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Special Issue Article

Comprehensive analysis of the regulatory network of blue-light-regulated conidiation and hydrophobin production in *Trichoderma guizhouense*

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Summary

Conidia of *Trichoderma guizhouense* (Hypocreales, Ascomycota) are frequently applied to the production of biofertilizers and biocontrol agents. Conidiation of some *Trichoderma* species depends on blue light and the action of different blue light receptors. However, the interplay between different blue-light receptors in light signalling remained elusive. Here, we studied the functions of the blue light receptors BLR1 and ENV1, and the MAP kinase HOG1 in blue light signalling in *T. guizhouense*. We found that the BLR1 dominates light responses and ENV1 is responsible for photoadaptation. Genome-wide gene expression analyses revealed that 1615 genes, accounting for ~13.4% of the genes annotated in the genome, are blue-light regulated in *T. guizhouense*, and remarkably, these differentially expressed genes (DEGs) including 61 transcription factors. BLR1 and HOG1 are the core components of the light signalling network, which control 79.9% and 73.9% of the DEGs respectively. In addition, the strict regulation of hydrophobin production by the blue light signalling network is impressive. Our study unravels the regulatory network based on the blue light receptors and the MAPK HOG pathway for conidiation, hydrophobin production and other processes in *T. guizhouense*.

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Introduction

Trichoderma (Hypocreales, Ascomycota) species have been widely used in agriculture as bio-fertilizers and biocontrol agents due to their remarkable abilities to antagonize plant pathogenic fungi and promote plant growth (Druzhinina *et al*., 2011). *Trichoderma guizhouense* NJAU 4742 has a superior capacity of plant growth promotion and can combat such ascomycetous phytopathogenic fungi as *Botrytis cinerea* (Helotiales), *Alternaria alternata* (Pleosporales), and hypocrealean *Fusarium fujikuroi* and *F. odoratissimum* (Zhang *et al*., 2016; Zhang *et al*., 2019; Zhu *et al*., 2021). For instance, the swollenin protein SWO from *T. guizhouense* can induce the cell wall extension in *Cucumis sativus* (cucumber) and thus facilitate root development (Meng *et al*., 2019). Moreover, *T. guizhouense* is known to be a versatile mycoparasite. For example, it can attack plant pathogenic fungi by producing reactive oxygen species (ROS) and use anti-oxidant secondary metabolites azaphilones for self-defence (Zhang *et al*., 2019; Pang *et al*., 2020). Furthermore, some mycoparasitic interactions involve the production of the neutral metallopeptidase and the short-chain dehydrogenase/reductase (Zhang *et al*., 2016; Zhu *et al*., 2021). Currently in China, conidia of *T. guizhouense* are used to produce biocontrol agents and bio-organic fertilizers, and the outstanding performance of these products endows *T. guizhouense* a high economic value. Thus, studying the conidiation of *T. guizhouense* is of great importance for maximizing the yield of conidia and hence minimizing the production cost.

Conidiation of fungi is genetically programmed by various developmental modifiers, of which the central regulatory pathway has been well studied in *A. nidulans* (Boylan *et al*., 1987; Adams *et al*., 1998; Etxebeste

et al., 2010). The central regulatory pathway consists of the C2H2 zinc-finger transcription factor (TF) BrlA, the transcriptional enhancer factor-1 family member AbaA and the developmental regulator WetA (Adams *et al*., 1988; Adams *et al*., 1990). The activation of BrlA is considered as the initiation step of conidiation, and then AbaA is induced followed by the activation of WetA (Marshall and Timberlake, 1991; Andrianopoulos and Timberlake, 1994). Previous studies have also identified the *fluffy* genes (*fluG* and *flbA-E*) as the upstream regulators of the central regulatory pathway, and deletion of these genes in *A*. *nidulans* caused the fluffy phenotype (Wieser *et al*., 1994; Adams *et al*., 1998). Taking examples among these genes, *fluG* encodes a protein similar to glutamine synthetase, which functions as the key activator of asexual development (Lee and Adams, 1994). The C2H2 zinc-finger transcription factor, FlbC, regulates conidiation by activating the expression of *brlA*, *abaA* and *vosA* (Kwon *et al*., 2010). Additionally, the transcription factors StuA and MedA are also required for precise spatial organization of the conidiophore (Adams *et al*., 1998). In *A*. *nidulans*, light regulates the expression of *brlA* and the *fluffy* genes to promote conidiation (Ruger-Herreros *et al*., 2011).

Light, as one of the most pervasive environmental cues, regulates many physiological and morphological processes in fungi (Yu and Fischer, 2019). Fungi utilize different photoreceptors to perceive light with specific wavelengths (Fischer *et al*., 2016). In *Neurospora crassa*, many biological processes are controlled by blue light, such as the circadian clock, carotenoids biosynthesis and reproduction (Idnurm *et al*., 2010; Dasgupta *et al*., 2015; Montenegro-Montero *et al*., 2015; Yu and Fischer, 2019). All these responses depend on the white collar complex (WCC) photosensory system comprised of the two GATA-type transcription factors white collar 1 (WC-1) (the blue light receptor) and white collar 2 (WC-2). The WCC exists as a heterodimer in nuclei, which binds to the light-responsive elements (LREs) in the promoters of light-regulated genes (Froehlich *et al*., 2002; Cheng *et al*., 2003; He and Liu, 2005). Upon blue light illumination, WCCs dimerize to regulate the early light-responsive genes including *vvd*, which encodes another photoreceptor VVD. VVD attenuates WCC-mediated light responses by disrupting the WCC dimers. This process is known as photoadaptation (Chen *et al*., 2010; Hunt *et al*., 2010; Malzahn *et al*., 2010).

The homologues of WC-1 and WC-2, BLR1 and BLR2, were also studied in *Trichoderma*, which are required for the photoconidiation of *T*. *atroviride* (Casas-Flores *et al*., 2004). The homologue of VVD in *T*. *reesei*, ENVOY, is essential for the photoadaptation and represses light responses (Castellanos *et al*., 2010). The stress-activated MAP kinase Tmk3 was reported to be participated in blue-light sensing in *T*. *atroviride*. Several light responsive genes were induced owing to the phosphorylation of Tmk3 in blue light (Esquivel-Naranjo *et al*., 2016). Although photoreceptors in *Trichoderma* species have been characterized several years ago, the regulatory network of blue-light sensing in *Trichoderma* is still unknown.

In this study, we investigated the interplay between the blue light receptors and the MAPK HOG pathway in blue light signalling in *T*. *guizhouense* through the functional genetic approach and the genome-wide gene expression analyses. The regulatory network based on blue light receptors and the MAPK HOG pathway for conidiation, production of the small secreted cysteine-rich protein hydrophobins (HFBs) and other processes were deciphered.

Results

Blue light triggers the conidiation of T. guizhouense *and alters the expression levels of light-responsive genes*

To study the photobiology of *T*. *guizhouense*, we first evaluated the light responses of this fungus. The wild type strain was incubated in darkness or blue light [1.7 μmol photons/(m^2 × s)] for 3 days on PDA medium at 28°C and the conidia production was quantified. In the dark, the white mycelia colonized the plate after 3 days of growth and no conidia were observed, whereas the colony formed in blue light was dark green and 3.6 × 10^9 conidia/plate were produced (Fig. 1A and B). After 6 days, only a few conidia (4.3 × 10^7 conidia/plate) were produced in the dark, still far less than that (4.4 × 10^9 conidia/plate) produced in blue light (Suppl. Fig. S1).

To analyse the blue light responses of this fungus on the gene level, we measured the expression levels of six putative light-regulated genes in response to different illumination times by quantitative real-time PCR (qPCR). These genes are the blue light receptor encoding genes *blr1* (OPB37744) and *env1* (OPB36488), the orthologue of the *wc-2* gene *blr2* (OPB44424), and the cryptochrome/photolyase family protein-encoding genes *cry-dash* (OPB38601), *phr1* (OPB37792) and *cry1* (OPB36878). After 24 h incubation in the dark, the samples of the wild type strain were illuminated with blue light for 5, 15, 30, 45, 60, 90 and 120 min respectively. The expression levels of *env1*, *cry-dash*, *phr1* and *cry1* increased significantly after 15 min illumination and peaked at 45 min and decreased thereafter, while the expression levels of *blr1* and *blr2* fluctuated slightly during the 120 min illumination (Fig. 1C).

