*) (KAF9886006.1), *Aspergillus affinis* (*A. affinis*) (KAI9036615.1), *Ustilago hordei* (*U. hordei*) (SYW80691.1), *Ustilago maydis* (*U. maydis*) (KIS66651.1).

3.4. BcCfaS *is involved in responses to stresses*

The stress-tolerance abilities of plant pathogens are essential to the establishment of a successful pathogenic lifecycle. CFA synthase has been shown to play an important role in mediating the adaptation of bacteria to multiple environmental stresses [22]. To investigate whether *BcCfaS* is involved in stress responses, the WT strain, Δ*BcCfaS*, and the complementary strain were inoculated on PDA medium containing various stress agents. After 3 days of incubation, the growth rates of each strain were compared and the inhibition rate of mycelial growth was calculated to evaluate the stress sensitivities. As shown in Fig. 4, the mycelial growth of the WT and complementary strains was significantly suppressed on PDA medium with the osmotic stressors NaCl or KCl, the acid stressors citric acid/disodium hydrogen phosphate buffer (pH 4), the oxidative stressor H_2O_2, and the hypoxic stressor $CoCl_2$ (Figs. 4 (a) and (b)). However, such suppression was compromised in Δ*BcCfaS*, showing a decreased relative mycelial growth inhibition (Fig. 4(c)). These results indicate that the deletion of *BcCfaS* in *B. cinerea* resulted in an increased tolerance to osmotic, acid, oxidative, and hypoxic stresses. In contrast, the *BcCfaS* mutant was more sensitive to cell wall stress, exhibiting much slower mycelial growth with the cell wall stressor Congo Red than the WT and complementary strains (Fig. 4(c)). Together, these phenotypes suggest that *BcCfaS* plays pleiotropic roles in regulating the tolerance of *B. cinerea* to various environmental stresses.

3.5. BcCfaS *regulates the lipid profile of* B.cinerea *in a light-dependent manner*

To uncover the function of *BcCfaS* in lipid metabolism, the lipid profiles of Δ*BcCfaS* and the WT grown under dark or light conditions were analyzed. In total, 789 lipid species (435 species in positive ion mode and 354 species in negative ion mode) belonging to 19 lipid subclasses were identified in *B. cinerea* (Table S2 in Appendix A). Among them, phosphatidylcholine (PC, 220 species) was found to be the most abundant lipid subclass, followed by triacylglycerol (TAG, 106 species), phosphatidic acid (PA, 76 species), fatty acid (FA, 73 species), phosphatidylethanolamine (PE, 65 species), ceramide (Cer, 41 species), cardiolipin (CL, 39 species), phosphatidylglycerol (PG, 38 species), phosphatidylserine (PS, 27 species), and diacylglycerol (DAG, 27 species) (Fig. 5(a); Table S2), suggesting a high coverage of lipid profiling by this LC-MS/MS-based lipidomics method. To ensure accuracy, all samples were analyzed in quadruplicate under both positive and negative ion models, which displayed a good reproducibility within each

