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The blue-light receptor CmWC-1 mediates fruit body development and secondary metabolism in *Cordyceps militaris*

Tao Yang[1] · **Mingmin Guo**[1,2] · **Huaijun Yang**[3] · **Suping Guo**[3] · **Caihong Dong**[1]

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Abstract Light is an essential factor for pigment formation and fruit body development in *Cordyceps militaris*, a well-known edible and medicinal fungus. *Cmwc-1*, a homolog of the blue-light receptor gene *white collar-1* (*wc-1*) in *Neurospora crassa*, was cloned from the *C. militaris* genome in our previous study. Here, *Cmwc-1* gene inactivation results in thicker aerial hyphae, disordered fruit body development, a significant reduction in conidial formation, and carotenoid and cordycepin production. These characteristics were restored when the Δ*Cmwc-1* strains were hybridized with wild-type strains of the opposite mating type. A genome-wide expression analysis revealed that there were 1042 light-responsive genes in the wild-type strain and only 458 in the Δ*Cmwc-1* strain. Among five putative photoreceptors identified, Vivid, cryptochrome-1, and cyclobutane pyrimidine dimer photolyase are strongly induced by light in a *Cmwc-1*-dependent manner, while phytochrome and cryptochrome-2 were not induced. The transcription factors involved in the fungal light reaction were mainly of the Zn_2Cys_6 type. CmWC-1 regulates adenylosuccinate synthase, an important enzyme for adenosine de novo synthesis, which could explain the reduction in cordycepin production. Some G protein-coupled receptors that control fungal fruit body formation and the sexual cycle were regulated by CmWC-1, and the cAMP pathway involved in light signal transduction in *N. crassa* was not critical for the photoreaction in the fungus here. A transcriptional analysis indicated that steroid biosynthesis was more active in the Δ*Cmwc-1* strain, suggesting that CmWC-1 might switch the vegetative growth state to primordia differentiation by suppressing the expression of related genes.

Keywords *Cordyceps militaris* · Cordycepin · Carotenoid · *Cmwc-1* · Fruit body

Introduction

Cordyceps militaris (L.) Fr., a well-known edible and medicinal fungus, is the type of *Cordyceps* species that generally parasitizes the larvae or pupae of lepidopteron insects. It has been widely used as an herbal drug and tonic food in East Asia and has also been studied in the West owing to various biological activities, such as antitumor (Jin et al. 2008), anti-influenza virus (Lee et al. 2014), radio-protection (Jeong et al. 2014), and anti-inflammatory (Smiderle et al. 2014). A series of pharmacologically active ingredients including cordycepin, cordycepic acids, polysaccharides, and carotenoids have been found in *C. militaris* (Holliday and Cleaver 2008; Dong et al. 2013). Among these compounds, cordycepin has so far only been reported in *C. militaris* (Zhou et al. 2009) and is currently being used in clinical trials against cancers (http://clinicaltrials.gov/show/NCT00709215). *C. militaris*' carotenoids are the most abundant of the known macrofungi and have a strong

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✉ Caihong Dong
dongch@im.ac.cn

[1] State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, No. 3 Park 1, Beichen West Road, Chaoyang District, Beijing 100101, China

[2] College of Chemistry and Life Science, Shenyang Normal University, Shenyang 110034, China

[3] Shanxi Research Institute for Medicine and Life Science, Taiyuan 030006, China

antioxidant activity (Yang et al. 2014). Thus, cordycepin and carotenoids are of particular interest as active components in *C. militaris*.

C. militaris is a typical heterothallic fungus, and its sexual reproduction (perithecia and ascospores) requires two mating types—mating 1-1 and 1-2 in separate strains, or a single strain containing both *MAT1-1* and *MAT1-2* information. However, this is the first ascomycete species reported to produce stroma (fruit body) without mature perithecia and ascospores from a strain with only single mating-type information (Zheng et al. 2011a).

As an edible and medicinal fungus, the fruit bodies of this fungus are the major form of interest for industrial production and commercialization. During the large scale cultivation and laboratory culturing, light is an essential environmental factor for *C. militaris* pigment formation and stroma production (Sato and Shimazu 2002). After exposure to light, the colony color changes from white to yellow or orange, and then the primordia begin to develop. There is no pigment or stromal production when cultured under darkness. Light also affects conidial production (Yang and Dong 2014) and metabolism, such as cordycepin and carotenoid formation, in this fungus (Dong et al. 2012; Lian et al. 2014a).