Blue light receptors are required for photoconidiation in T. guizhouense

Next, we knocked out *blr1*, *blr2*, *env1* and the orthologue of the *tmk3* gene, *hog1* (OPB38173) in *T*. *guizhouense* and eventually obtained at least three positive

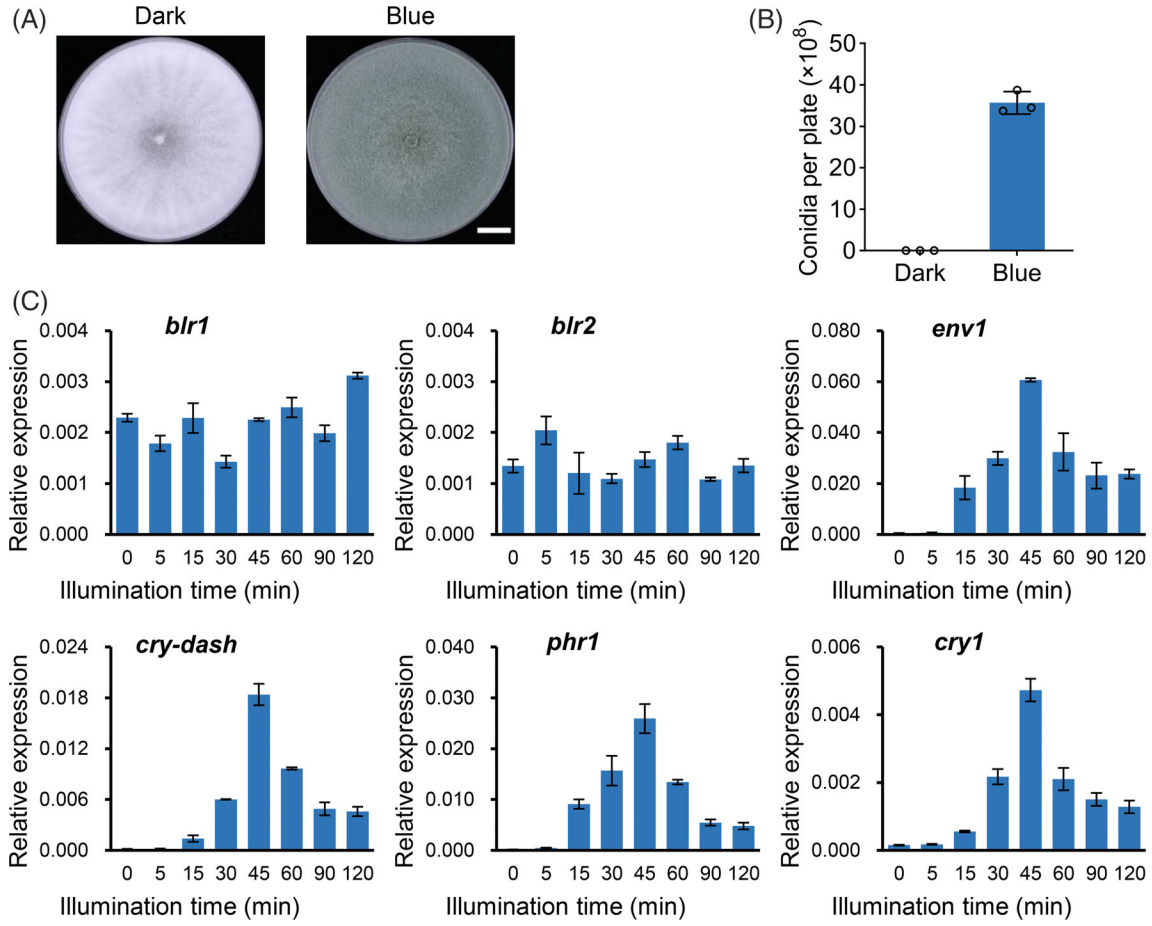


Fig 1. Light responses of *T. guizhouense* upon blue light illumination.
A. Phenotype of the wild type (WT) strain in blue light. Wild type was grown on PDA medium at 28°C for 3 days. Scale bar, 1 cm.
B. Quantification of conidia of wild type produced in darkness and blue light. Three plates of each strain were analysed, and the mean values for the three samples were displayed. The error bar represents the standard deviation (SD).
C. Expression levels of six putative blue-light-responsive genes in wild type upon different times of blue light illumination. The wild type strain was cultured on PDA medium at 28°C for 24 h and then kept in the dark or exposed to blue light for 5, 15, 30, 45, 60, 90, and 120 min. The expression level of each gene was normalized to the translation elongation factor 1 alpha (*tef1*) gene, and the error bar represents SD of three biological replicates.

transformants with identical phenotype for each gene. The positive mutants were confirmed by diagnostic PCR as well as Southern blot analysis (suppl. Fig. S1). To analyse the phenotypic differences of these strains, wild type, the *Δblr1*-, *Δblr2*-, *Δenv1*- and *Δhog1*-mutants were cultured on PDA medium at 28°C in darkness or blue light for 3 days and the conidia production of each strain was quantified. In the dark, all the strains formed white colonies and no conidia were observed. In blue light, wild type yielded large quantities of green conidia, while the *Δblr1*-mutant was unable to sporulate (Fig. 2A and B). The *Δblr2*-mutant phenocopied the *Δblr1*-mutant. Interestingly, the vegetative growth of the *Δenv1*-mutant was highly sensitive to blue light, which was strongly inhibited. The *Δhog1*-mutant also formed conidia in blue light, but the amount was only 69.9% of that of wild type.

To further analyse the regulatory effect of the photoreceptors on genes essential for conidiation in *T. guizhouense*, we measured the relative expression of *brl1* (OPB45677), *aba1* (OPB44327), *wet1* (OPB37120), *med1* (OPB40389), *fluG* (OPB45752) and *flbC* (OPB42921) in wild type, the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains by qPCR. Strains were incubated in darkness or constant blue light for 24 or 48 h. In wild type, all genes tested were significantly upregulated, especially after 48 h illumination (Fig. 2C). In comparison to wild type, the relative expression of these genes decreased in the *Δblr1*-, but increased in the *Δenv1*-mutant strain. However, in the *Δhog1*-mutant the relative expression of *brl1*, *aba1*, *wet1* and *med1* in blue light was similar to that in wild type, while the expression levels of *fluG* and *flbC* decreased.