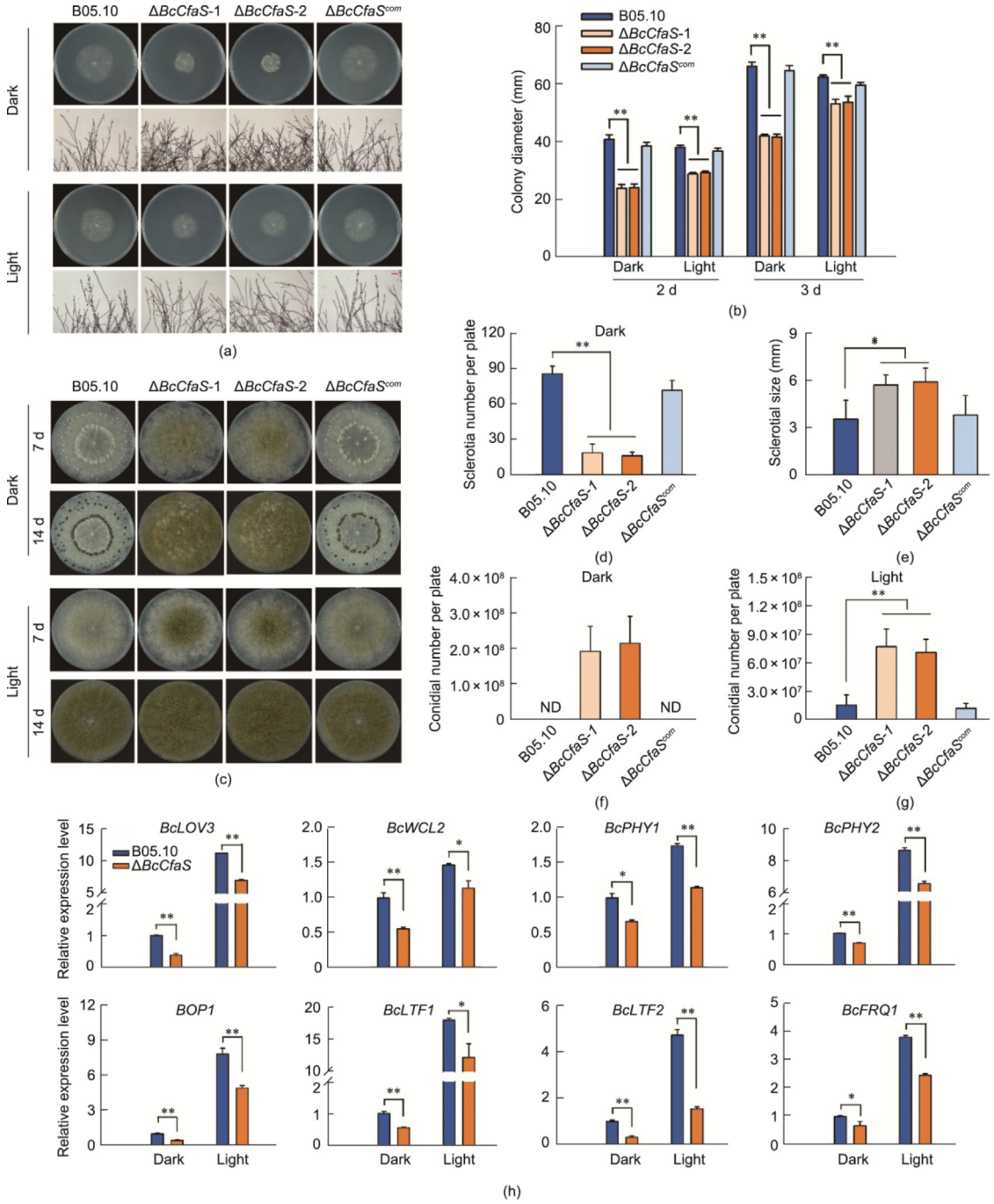

Fig. 2. Deletion of *BcCfaS* affects the photomorphogenesis of *B. cinerea*. (a) Colonies of the WT, Δ*BcCfaS* and the complementary strain (Δ*BcCfaS*^{com}) grown on PDA medium for 2 days at 22 °C. Red bar = 100 μm. (b) Statistical analysis of colony diameter. (c) *BcCfaS* negatively regulates conidiation and promotes sclerotial development. (d) Sclerotia number of the indicated strains on PDA medium in dark for 14 days. (e) Sclerotial size. (f) Conidia number in dark for 14 days. ND: not detected. (g) Conidia number in light for 7 days. (h) Expression levels of several photoreceptor-encoding genes and light-responsive genes related to development were significantly downregulated in Δ*BcCfaS* mutant in darkness and after light exposure. *BcLOV3, BcWCL2, BcPHY1, BcPHY2 and BcBOP1* are photoreceptor-encoding genes. *BcLTF1, BcLTF2 and BcFRQ1* are light-responsive genes that have been shown to regulate developmental processes in *B. cinerea*. The WT strain and Δ*BcCfaS* mutant were grown in dark for 60 h, and then transferred to white light for 60 min. Each bar indicates the mean ± standard deviation (SD) of three repeated experiments (*P < 0.05, **P < 0.01, Student's t test).

group and a clear separation among groups in the partial least-squares discriminant analysis (PLS-DA) scores (Fig. 5(b)).

To identify light-responsive lipid metabolites, we initially analyzed lipid profile changes in the WT strain or Δ*BcCfaS* after light exposure (Fig. 6(a)). In the WT strain, light treatment resulted in 19 differential lipid metabolites in comparison with darkness. In Δ*BcCfaS*, a total of 33 lipid metabolites exhibited light-responsive characteristics, among which only three lipid metabolites were shared with the WT, indicating that *BcCfaS* may play important roles in the light-regulated lipid metabolism of *B. cinerea*. Subsequently, we focused on the differences in lipid composition caused by the deletion of *BcCfaS* (Fig. 6(b)). Compared with the WT strain, 84 differential lipid metabolites under dark conditions and 68 differential lipid metabolites under light conditions were found to be significantly different in Δ*BcCfaS*. To better understand the lipid profile changes between Δ*BcCfaS* and the WT, differential lipids