Sensing light as a signal for morphogenesis and metabolite production has been documented in several fungi. The best-characterized model system was discovered in *Neurospora crassa*, and the white collar (WC) complex consisting of the WC-1 and WC-2 proteins (Ballario et al. 1996) is the sensor for blue light. As a transcription factor (TF), WC-1 is an essential component of all known blue light responses, including mycelial carotenogenesis, perithecial beak phototropism, circadian rhythms of conidiation, sexual development, and circadian clock resetting (for reviews, see Linden et al. 1997; Linden 2002; Dunlap 2006; Corrochano 2007; Chen et al. 2010; Idnurm et al. 2010). WC-1 contains a zinc finger DNA-binding domain, glutamine-rich putative transcription activation domains, protein-protein interaction domains, a nuclear localization signal, and a chromophore-binding domain (Ballario et al. 1996). WC-1 and WC-2 interact through the protein-protein interaction domains to form the functional white collar complex (WCC) that binds to the promoters of light-regulated genes to rapidly activate transcription in response to light (Talora et al. 1999). WC-1 homologs have been found in various fungal species (for a review, see Corrochano 2007). The photoreceptor orthologs BLR1 and BLR2 are known to mediate nearly all known light responses in some species of the genus *Trichoderma* (Schmoll et al. 2010). The blue-light receptor LreA (WC-1) in *Alternaria alternata* has a repressing function in the dark as well as an activating function in the light to control the secondary metabolism and sporulation (Pruß et al. 2014). Morphological and physiological differentiation in *Aspergillus nidulans* are mediated through a network, consisting of FphA (phytochrome), LreA (WC-1), and LreB (WC-2), that senses red and blue light (Purschwitz et al. 2008). However, the photoresponses of *Aspergillus fumigatus* differ in notable ways from the well-studied model *A. nidulans* (Fuller et al. 2013).

There have been only a few studies on the photoreceptors in macrofungi. A photoreceptor gene (*Le.phrA*) from the basidiomycete *Lentinula edodes* was cloned and sequenced (Sano et al. 2007), and the transcriptome data analysis suggested that the mechanism of brown film formation in the *L. edodes* mycelium was dependent on photoreceptors (Tang et al. 2013). Two genes, *dst1* and *dst2*, homologs of *wc-1* and *wc-2*, are involved in the mushroom photomorphogenesis of *Coprinopsis cinerea* encoding putative photoreceptors for blue light (Terashima et al. 2005; Kamada et al. 2010). There was another study on the gene function of the macrofungal blue-light receptor complex WC-1/2 in fruit body formation for *Schizophyllum commune* (Ohm et al. 2013).

The essential role of light in fruit body development and certain metabolite production in *C. militaris* was demonstrated in our previous study (Lian et al. 2014a). The gene *Cmwc-1*, homologous to *N. crassa wc-1*, from the genome of *C. militaris* has been cloned, and its structure and expression in different strains were compared (Yang and Dong 2014). The size and sequence of the predicted CmWC-1 protein are similar to those of WC-1. *Cmwc-1* mRNA is also light-inducible, like *wc-1* in *N. crassa* (Ballario et al. 1996), and the expression level increased significantly after irradiation in all of the tested strains. In the current study, we characterized the *Cmwc-1* gene and the biological role of CmWC-1 in *C. militaris*. The knockout of the *Cmwc-1* gene resulted in disordered fruit body development and a significant reduction in carotenoid and cordycepin formation. The putative genes regulated by CmWC-1 were identified through an RNAseq analysis and their functions studied in this fungus.

Materials and methods

Fungal and bacterial strains, vectors, and other reagents

All of the microbial strains and plasmids used in this study are listed in Table 1. Single ascospore strains were obtained from *C. militaris* strain 40 (CGMCC 3.16322), which contains both *MAT1-1* and *MAT1-2*. Fresh stromata formed by CGMCC 3.16322 were attached to the inner side of a Petri dish lid containing 1.5 % water agar and were incubated at 22 °C under light. Single ascospores were forcibly discharged and randomly selected from Petri dishes under an inverted microscope (Axio Observer A1, Zeiss, Oberkochen, Germany) using a sterile pin. They were then inoculated on potato dextrose agar (PDA) plates at 20 °C. The mating type was identified by following previously described procedures (Yang