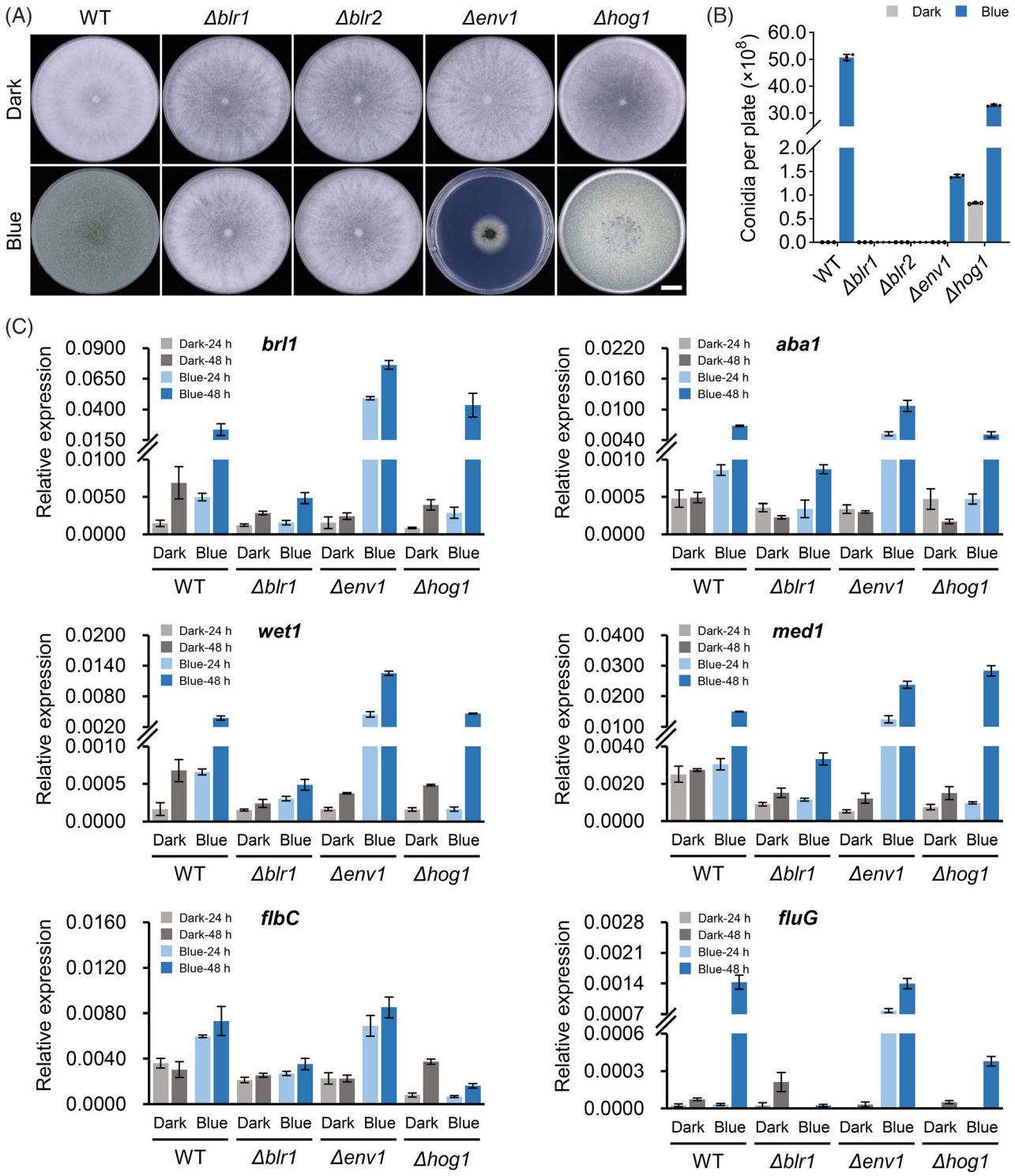


Fig 2. Impact of photoreceptors on photoconidiation in *T. guizhouense*. The wild type, and the *Δblr1*-, *Δblr2*-, *Δenv1*- and the *Δhog1*-mutant strains were grown on PDA medium at 28°C for 3 days.

A. Phenotype of wild type and mutants in blue light. Scale bar, 1 cm.

B. Quantification of conidia of each strain produced in blue light. Three plates of each strain were analysed, and the mean values for the three samples are displayed. The error bar represents SD of three biological replicates.

C. Expression of the conidiation regulation genes upon a blue light stimulus in wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains. All strains were cultured on PDA medium covered with cellophane at 28°C in the dark or in blue light for 24 and 48 h. The expression level of each gene was normalized to the *tef1* gene. Error bars indicate SD of three biological replicates.

The HOG pathway is activated by blue light through the BLR1/2 complex

In *A. nidulans*, the MAP kinase HogA/SakA is phosphorylated and then translocated into the nucleus upon a red-light stimulus or salt stress (Yu *et al*., 2016). To investigate the subcellular localization of HOG1 in *T. guizhouense* upon exposure to blue light, we constructed an eGFP-tagged HOG1 strain. Salt stress (0.5 M NaCl) was imposed for 3 min in the dark as a positive control. In darkness, HOG1 was evenly distributed in nuclei and the cytoplasm (Fig. 3A). However, HOG1 accumulated in nuclei after 3 min of blue light exposure.

Next, we performed an immunostaining assay with the antibody against phosphorylated HOG1 to analyse the phosphorylation level of HOG1 in wild type, and the *Δblr1*-, *Δblr2*- and *Δenv1*-mutant strains. In the dark, the phosphorylation signal was not detected in all tested strains (Fig. 3B). However, strong red fluorescence was detected in wild type after 3 min of blue-light illumination. The phosphorylation level of HOG1 in the *Δenv1*-mutant was similar to wild type upon blue light exposure, whereas the corresponding signal was weak in both the *Δblr1*- and *Δblr2*-mutant strains.

The BLR1/2 complex and the HOG pathway play a major role in the regulation of blue-light-responsive genes

To globally analyse the blue-light-regulated genes in *T. guizhouense*, we performed RNA sequencing analyses of wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains. Since the expression of the light-responsive genes peaked at 45 min illumination (Fig. 1C), to get the maximum light responses on the gene level, the samples were exposed to blue light for 45 min. After RNA sequencing, with the thresholds of $|\log_2(\text{fold change})| \geq 1$ and false discovery rate (FDR) <0.05, we identified 1615, 424, 1492 and 1262 differentially expressed genes (DEGs) in wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains respectively.

To gain insight into the regulatory network of blue-light receptors in *T. guizhouense*, we compared DEGs in Venn diagrams (Fig. 4A). In wild type, we found 829 genes were upregulated and 786 genes were downregulated, whereas in the *Δblr1*-mutant only 218 upregulated and 206 downregulated genes were identified. 79.9% (1290/1615) of DEGs in wild type were dependent on BLR1. In the *Δenv1*-mutant, 1492 genes, 729 upregulated and 763 downregulated, were differentially expressed, but only 887 (54.9%) of them overlapped with the DEGs identified in wild type. Additional 605 genes were differentially expressed upon blue light when ENV1 was absent. In the *Δhog1*-mutant strain, 1262 genes, 423 upregulated and 839 downregulated, were identified, whereas surprisingly only 422 (26.1%) of them overlapped with the DEGs in wild type. 1193 (73.9%) of DEGs in wild type depended on HOG1 and 840 genes were additionally differentially expressed when HOG1 was absent. This means a total of 2033 genes (∼16.8% of the genes in the genome) were under the control of HOG1 in this condition. The differences of transcription profiles among wild type and mutant strains demonstrated that HOG1 plays a pivotal function in the regulation of blue-light-responsive genes.

We then performed Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs in wild type. We found that blue light affects many aspects of fungal metabolism, such as carbon metabolism, nitrogen metabolism and amino acid metabolism (Fig. 4C). This result was consistent with previous studies in *Trichoderma*, which suggests that light signalling impacts diverse pathways such as vegetative growth, and carbon and sulfur metabolism (Schmoll *et al*., 2010). Next, we used gene ontology (GO) assignments to classify the functions of DEGs in all strains. The DEGs were mainly associated with the enriched GO terms 'catalytic activity' and 'metabolic process' (Fig. 4B). The number of DEGs associated with enriched GO terms in wild type was significantly higher than that in the *Δblr1*-mutant, while the numbers in the *Δenv1*- and the *Δhog1*-mutant strains were similar to that in wild type.

Induction of ENV1, HOG1 and the cryptochrome/photolyase family protein-encoding genes by light depends on the BLR1/2 complex

Through transcriptome analyses, we found the transcript abundances of *env1*, *hog1*, *cry-dash*, *phr1*, and *cry1* were upregulated in wild type, but none of them was differentially expressed in the *Δblr1*-mutant strain (Fig. 5A). The transcript abundance of *cry1* was also dependent on HOG1. The reliability of these results was validated by qPCR (Fig. 5B). Indeed, upon a short-time blue light stimulus, the expression of these genes increased significantly, but they could not be induced anymore by blue light when BLR1 was absent.

Since the expression of *env1*, *cry-dash*, *phr1*, and *cry1* would decrease after 45 min illumination, we further asked if BLR1 still modulates the expression of these genes in constant light (24 or 48 h blue light illumination). Likewise, BLR1 was essential for the induction of *env1*, *cry-dash*, *phr1*, and *cry1* in response to blue light (Fig. 5C). Notably, the expression of *hog1* did not respond to blue light in the wild type and the *Δblr1*-mutant strain unless ENV1 was absent. In the *Δenv1*-mutant strain, the induction of *phr1* and *cry1* was stronger than