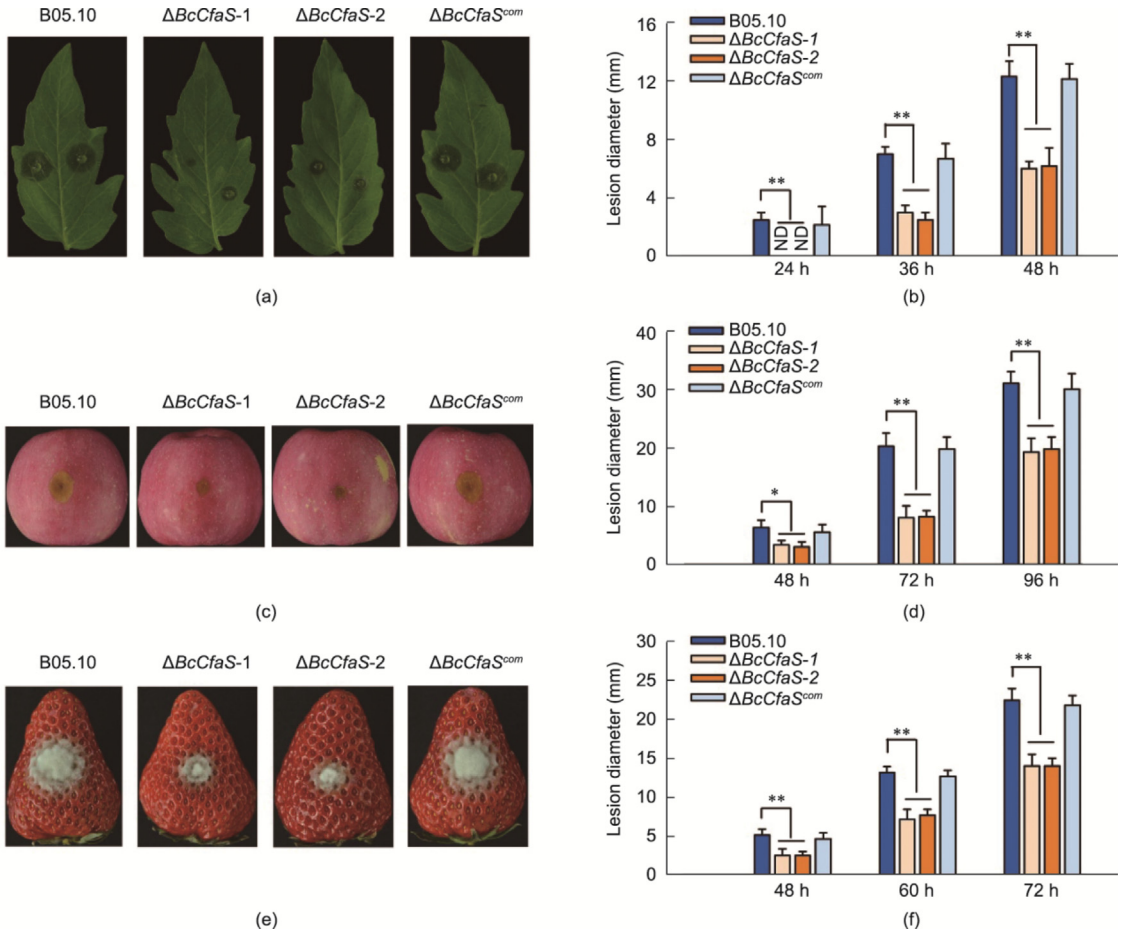

Fig. 3. Deletion of *BcCfaS* impairs the virulence of *B. cinerea*. Tomato leaves and fruits were inoculated with conidia suspensions (5 μL, 2 × 10^5 conidia·mL^{-1}) from the indicated strains and stored at 22 °C. Disease symptoms on (a) tomato leaves at 48 hpi, (c) wounded apple fruits at 72 hpi and (e) wounded strawberry fruits at 72 hpi. Statistical analysis of lesion diameters of (b) tomato leaves, (d) apple fruits and (f) strawberry fruits. Each bar indicates the mean ± SD of three repeated experiments (**$P < 0.01$, Student's t test).

were classified and visualized using heatmap analysis with TBtools software. As shown in Fig. 6(c), these differential metabolites were mainly enriched in seven lipid subclasses, including CL (10 species), PC (21 species), PG (5 species), FA (19 species), PA (24 species), Cer (12 species), and DAG (5 species). Taken together, these results demonstrate that *BcCfaS* disruption widely alters the lipid composition of *B. cinerea* in a light-dependent manner.

3.6. BcCfaS-mediated MeJA synthesis is required for light-dependent development

A link between FA composition and developmental regulation has been well established in filamentous fungi [37–39]. Lipidomic analyses revealed that the levels of 26% of the FAs in *B. cinerea* were significantly changed upon *BcCfaS* deletion (Fig. 6(c)). In terms of biological function, most of these FAs are largely unknown in fungi. Among them, MeJA, a well-known fatty-acid-derived signaling molecule, attracted our attention because of its unique physiological functions in many organisms [40–42]. Accordingly, we quantified the intracellular levels of MeJA in the WT and Δ*BcCfaS* strains grown under dark or light conditions by means of LC-MS/MS (Fig. 7(a)). The results revealed that MeJA could be detected in both Δ*BcCfaS* and WT strains, but its contents were significantly reduced in the *BcCfaS* deletion mutant, regardless of the presence or absence of light (Fig. 7(b)). Furthermore, the level of MeJA was induced by 1.37 fold in the WT strain after light exposure but this induction was largely abolished in Δ*BcCfaS* (Fig. 7(b)). Here, we provide the first evidence that *BcCfaS* plays a role in MeJA synthesis in *B. cinerea*.

To test whether the photomorphogenesis of *B. cinerea* is associated with MeJA level, the WT strain and Δ*BcCfaS* were cultured on PDA medium containing different concentrations of exogenous MeJA. The results showed that MeJA at low concentration had little impact on vegetative growth but significantly affected the conidiation and sclerotial development of the WT strain (Fig. S3 in Appendix A). As shown in Fig. 8, MeJA treatment could not rescue the growth deficiency of the *BcCfaS* mutant in darkness (Figs. 8(a) and (b)) or light (Figs. 8(a) and (c)). However, interestingly, the exogenous addition of MeJA recovered the mutant defects in sclerotial development and conidia production in a concentration-dependent manner (Fig. 8(h)). When the concentration of MeJA increased to 100 μmol·L^{-1}, the number of sclerotia and conidia under dark conditions (Figs. 8(d) and (e)), as well as the conidia under light conditions (Fig. 8(f)), were almost restored to the levels of the WT strain. Moreover, MeJA treatment recovered the expression levels of important components in the light-signaling pathway in the Δ*BcCfaS* mutant, such as *BcLTF1*, *BcWCL2*, and *BcPHY2* (Fig. 8(g)). These results indicate that the light-dependent developmental defects of the *BcCfaS* mutant were largely due to the lack of MeJA, and that an increase in exogenous MeJA could restore the mutant phenotypes.

4. Discussion

Fungi can perceive and transduce light signals from the environment and coordinate transcriptional regulation to adjust their

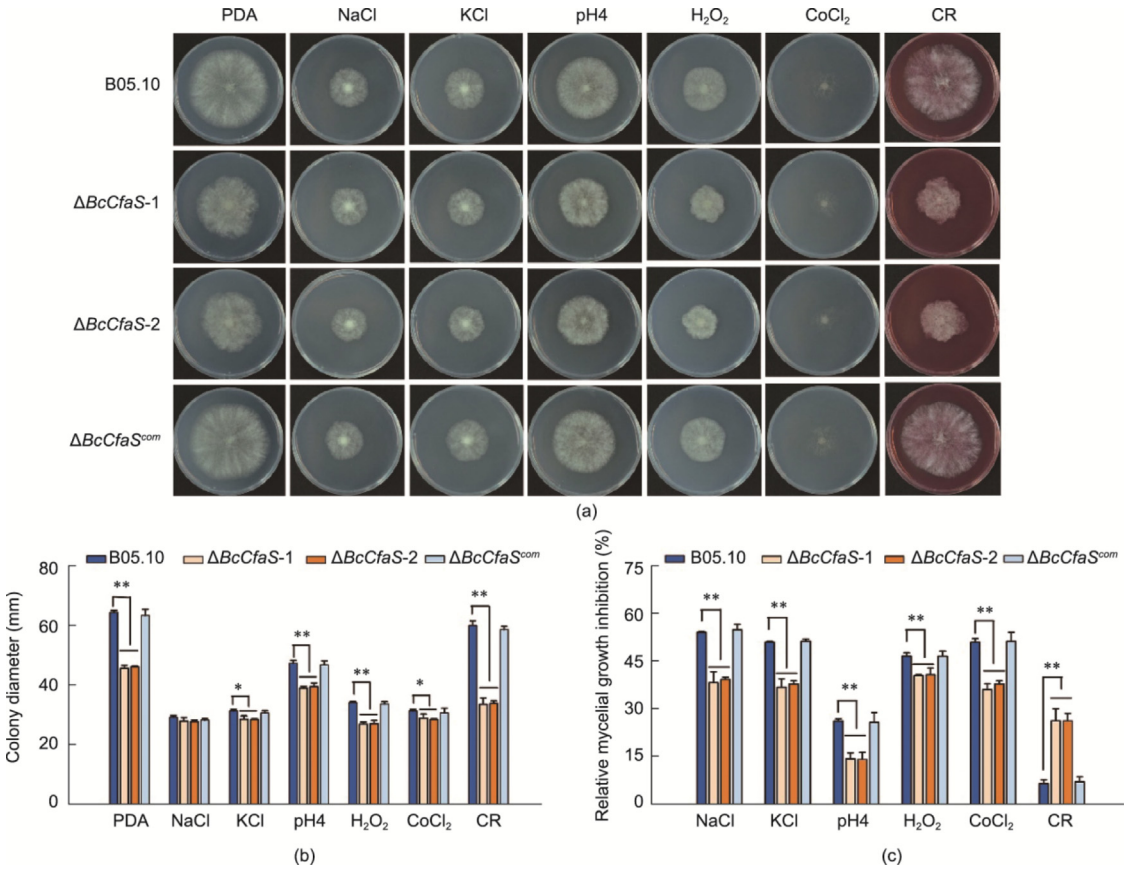

Fig. 4. *BcCfaS* regulates *B. cinerea* stress adaptation. (a) Colony morphologies of the WT, Δ*BcCfaS* and complementation strains cultured on PDA amended with osmotic stressors NaCl and KCl, acid stressor, oxidative stressor H_2O_2, hypoxic stressor $CoCl_2$ or cell wall-perturbing agent Congo Red (CR) at 3 dpi. (b) Colony diameters at 3 dpi, pH4: the acid stressors citric acid/disodium hydrogen phosphate buffer. (c) Relative mycelial growth inhibition. Each bar indicates the mean ± SD of three repeated experiments (*P < 0.05, **P < 0.01, Student's t test).

growth and development to the prevailing environmental conditions [2,4]. Over the past few decades, a large number of light-responsive genes and their functions during light responses have been investigated in *Neurospora crassa* and *Aspergillus nidulans* [43–45]. In contrast, the components and detailed mechanism underlying light responses in *B. cinerea*, a model necrotrophic fungus for studying host-pathogen interaction, are largely unknown. In this study, we identified a novel light-responsive gene *BcCfaS* in *B. cinerea* that encodes a putative CFA synthase family protein. In the WT strain, light exposure strongly induced the expression of *BcCfaS* (Figs. 1(a) and (b)). Similar results have been observed in other microorganisms. For example, in the bacterial human pathogen *Acinetobacter baumannii* and in the mushroom fungus *C. cinerea*, genes encoding for CFA synthase were found to be transcriptionally triggered by light [46,47]. Recently, CFA synthase gene was found to be a direct target of the light-responsive transcription factor NsdD and to cooperate with NsdD to regulate photomorphogenesis in *C. cinerea* [48]. These findings led to the hypothesis that CFA synthase may be a conserved and important light signaling component in different organisms. Here, our findings provide the first genetic and phenotypic evidence that *BcCfaS* is a key factor in the light-regulated physiological and morphological processes of the necrotrophic fungal pathogen *B. cinerea*.

Light plays a critical role in coordinating fungal growth and the balance between asexual and sexual development [4]. As previously reported, light exposure is a source of stress for *B. cinerea* that leads to a slight reduction in hyphae growth rate [18]. However, this light-dependent inhibition of vegetative growth was compromised in the Δ*BcCfaS* mutant, which exhibited much faster hyphal growth with light than without light (Figs. 2(a) and (b)). We therefore propose that *BcCfaS* disruption may cause a decreased sensitivity to light. This hypothesis could be supported by the "blind" phenotype in light-regulated development and the downregulated transcripts of numerous light-regulated genes in the Δ*BcCfaS* mutant. Although conidia are important in the pathogenic life cycle of fungi, excessive conidiation is disadvantageous, as it may prevent vegetative growth, sexual development, and even virulence. *B. cinerea* initiates conidiation in response to light, as the presence of light is an indicator of hyphal exposure to air, where the transition from vegetative growth to conidiation for dispersal is optimal [49]. Conversely, in the absence of light, such as in soil or inside decaying plant material, the production of asexual conidia is pointless, as they cannot be dispersed; thus, *B. cinerea* prefers to devote its resources to producing sclerotia [18,49]. Therefore, in balance with conidiation activators, *B. cinerea* also possesses several light-regulated conidiation repressors for proper development, such as *BcWCL1*, *BcLTF1*, *BcLTF3*, *Bcmads1*, and *Bcvel1* [50–53]. The inactivation of these genes led to mutants that were "blind" to light, in which development was shifted toward the overproduction of conidia independent of the light conditions, accompanied by an almost complete loss of sclerotia production. Our studies demonstrated that disrupting *BcCfaS* resulted in a similar "blind" phenotype with increased conidia formation in both light and darkness, as well as severely impaired sclerotial development in darkness (Fig. 2(c)). This result indicates that *BcCfaS* acts as an important regulator mediating light-dependent developmental processes. Accompanied by the light-dependent developmental defects, eight light-regulated genes were remarkably downregulated under both dark and light conditions (Fig. 2(h)). Of these, the gene *BcLTF1*—an important light-regulated signaling compo-