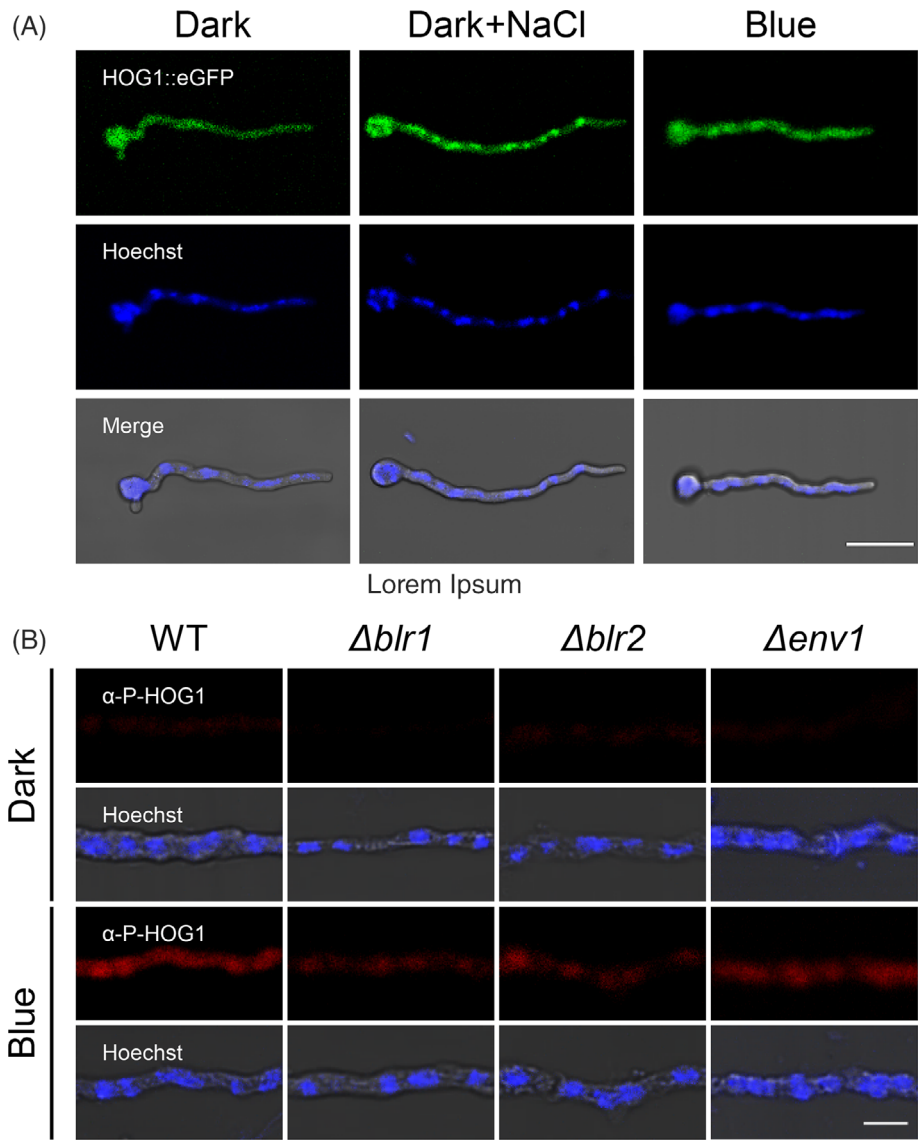


Fig 3. Subcellular localization and phosphorylation of HOG1 after blue light exposure.
A. The localization of HOG1 in the dark or in blue light. Fresh conidia of the HOG1::eGFP strain were incubated on microscope coverslips supplemented with 400 μl PDB overnight in darkness at 28°C. Afterwards, samples were exposed to blue light or PDB medium with 0.5 M NaCl for 3 min. The mycelium was then fixed with 4% formaldehyde in the dark (dim-green light) for 10 min. Scale bar, 20 μm.
B. Phosphorylation levels of HOG1 in different strains. Conidia of wild type, and the *Δblr1*-, *Δblr2*- and *Δenv1*-mutant strains were germinated on coverslips with PDB medium for 14 h at 28°C in the dark. Samples were then exposed to blue light for 3 min and fixed immediately before immunostaining. Antiphospho-p38 MAP kinase (Thr180/Tyr182) antibody and Cy3-conjugated anti-rabbit IgG second antibody were used to detect the phosphorylation level of HOG1. Nuclei were stained with Hoechst. Scale bar, 5 μm.

that in wild type regardless of upon 24 or 48 h illumination.

Expression of light-regulated transcriptional factors including BLU7 depends on the HOG pathway

In *N. crassa*, several light-responsive TFs were identified upon white light illumination, which control the hierarchical expression of light-responsive genes (Chen *et al*., 2009). Surprisingly, we screened 61 transcriptional factors (TFs) including 45 upregulated and 16 downregulated from 1615 DEGs of wild type (Fig. 6A). Among these TFs, 57 were regulated by BLR1, 46 by HOG1 and 43 by both (Fig. 6B). The gene OPB36480 encoding the homologue of transcription factor Blu7, which regulates conidiation and nitrogen metabolism in *T. atroviride* (Cetz-Chel *et al*., 2016), was upregulated 11.4-folds after blue light exposure. In the *Δhog1*-mutant strain, the expression level of *blu7* increased only 2.9-folds in blue light and in the *Δblr1*-mutant strain the expression of this gene did not change. The reliability of these data was validated by qPCR (Fig. 6C). The expression of *blu7* in the *Δhog1*-mutant strain significantly decreased in comparison to that in the wild type strain.

Hydrophobin production is controlled by blue-light receptors

As described above, one characteristic phenotype of the *Δenv1*-mutant strain in blue light was the severely inhibited vegetative growth, and a similar phenotype was also reported in *T. reesei env1*-deletion mutant (Schmoll

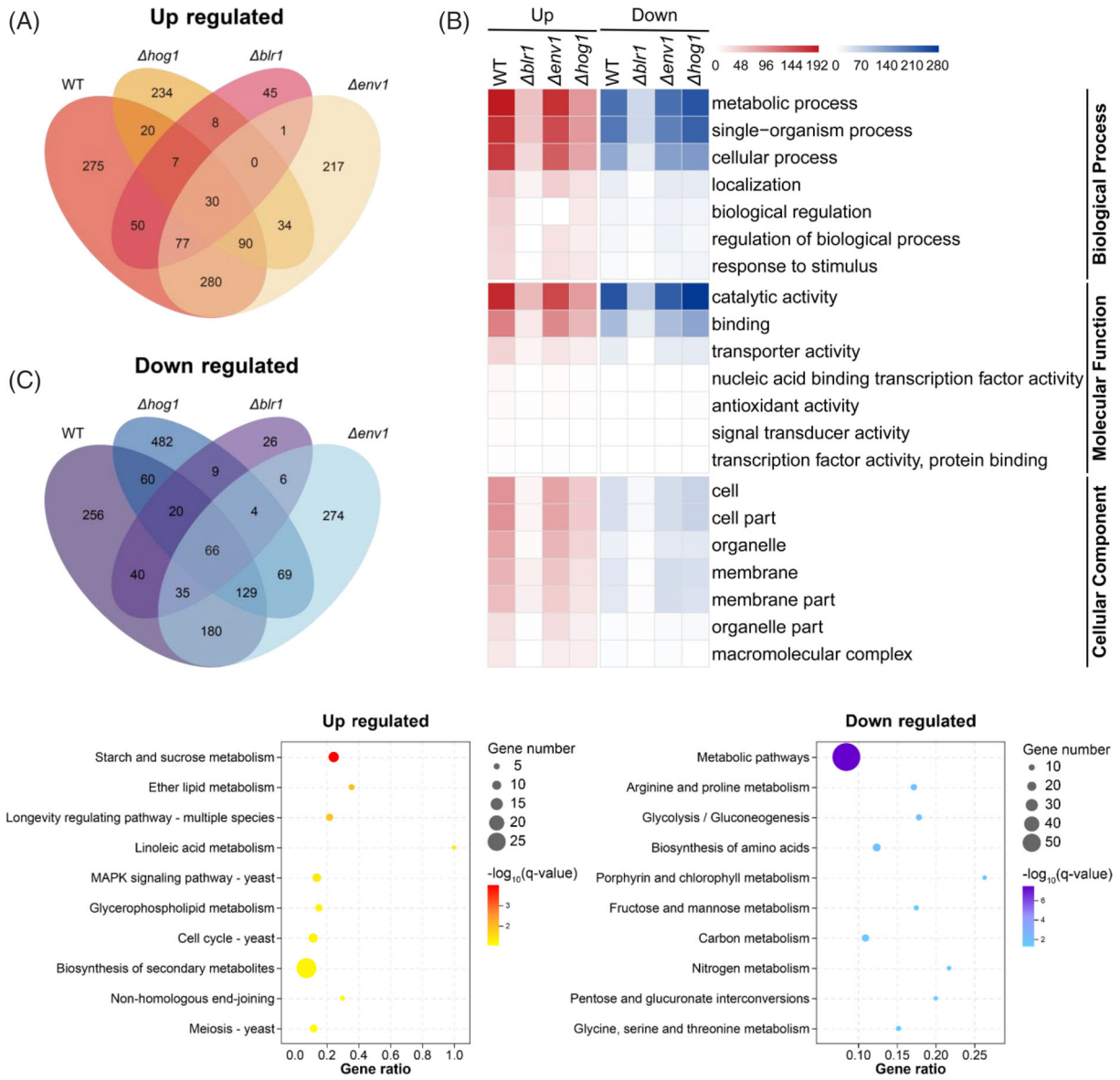
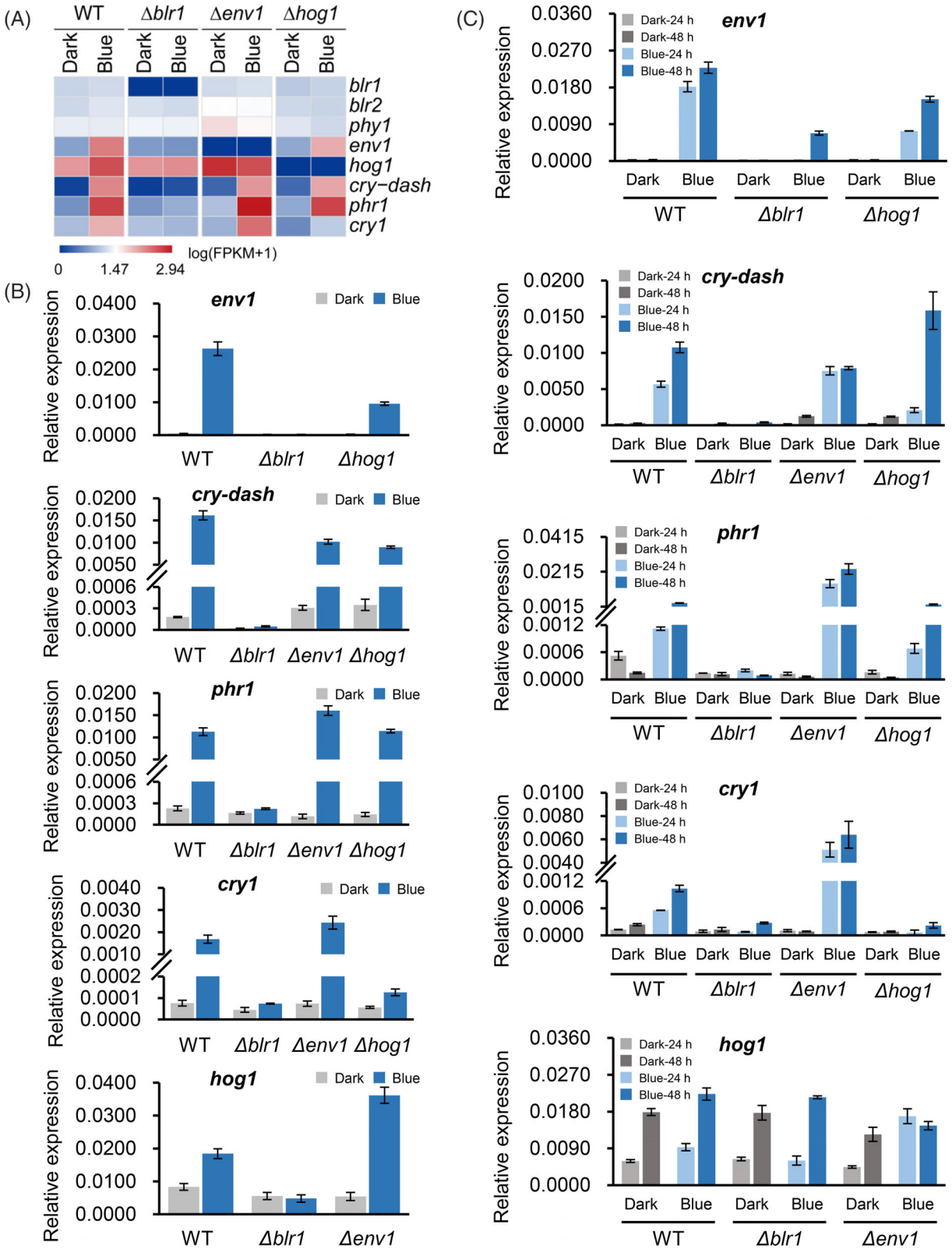