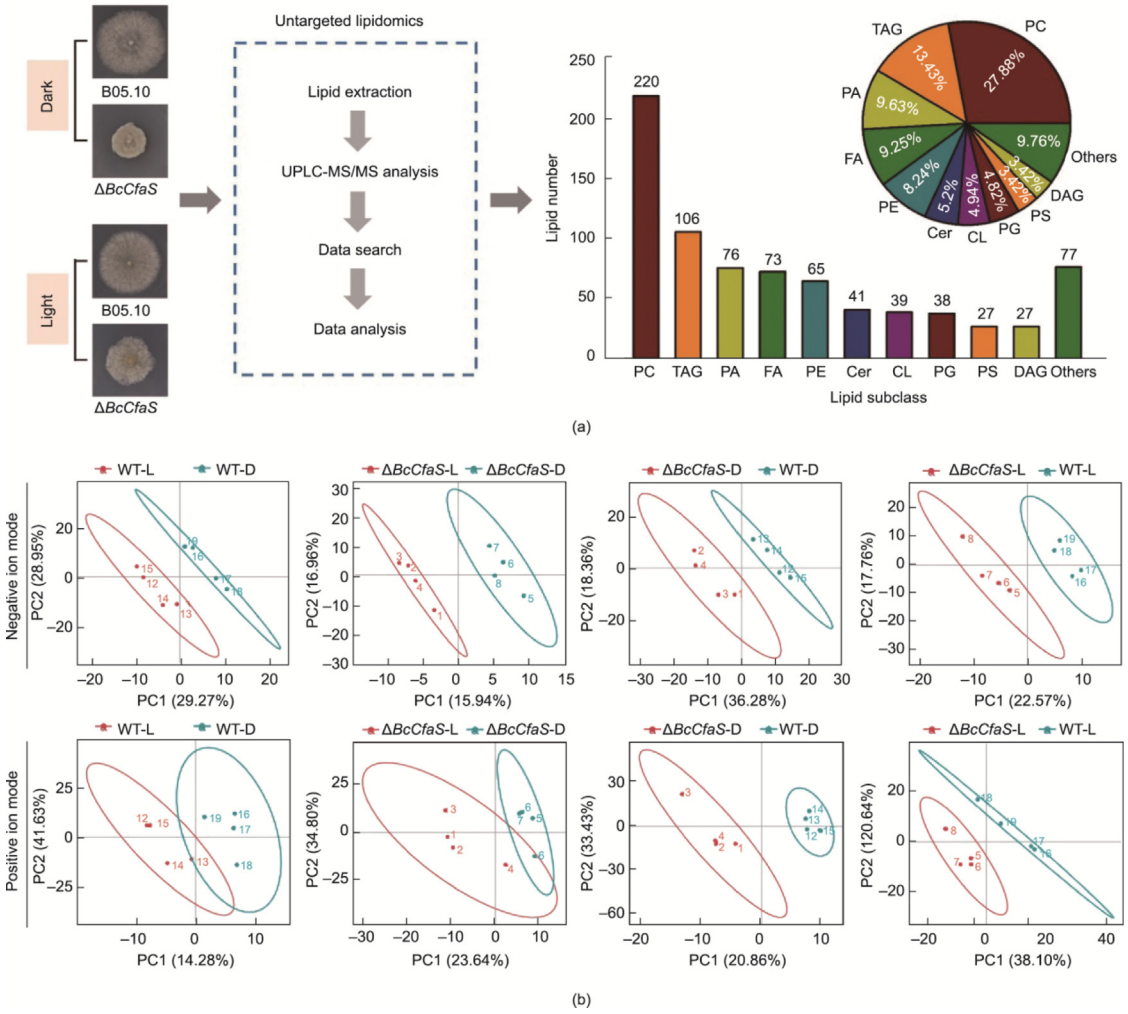

Fig. 5. (a) Subclass distribution and (b) PLS-DA score plots of lipid metabolites analyzed by UPLC-MS/MS in WT grown under dark (WT-D) and light (WT-L) conditions, and Δ*BcCfaS* grown under dark (Δ*BcCfaS*-D) and light (Δ*BcCfaS*-L) conditions.

nent—exhibited a similar function [50], suggesting that *BcCfaS* affects light-dependent development at least partially through the regulation of *BcLTF1*.

In fungi, several light-responsive genes have been reported to regulate the stress response in order to successfully adapt to stresses caused by light, although the regulatory mechanism remains unclear [50,54,55]. In this work, we found that the Δ*BcCfaS* mutants displayed an increased sensitivity to cell wall stress but a decreased sensitivity to osmotic, acid, oxidative, and hypoxic stresses (Fig. 4). BcCfaS was predicted to function as a CFA synthase that has been reported to regulate lipid metabolism in bacteria, plants, and parasites [19,20,56]. Consistent with previous findings, we observed that the deletion of *BcCfaS* had a global impact on the lipid composition of *B. cinerea* under both dark and light conditions. Analysis of the differential lipid metabolites showed that the deletion of *BcCfaS* significantly changed the levels of phospholipids, including PC, PG, CL, PE, and PS (Fig. 6(c)). Phospholipids are major structural components of cellular membranes. The fatty acyl chain composition of membrane phospholipids is a key factor in determining membrane properties, which in turn influences many crucial membrane-associated functions [57]. In *Escherichia coli* (*E. coli*), CFA synthase-mediated cyclopropanation causes a shift from unsaturated to saturated fatty acyl groups in membrane phospholipids, and thus decreases membrane permeability and regulates the responses of *E. coli* to multiple stresses [22,23]. These observations suggest that the function of *BcCfaS* in stress response may be through its regulation of the membrane phospholipids composition of *B. cinerea*. Pathogens may encounter many stresses imposed by hosts during infection. Therefore, the adaptation of pathogens to host-imposed stresses, such as acid stress, cell wall stress, and oxidative stress, is important for virulence [58,59]. Studies of the human pathogen *H. pylori* have indicated that CFA synthase can contribute to gastric colonization by regulating tolerance to acid stress [25]. In this study, we found that the deletion of *BcCfaS* resulted in the reduced virulence of *B. cinerea* on different plant hosts (Fig. 3). Therefore, the reduced pathogenicity upon *BcCfaS* deletion may partially result from the stress response mediated by *BcCfaS*. Overall, our results suggest a conserved function for *BcCfaS* in lipid metabolism, stress response, and pathogenicity.

MeJA, a lipid-derived cyclopentanone compound, is known to function as a signal molecule and regulate diverse physiological processes in plants such as growth, development, and responses to biotic and abiotic stresses [42]. In fungi, intrinsic MeJA has only sporadically been reported to occur in the rice blast fungus *M. oryzae*, which plays an important role in plant-pathogen interaction [60]. Nevertheless, its free acid-namely, jasmonic acid, and derivatives have been identified in various fungal species, although their roles have not been studied thus far [61–63]. *In vitro* studies have shown that MeJA treatment can affect fungal growth, conidiation, and spore germination [64–67]. Recently, it was shown that