Fig 4. Transcriptome analysis of different strains upon blue light.
A. Differentially expressed genes (DEGs) in wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains are compared in Venn diagrams.
B. The number of up- and down-regulated DEGs in enriched gene ontology (GO) terms in wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains. DEGs were derived from the comparison between the dark and blue, with the thresholds of |log2(FC) | ≥1.0, FDR <0.05. FC, fold change; FDR, false discovery rate.
C. Top 10 significantly enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways of up- and down-regulated DEGs in wild type. The gene ratio represents the ratio of the proportion of genes annotated to the pathway among DEGs to the proportion of genes annotated to the pathway among all genes.

et al., 2010). However, more intriguingly, we observed that in blue light crystal dewdrops formed on *Δenv1*-mutant colonies after 3 days incubation at 28°C (Fig. 7A), which implies the enhanced hydrophobicity of the *Δenv1*-mutant in comparison to wild type. This phenomenon prompted us to investigate the relationship between blue light sensing and HFB production in *T. guizhouense*. We first analysed the transcript abundances of seven HFB-encoding genes in our transcriptome data and found that the expression level of *hfb10* was the highest (Fig. 7B). After blue light illumination, the expression level of *hfb10* was upregulated significantly in wild type and the *Δenv1*-mutant strain, and the expression level in the *Δenv1*-mutant was even higher than that in wild type, while the expression in the *Δblr1*-mutant was lower than that in wild type. The expression



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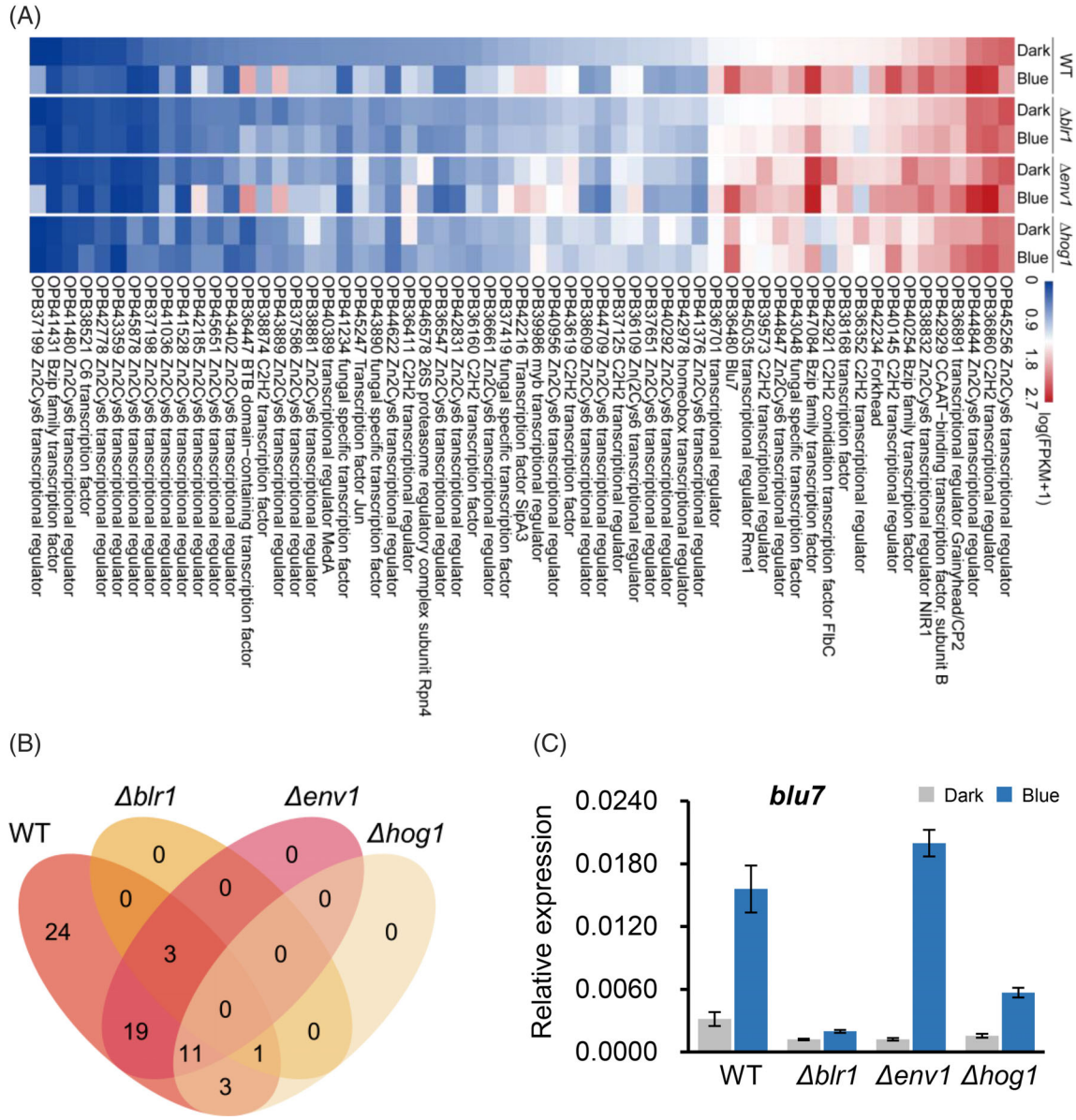

Fig 6. Blue-light-regulated transcription factors (TFs) identified in *T. guizhouense*.
A. Transcriptional profiles of the TFs screened from the DEGs.
B. Venn diagram of the 61 differentially expressed TFs in wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains.
C. The expression levels of the TF encoding gene *blu7* in wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains. All strains were cultured on PDA medium at 28°C for 24 h and then kept in the dark or exposed to blue light for 45 min. The expression level of the gene was normalized to *tef1* gene. Error bars indicate SD of three biological replicates.

level of *hfb10* showed no significant change in the *Δhog1*-mutant strain.

To evaluate the effect of constant blue light on HFB-encoding genes, we measured the expression levels of *hfb10*, *hfb4*, *hfb2* and *hfb3* in each strain grown in darkness or constant blue light for 24 or 48 h by qPCR. The other HFB-encoding genes were not chosen for investigation here due to their low transcript abundances. In

Fig 5. Analysis of transcript abundances of *blr1*, *blr2*, *env1*, *hog1*, *cry-dash*, *phr1* and *cry1* in different strains.
A. Transcriptional profiles of *blr1*, *blr2*, *phr1*, *env1*, *hog1*, *cry-dash*, *phr1* and *cry1* from the transcriptome data.
B, C. Expression levels of *env1*, *hog1*, *cry-dash*, *phr1* and *cry1* in wild type, and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains after short-time (45 min, B) or constant (24 and 48 h, C) blue light exposure. The expression level of each gene was normalized to *tef1* gene. Error bars indicate SD of three biological replicates.

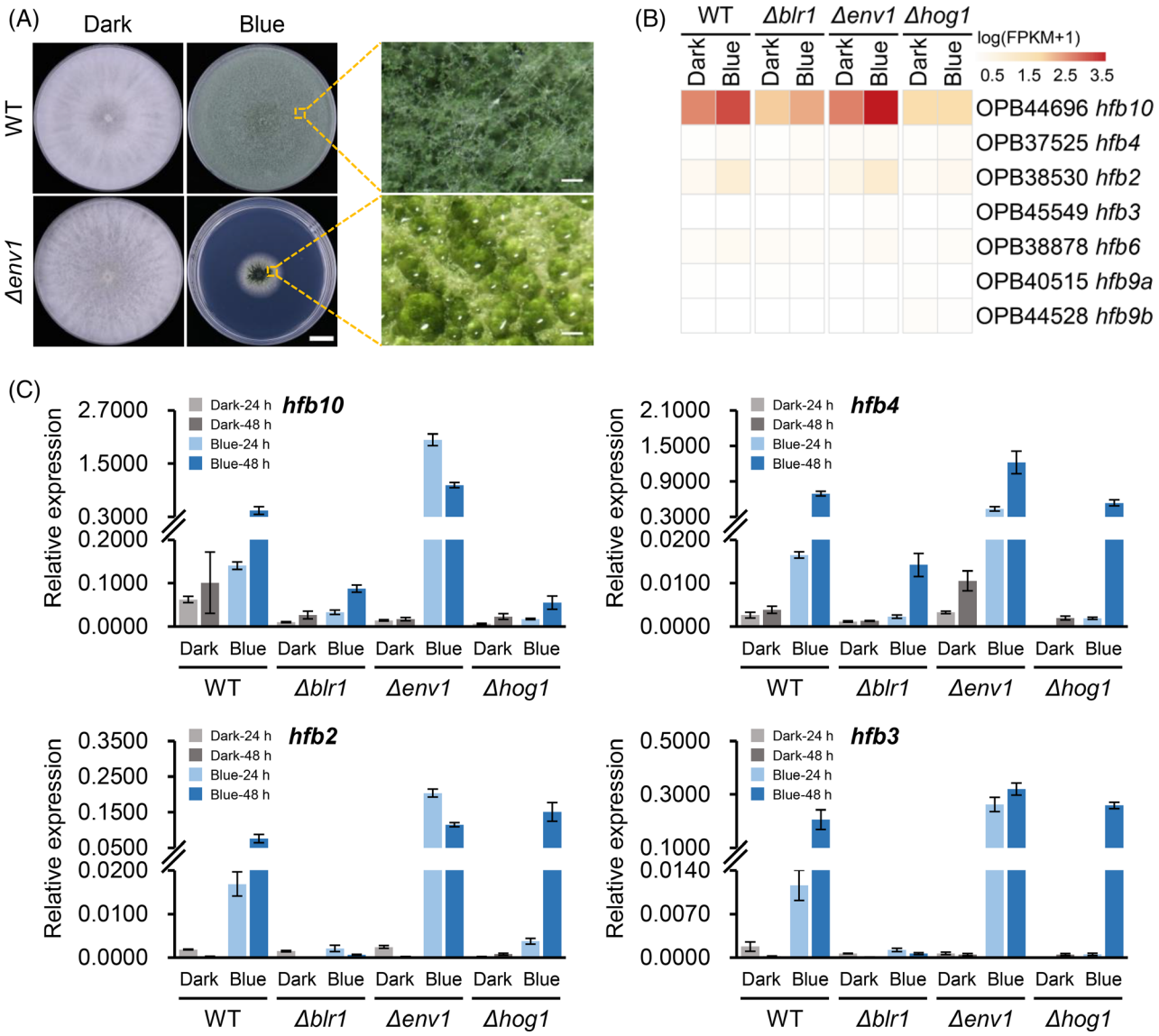

Fig 7. Crucial role of blue light receptors in hydrophobin production.
A. High hydrophobicity of the *Δenv1*-mutant. Left: Colony surface of wild type and the *Δenv1*-mutant in dark or blue light. Scale bar, 1 cm. Right: Stereoscopic observation of the corresponding colony surface after 3 days of cultivation in blue light. Scale bar, 300 μm.
B. Transcriptional abundances of all HFB-encoding genes derived from the transcriptome data.
C. Expression levels of main HFB-encoding genes in different strains upon constant blue light stimulation. Strains were cultured on PDA medium covered with cellophane at 28°C in the dark or in blue light for 24 and 48 h. The expression level of each gene was normalized to *tef1* gene. Error bars indicate SD of three biological replicates.

accordance with the former result, the expression of *hfb10* was the highest, followed by *hfb4* (Fig. 7C). The expression levels of all genes at 48 h were higher than those at 24 h, which is also consistent with previous studies (Cai *et al*., 2020; Zhao *et al*., 2021). In blue light, *hfb10*, *hfb4*, *hfb2* and *hfb3* were all upregulated in wild type and the *Δenv1*-mutant strain, and the expression level of each gene in the *Δenv1*-mutant was higher than that in wild type. However, the increase was not observed when BLR1 was deleted. We also investigated if the HOG pathway is involved in the light-dependent regulation of HFB production. Although the expression levels of *hfb4*, *hfb2* and *hfb3* were similar to those in wild type, the expression of *hfb10* in the *Δhog1*-mutant was significantly lower than in wild type.

Putative LREs present in the promoters of the light-regulated genes

In *N. crassa*, WCC binds to the LREs in the promoters of some light-responsive genes, which possess the consensus sequence GATNC------CGATN (He and Liu, 2005).

After scanning the promoters (1000 bp before open reading frame) of the aforementioned genes, we identified two putative LREs in *env1*, *hog1* and *hfb4* promoters, and one LRE in *hfb2*, *brl1*, *wet1*, *blu7*, *med1* and *fluG* promoters respectively (Suppl. Fig. S3). This suggests that the BLR1/2 complex probably binds to the promoters of these genes directly to activate the gene expression. In contrast, LREs were not found in the promoters of *hfb10*, *aba1* and *blu7*, which is consistent with our finding that these genes were regulated by BLR1/2 complex through the HOG pathway. However, an LRE was identified in the *flbC* promoter (Suppl. Fig. S3), although the expression of *flbC* and *blu7* seemed to depend on the HOG pathway (Fig. 2C).

Discussion

A large proportion (~13.4%) of the genes in genome regulated by blue light and the significant phenotypic alterations of the respective mutants suggest that *T. guizhouense* can be a good model organism for the study of photobiology. In *T. guizhouense*, BLR1 and BLR2 predominate asexual development, which is consistent with the critical role of them in photoconidiation in *T. atroviride* (Casas-Flores *et al*., 2004). While HOG1 is also involved in asexual development, it is not as important as BLR1 and BLR2 in terms of conidiation. The *Δhog1*-mutant was still able to sporulate in blue light although the conidia production of it was less than wild type (Fig. 2). This is because BLR1 and BLR2 strictly control the conidiation-related genes, but HOG1 does not regulate all of them. ENVOY was initially known as the cellulase gene regulator in *T. reesei* (Schmoll *et al*., 2004), but its indispensable function on fungal photoadaptation suggested that ENVOY has the same function as VIVID in *N. crassa* (Castellanos *et al*., 2010). The deletion of *env1* in *T. reesei* led to retarded vegetative growth, whereas the *Δenv1*-mutant of *T. guizhouense* had an even stronger phenotype grown in blue light. The severe growth defect and the impressively high hydrophobic property of the colony surface in blue light make the mechanism of ENV1-dependent fungal photoadaptation more enigmatic. The additional 605 genes differentially expressed in the *Δenv1*-mutant indicates that ENV1 functions to repress the activity of BLR1/2 complex to achieve photoadaptation.