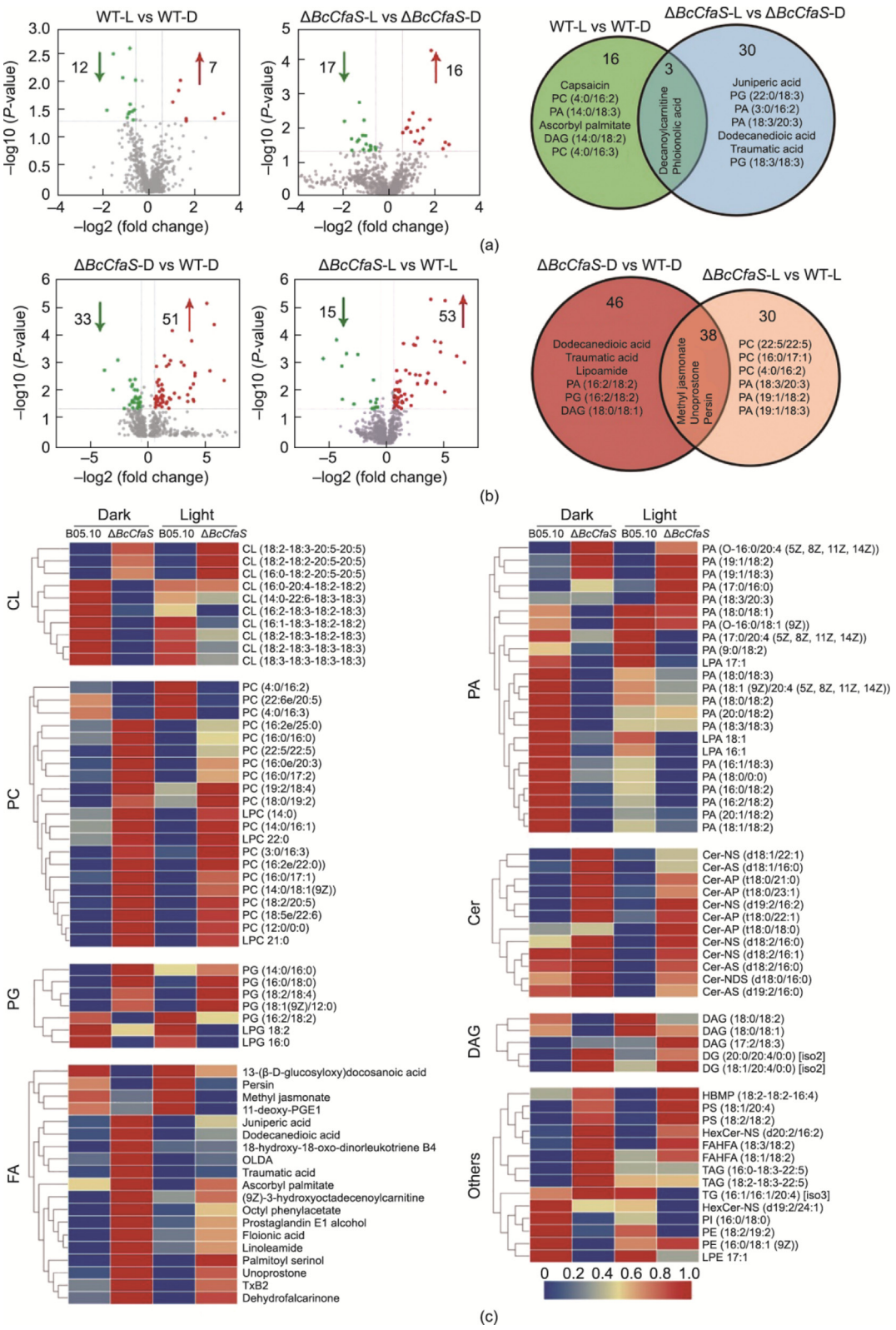

Fig. 6. Lipidomics analysis reveals that *BcCfaS* regulates lipid metabolism in a light-dependent manner. (a) Volcano plot and venn diagram showing the lipid profile changes in WT treated with light versus WT grown under dark (WT-L vs WT-D), Δ*BcCfaS* treated with light versus Δ*BcCfaS* grown under dark (Δ*BcCfaS*-L vs Δ*BcCfaS*-D). (b) Volcano plot and venn diagram showing the lipid profile changes in Δ*BcCfaS* grown under dark versus WT grown under dark (Δ*BcCfaS*-D vs WT-D) and Δ*BcCfaS* treated with light versus WT treated with light (Δ*BcCfaS*-L vs WT-L). (c) Heatmaps depicting the differential lipid metabolites between WT and Δ*BcCfaS* in dark and light. Different heatmaps represent different lipid subclasses. OLDA: N-Oleoyldopamine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; LPE: lysophosphatidylethanolamine; LPA: lysophosphatidic acid; TxB2: thromboxane B2; iso: isomers; DG: diglyceride; FAHFA: fatty acid ester of hydroxyl fatty acid. Each rectangle represents a lipid colored by its normalized intensity scale from red (increased level) to blue (decreased level).

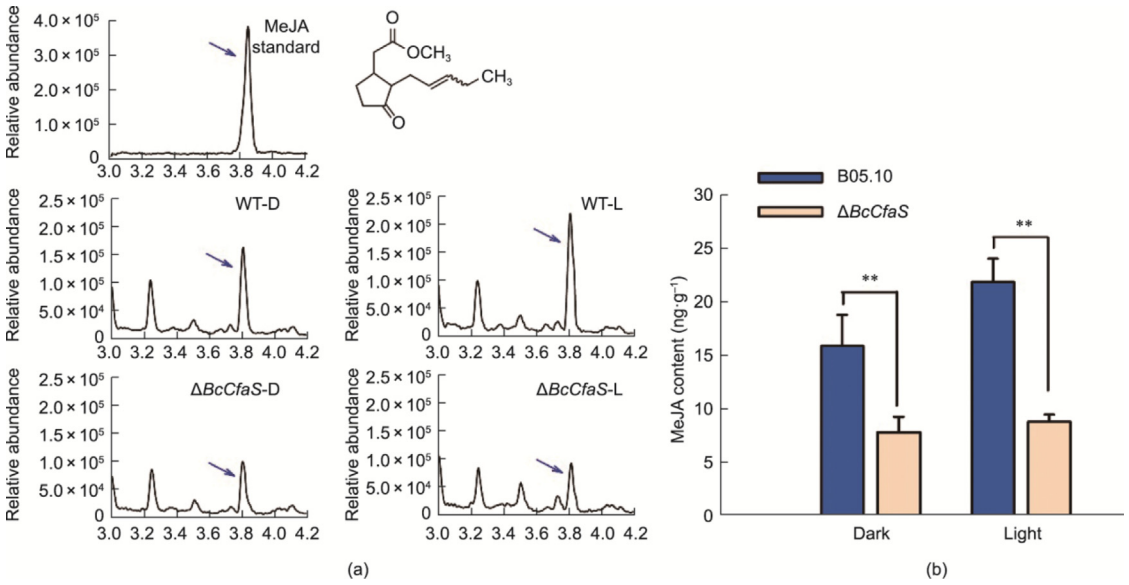

Fig. 7. *BcCfaS* regulates MeJA synthesis under dark and light conditions. (a) LC-MS/MS chromatograms of MeJA in WT and Δ*BcCfaS* under different conditions. Selective ion monitoring for the ion m/z = 225.04 was utilised for detection of MeJA. (b) MeJA content in the indicated strains based on LC-MS/MS analysis. Each bar indicates the mean ± SD of three repeated experiments (**P < 0.01, Student's t test).