Although in *N. crassa* WCC-dependent blue light signalling is well studied, the importance of the MAPK pathway for blue light signalling has not been evaluated yet. However, it has been proved that the circadian clock regulates rhythmic phosphorylation of MAPK OS-2 in *N. crassa*, which implies the involvement of the MAPK pathway in blue light signalling in *N. crassa* (Vitalini *et al*., 2007; de Paula *et al*., 2008). In *T. atroviride*, the MAP kinase Tmk3, involved in photoconidiation and the regulation of the blue-light-responsive genes *blu1*, *env1* and *phr1*, can be phosphorylated in blue light (Esquivel-Naranjo *et al*., 2016). While in *A. nidulans* a short-time (5 min) red-light illumination or heat shock can trigger the phosphorylation and the translocation from the cytoplasm into nuclei of SakA/HogA to activate light-inducible genes, a short-time illumination of blue light has no effect on the action of HogA/SakA (Yu *et al*., 2016). The most striking finding in this study is that a large proportion (~16.8%) of genes in the genome is under the control of HOG1 and these genes must involve in many physiological and morphological processes. We think that the activation of HOG pathway is an important step for the quick photoadaptation of fungi. The effect of ENV1 on the activation of HOG pathway is worthy of further study in the future.

In *A. nidulans*, the MAPK HogA/SakA pathway is responsible for the sensing of oxidative and osmotic stresses, red light and heat shock (Furukawa *et al*., 2005; Yu *et al*., 2016; Garrido-Bazan *et al*., 2018; Yu *et al*., 2019). In *T. atroviride*, Tmk3 is also involved in oxidative stress sensing (Esquivel-Naranjo *et al*., 2016). The pivotal role of HOG1 in *T. guizhouense* raises the question of how the fungus distinguishes so many different environmental signals using the same highly conserved MAPK HOG pathway. In *A. nidulans*, upon light or oxidative stress, the transcript factor AtfA interacts with HogA/SakA to promote gene expression. The 61 TFs regulated by light provide more possibilities for the direction of light signal transmission. In *Saccharomyces cerevisiae*, HOG1 can modify histone with the assistance of histone modifiers to regulate osmoresponsive genes (De Nadal *et al*., 2004). In *N. crassa*, WC-1 modulate the acetylation of histone 3 lysine 14 (H3K4) to induce gene expression by directly interacting with the histone acetyltransferase NGF-1 (Grimaldi *et al*., 2006) and in *A. nidulans*, the red light receptor phytochrome and the blue light receptor LreA modulate gene expression also through modifying H3 acetylation (Hedtke *et al*., 2015). These suggest that fungi may recognize different signals through the crosstalk between histone remodelling and HOG pathway or transcription factors. We speculate that different signals converging on the MAPK cascade can diverge at HOG1 to regulate the specific genes.

HFBs are the small cysteine-rich proteins putatively located on the outer surface of the fungal cell wall (Wosten, 2001). *Trichoderma* HFBs drew more attention because genomes of these fungi are enriched in respective genes (Kubicek *et al*., 2008; Kubicek *et al*., 2011; Seidl-Seiboth *et al*., 2011; Schmoll *et al*., 2016; Kubicek *et al*., 2019). Recent studies in *Trichoderma* suggested that HFBs are usually considered as useful conidiation markers because they are highly expressed during the formation of aerial mycelium and remained highly active during conidiation (Schmoll *et al*., 2016; Cai *et al*., 2020). Although intensive studies for HFBs are focused on their

function and application potential, the regulatory mechanism for HFB production is still unclear. In *T. guizhouense*, the expression level of *hfb10* is higher than *hfb4* during asexual development, but HFB4 is more important for the spore surface hydrophobicity (Cai *et al*., 2020). We showed evidence that HFBs are regulated by blue light. The expression of *hfb10* was always the highest no matter in constant blue light or after short-time light stimulus, which was controlled by BLR1, ENV1 and the HOG pathway. In contrast, *hfb2*, *hfb3* and *hfb4* were only regulated by BLR1 and ENV1. BLR1/2 complex probably binds to the LREs in the promoters of *hfb2* and *hfb4* to regulate their expression directly, but the regulation of *hfb3* could be indirect. Previous study has shown that production of different HFBs varies during the whole life cycle of *Trichoderma* (Cai *et al*., 2020). The distinct regulatory mechanisms for different HFB-encoding genes may correspond to different developmental stages of fungi.

It is worth noting that there were 208 genes (12.9%) in the DEGs identified from wild type that were regulated by neither BLR1 nor ENV1 (Fig. 4A). However, 112 of them were still controlled by HOG1. The different expression of these genes upon blue light we think are caused by ROS generated by blue light or the regulation from other light receptors. The fungus *S. cerevisiae*, not equipped with photoreceptors, can convert blue light to hydrogen peroxide by a peroxisomal oxidase, which will alter the localization of the stress-responsive transcription factor Msn2 to affect the gene expression (Bodvard *et al*., 2017).

While the blue light signalling is complex, BLR1 and HOG 1 are the core components of the whole regulatory network in *T. guizhouense* (Fig. 8). Upon short-time blue light illumination, BLR1/2 complex, on the one hand, activates the HOG pathway to control 61.4% of the DEGs identified in wild type including *hfb10*, *flbC*, *cry1*, *blu7* and other transcription factor encoding genes. On the other hand, BLR1/2 complex modulates 18.5% of the DEGs, which include ENV1, HOG1, HFB2/3/4, MED1, PHR1 and CRY-DASH encoding genes. ENV1 repress the light responses on the DNA level through inhibiting the activity of the BLR1/2 complex to fulfil photoadaptation. Additionally, other light receptors or ROS generated by blue light also trigger the different expressions of some genes. But, even so, more than half of these genes are still regulated by the HOG pathway.

Experimental procedures

Strains and culture condition

The wild type strain used in this study is *T. guizhouense* NJAU 4742, which was cultivated on PDA medium (BD Difco, Germany) at 28°C. To evaluate blue light responses, wild type and all mutants were exposed to blue light with an intensity of 1.7 μmol photons/($m^2 \times s$). All strains used in this study are listed in Suppl. Table S1.

The *Δblr1*-, *Δblr2*-, *Δenv1*- and *Δhog1*-mutants were constructed following the method described previously (Zhang *et al*., 2019). In brief, the hygromycin phosphotransferase gene (*hph*) cassette, upstream and downstream fragments of the targeted gene were amplified via PCR and then were cloned into the vector pUC19 by using the ClonExpress MultiS One Step Cloning Kit (Vazyme, China), yielding the plasmid for gene knockout. The deletion cassettes were then amplified from the plasmids and used for transformation. The positive transformants were confirmed by PCR and Southern blot. The genes encoding HOG1 and eGFP (green fluorescent protein) were amplified separately via PCR, and a synthetic linker ($3 \times$ GGGGS) was amplified and used to conjugate *hog1* and *gfp* together at the C-terminus of *hog1*. Then the *hog1::egfp* cassette was amplified and transformed into wild type. The positive transformants were confirmed by PCR and Southern blot. All primers used in this study are listed in Suppl. Table S2.

Quantification of conidia

The wild type and all mutants were inoculated on PDA medium (90 mm plate) and cultured in darkness or constant blue light for 72 h. Conidia were collected with distilled water and quantified with the haemocytometer. Three biological replicates were performed for each culture, and the assay was repeated three times.

DNA extraction

Mycelium of each strain was harvested from PDA plates covered with cellophane after 3 days incubation and then ground in liquid nitrogen. Afterwards, DNA was extracted using the Fungal DNA kit (OMEGA, USA). Standard methods were used for electrophoresis, blotting and hybridization of nucleic acids.