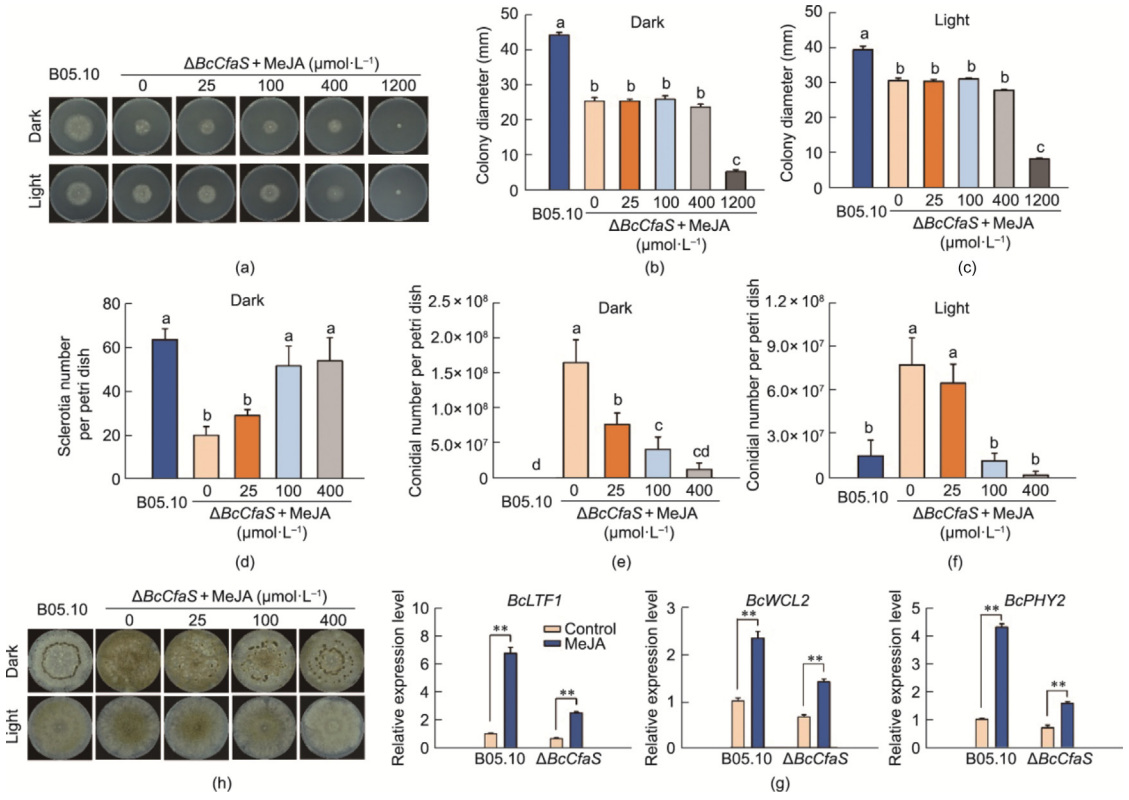

Fig. 8. MeJA is required for light-dependent development. (a) Effect of MeJA on vegetative growth of Δ*BcCfaS* strain in dark and light. Colony diameter of the indicated strains cultured on PDA supplemented with various concentrations (0, 25, 100, and 400 μmol·L^{-1}) of MeJA in (b) dark and (c) light. Effect of MeJA on (d) sclerotia number and (e) conidiation at 14 dpi in dark, and (f) conidiation at 7 dpi in light of Δ*BcCfaS* strain. Letters above the bars denote statistical differences (Duncan's test, P < 0.05). (h) MeJA rescues defects in conidiation and sclerotial development of Δ*BcCfaS* strain. (g) Effect of MeJA on the expression levels of *BcLTF1*, *BcWCL2* and *BcPHY2* in indicated strains. Each bar indicates the mean ± SD of three repeated experiments (*P < 0.05, **P < 0.01, Student's t test).

exogenous MeJA can restore germ tube development and appressorium formation in a Δ*pth11* mutant that lacks G protein/cAMP signaling [68]. These observations imply that MeJA may function as a key factor in various developmental processes in fungi. In the present study, by means of lipidomic and targeted analysis using LC-MS/MS, we confirm that *B. cinerea* is able to form MeJA, and that *BcCfaS* disruption results in decreased MeJA accumulation under both dark and light conditions (Fig. 7), indicating that *BcCfaS* is required for the biosynthesis of MeJA in *B. cinerea*. More interestingly, the addition of exogenous MeJA can activate the transcription of light-signaling components and rescue the light-dependent developmental defects of the *BcCfaS* mutant (Fig. 8).

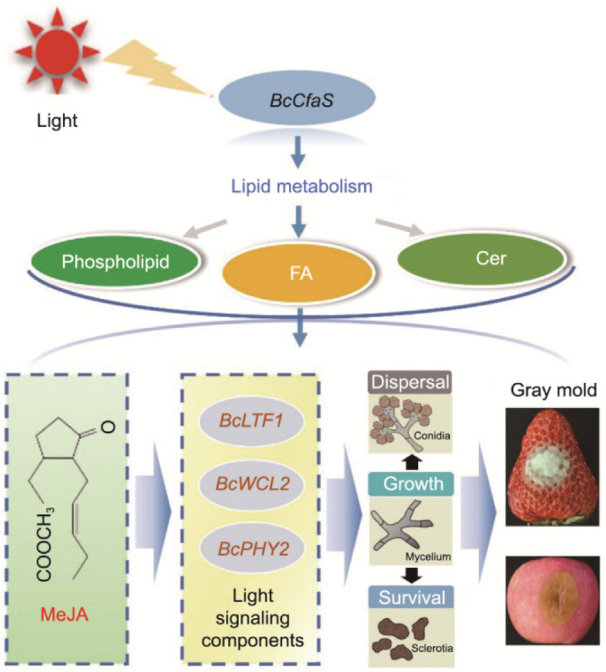

Fig. 9. A proposed working model illustrating the role of *BcCfaS*. Expression of *BcCfaS* is controlled by surrounding light environment, which further affects the accumulation of lipid metabolites, such as phospholipids, fatty acids and ceramides, in a light-dependent manner. *BcCfaS* positively regulates the MeJA synthesis to activate the transcription of light signaling components, thereby controlling the developmental decisions from vegetative growth to either asexual spores for dispersal or sclerotia for survival, which together may affect the prevalence and severity of gray mold disease.

These results suggest that *BcCfaS* functions in the repression of conidiation and the activation of sclerotial development mainly by regulating MeJA synthesis, which is essential for a proper switch-over from vegetative growth to reproductive development in *B. cinerea*. This regulatory mechanism has not been previously reported in fungi and thus may be a newly discovered mechanism for the light control of fungal development.

In conclusion, a novel light-signaling component *BcCfaS* is identified as an essential factor for the photomorphogenesis, pathogenesis, stress response, and lipid metabolism of the necrotrophic fungal pathogen *B. cinerea*. Based on our results, we propose a working model for the role of *BcCfaS* in controlling the balance between conidiation and sclerotia development (Fig. 9) and consider that *BcCfaS* serves as an integration node for light and lipid metabolism. These findings provide not only a regulatory mechanism by which fungi adapt their development to a changing light environment but also an important target for antifungal design for the control of fungal diseases.

Acknowledgements

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Compliance with ethics guidelines

Guangjin Li, Yong Chen, Tong Chen, Boqiang Li, Zhanquan Zhang, and Shiping Tian declare that they have no conflict of interest or financial conflicts to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eng.2023.08.015.

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