RNA isolation and qPCR

Strains were grown on PDA medium covered with cellophane in Ø 6 cm Petri dishes at 28°C in the dark or constant light for 24 or 48 h. For short time illumination (45 min), blue light was imposed after 24 h incubation in the dark. Mycelium on cellophane was harvested in dim-green light and frozen immediately in liquid nitrogen. Total RNA was extracted from each sample with the SteadyPure® Plant RNA Extraction Kit (Accurate Biotechnology, China), and ~1 μg of total RNA was used for cDNA synthesis with HiScript® II Q RT SuperMix for

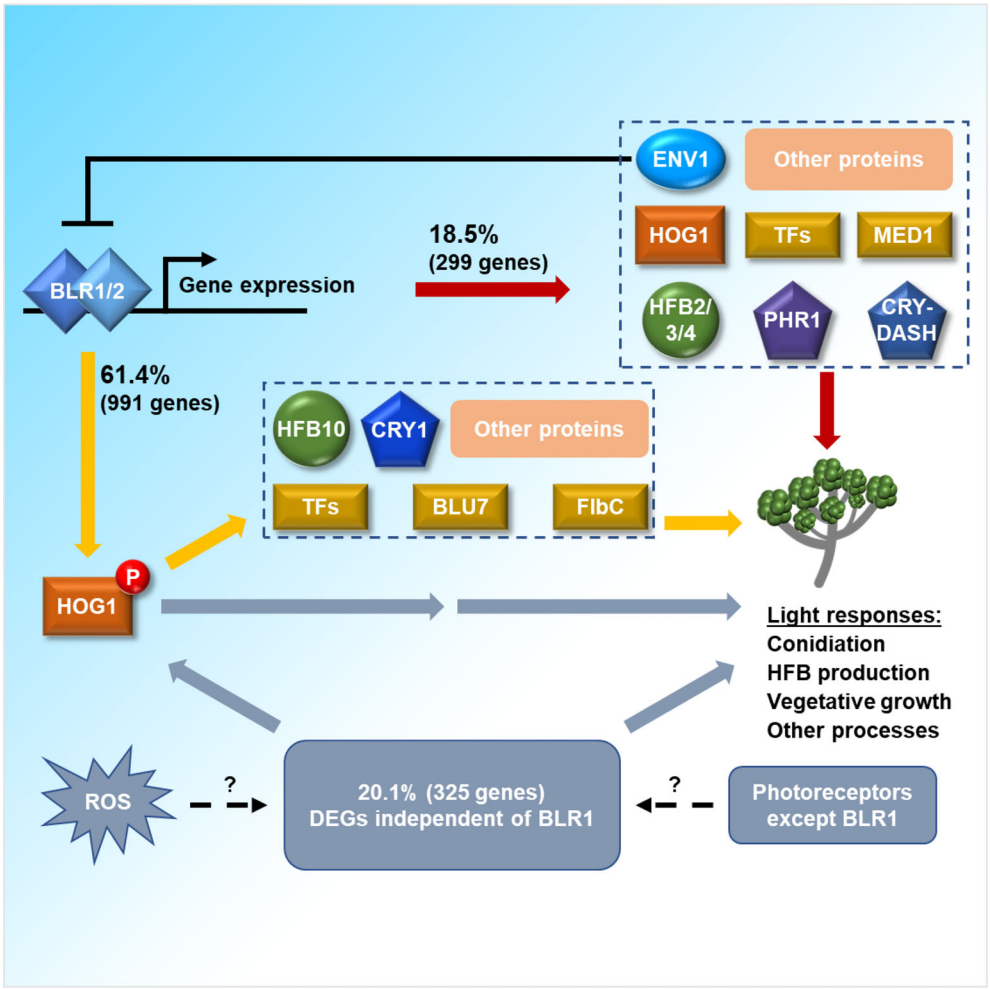

Fig 8. Model for blue light signalling in *T. guizhouense*. Short-time blue light illumination causes 1615 genes differentially expressed in wild type, and 79.9% of these DEGs are dependent on BLR1. Among them, 18.5% of the DEGs including *env1*, *hog1*, *hfb2/3/4*, *med1*, *phr1* and *cry-dash* are directly modulated by BLR1/2 complex, and 61.4% of the DEGs including *hfb10*, *flbC*, *cry1*, *blu7* and other transcription factor encoding genes are controlled by BLR1/2 complex through the HOG pathway. The blue light receptor ENV1 inhibits the activity of the BLR1/2 complex to fulfil photoadaptation on the DNA level. Furthermore, other photoreceptors or ROS generated by blue light can also trigger some genes differentially expressed.

qPCR (+gDNA wiper) (Vazyme). qPCR was performed with the ChamQ SYBR qPCR Master Mix (Vazyme) and the expression level of each gene was normalized to the translation elongation factor 1 alpha (*tef1*) gene. Primers used in this study are listed in Suppl. Table S2. Statistical analyses were performed based on three biological replicates.

RNA-Seq analysis

The wild type and the *Δblr1*-, *Δenv1*- and *Δhog1*-mutant strains were incubated on PDA medium covered with cellophane in Ø 6 cm Petri dishes at 28°C in the dark for 24 h. The mycelium was harvested in dim-green light immediately or after 45 min illumination of blue light and frozen immediately in liquid nitrogen. Transcriptome sequencing was performed on the Novaseq by Gene Denovo Biotechnology (Guangzhou, China). Three biological replicates for each group were used for transcriptome sequencing.

After quality control, clean reads were mapped to the reference genome of *T. guizhouense* using HASAT2 v2.1.0. For each transcript, a fragment per kilobase of transcript per million mapped reads value was calculated to quantify its expression abundance and variations, using the StringTie v1.3.1. DEGs were identified using DEGseq2 with thresholds of $|\log_2(\text{fold change})| \geq 1$ and FDR <0.05. All DEGs were mapped to GO terms in the GO database (http://www.geneontology.org/) and subjected to GO analyses. To identify the pathways significantly associated with DEGs, the same method was used to blast the DEGs against the KEGG database

(https://www.genome.jp/kegg/). Venn diagram and heatmap were generated in R environment.

The sequencing data are available in the NCBI database Sequencing Read Archive under the accession number PRJNA743899.

Microscopy

To analyse the subcellular localization of HOG1 upon blue light exposure, fresh conidia of the strain HOG1::eGFP were inoculated on microscope coverslips with 400 μl supplemented PDB. After 14 h incubation in the dark at 28°C, samples were exposed to blue light for 3 min or kept in the dark. Then samples were fixed immediately with 4% formaldehyde for 10 min and washed twice with 1× PBS (pH 7.4). Nuclei were stained with Hoechst (no. C0031, Solarbio) before microscopy. The samples were observed with the confocal laser scanning microscope (Leica TCS SP8, Germany). The excitation wavelength was set at 488 nm, and GFP emission was set at a wavelength range of 501–530 nm.

Immunofluorescence

Fresh conidia were inoculated onto coverslips with 400 μl PDB medium and cultivated overnight in the dark at 28°C. Then the samples were exposed to blue light for 3 min or kept in the dark. Medium on coverslips was discarded and then 400 μl fixation solution containing 8.6% formaldehyde was added immediately. Afterwards, the samples were incubated at room temperature for 30 min. The coverslips with the adhered germlings were washed three times with 1× PBS buffer (pH 7.4) and incubated with 150 μl digestion solution (100 mg driselase (Sigma-Aldrich), 20 μl of 5 U ml^{-1} zymolyase, and 800 mg lysing enzyme from *Trichoderma* diluted in 2.5 ml of 50 mM Na citrate (pH 5.8) and mixed with 2.5 ml egg white for 1 h at room temperature. Then the coverslips were washed three times with 1× PBS buffer (pH 7.4). Next, the samples were incubated with precooled methanol at −20°C for exactly 10 min and then washed two times with 1× PBS buffer (pH 7.4). Samples were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 15 min. Afterwards, the samples were incubated with antiphospho-p38 MAP kinase (Thr180/Tyr182) antibodies (no. 9211, 1:400 dilution; Cell Signalling Technology) in TBST buffer with 5% BSA overnight at 4°C and then washed three times with TBST before incubation with the Cy3-conjugated anti-rabbit IgG secondary antibody (Sangon Biotech, Shanghai, China) at a 1:100 dilution in 5% BSA in TBST. After 1 h of incubation at room temperature, the coverslips were washed three times with TBST. Nuclei were stained with Hoechst and the samples were sealed with nail polish before microscopy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Fig. S1. Conidia production of wild type after blue light exposure for six days. Quantification of conidia of wild type produced in darkness and blue light after six days incubation. Error bars indicate SD of three biological replicates.

Supplementary Fig. S2. Construction of the corresponding gene deletion mutants.

(A) Schematic diagram of each gene deletion. The construction strategy is based on homologous recombination. The grey rectangles represent the upstream and the downstream homology arm respectively. *Hph*, hygromycin B phosphotransferase gene cassette. (B) Southern blot analysis of each mutant digested with the restriction enzymes indicated in (A) and hybridized with the probes amplified by primers listed in suppl. Table S2.

Supplementary Fig. S3. Putative light response elements (LREs) identified in the promoters of the light responsive genes. Putative light response elements (LREs) location in the promoters of genes *env1*, *hog1*, *hfb4*, *hfb2*, *brl1*, *wet1*, *med1*, *flbC* and *fluG*. Positions are relative to the translation start codon. GATNC------CGATN is the consensus sequence of LREs.

Supplementary Table S1. Strains used in this study.

Supplementary Table S2. Oligonucleotides used in this study.