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or reference
Cordyceps militaris 40 (CGMCC 3.16322)	Wild-type strain with *MAT1-1* and *MAT1-2* information	Isolated from Shenyang, China
Cordyceps militaris 40d8	Wild-type strain with *MAT1-1*	Single ascosporic strain isolated from strain 40
Cordyceps militaris 40d26	Wild-type strain with *MAT1-2*	Single ascosporic strain isolated from strain 40
Agrobacterium tumefaciens AGL-1	Strain used for fungal transformation	Khang et al. (2007)
pAg1-H3	Vector used for ATMT of fungi	Zhang et al. (2003)
PAg1-H3-Cmwc1	Plasmid used for disruption of *Cmwc-1*	This work
Escherichia coli strain DH5α	Strains used for plasmid manipulations	Tiangen, China
pUMT	Vector used for PCR cloning and subcloning	Bioteke, China
pUMT-Cmwc1	Vector used for complementation	This work
pAgG-Cmwc1	Vector used for complementation	This work
pBN50	Plasmid containing geneticin resistant gene *kanMX4*	Hartzog et al. (2005)
pAg1-G	Vector based on pAg1-H3 by replacing hygromycin resistance gene with geneticin resistant gene from pBN50	This work

and Dong 2014). Wild-type strains 40d8 (*MAT1-1*) and 40d26 (*MAT1-2*) were selected for transformations.

Media and growth conditions

The mycelial growth of *C. militaris* strains were measured on PDA plates under a 12 h:12 h light/dark that included white and blue lights in an illumination incubator (MGC-450BP, Yiheng, Zhabei District, Shanghai, China). The blue light was produced by Samvol powered 12-W light-emitting diodes (LEDs, Zhongshan, China). The distance between the LEDs and the agar plates was 50 cm with a light intensity of 50 lux. The colony diameter was measured after 2 weeks of incubation, and three replicates for each strain were applied.

Conidial production was determined by scraping mycelia of colonies from PDA plates incubated for 14 days and placing them into 15-mL centrifuge tubes containing 10 mL Tween 80 solution (20 % *w/v*). After filtration, the conidial suspensions were appropriately diluted and counted using a hemocytometer under a microscope. The conidial production was calculated from six replicate plates with three counts for each strain.

The fruit bodies were cultivated according to the method of Zhan et al. (2006). Two strains with different mating types were simultaneously inoculated in equivalent proportions in the seed medium for strain cross-mating.

Escherichia coli and *Agrobacterium tumefaciens* were grown in Luria-Bertani (LB) broth (1 % NaCl, 0.5 % yeast extract, and 1 % tryptone) or LB agar. Induction medium and co-cultivation medium (IMAS) were used for *A. tumefaciens*-mediated transformation (ATMT) of *C. militaris* (Khang et al. 2007).

Disruption of *Cmwc-1* in *C. militaris*

Genomic DNA was prepared using the cetyltrimethylammonium bromide method (Doyle and Doyle 1987). Primers Cmwc1-F and Cmwc1-R were used to amplify the full-length *Cmwc-1* gene. Details of the primers used in this study are listed in the Supplementary Material Table S1.

A strategy of homologous recombination was employed to disrupt *Cmwc-1* in *C. militaris* (Fig. 1a). The 1311- and 1287-bp DNA fragments upstream and downstream of *Cmwc-1* were amplified from genomic DNA with primers Cmwc1-up-*Sb*/*As* and Cmwc1-down-*Ap*/*Kp*, respectively (in the Supplementary Material Table S1). After being cloned into vector pUMT, the amplified DNA fragments were digested with *Sbf*I-*Asc*I or *Apa*I-*Kpn*I and inserted into the corresponding sites of vector pAg1-H3 to generate pAg1-H3-Cmwc1. In total, 1168 base pairs of the Cmwc-1 coding region were deleted. The constructs were introduced into *C. militaris* by ATMT using the method reported by Zheng et al. (2011b) with slight modifications. Conidia for transformation were harvested and suspended into sterile 0.05 % Tween 80 and adjusted to a concentration of 10^5 spores mL^{-1}. Then, 100 μL of *C. militaris* conidial suspensions and 100 μL of *A. tumefaciens* (OD_{660}= 0.6–0.8) were mixed and spread on the IMAS agar plate and co-incubated at 23 °C for 4–5 days. The co-culture of *A. tumefaciens* and *C. militaris* was covered with PPD agar supplemented with 300 μg mL^{-1} cefotaxime and 500 μg mL^{-1} hygromycin B (hygB) and incubated at 23 °C for 10 days before isolating hygB-resistant colonies. Three polymerase chain reaction (PCR) primer pairs (in the Supplementary Material Table S1) were used to verify the transformants: Cmwc1-up-*Sb*/Cmwc1-down-*Kp*, P1985/phph4514, and Phph6096/P7604R.

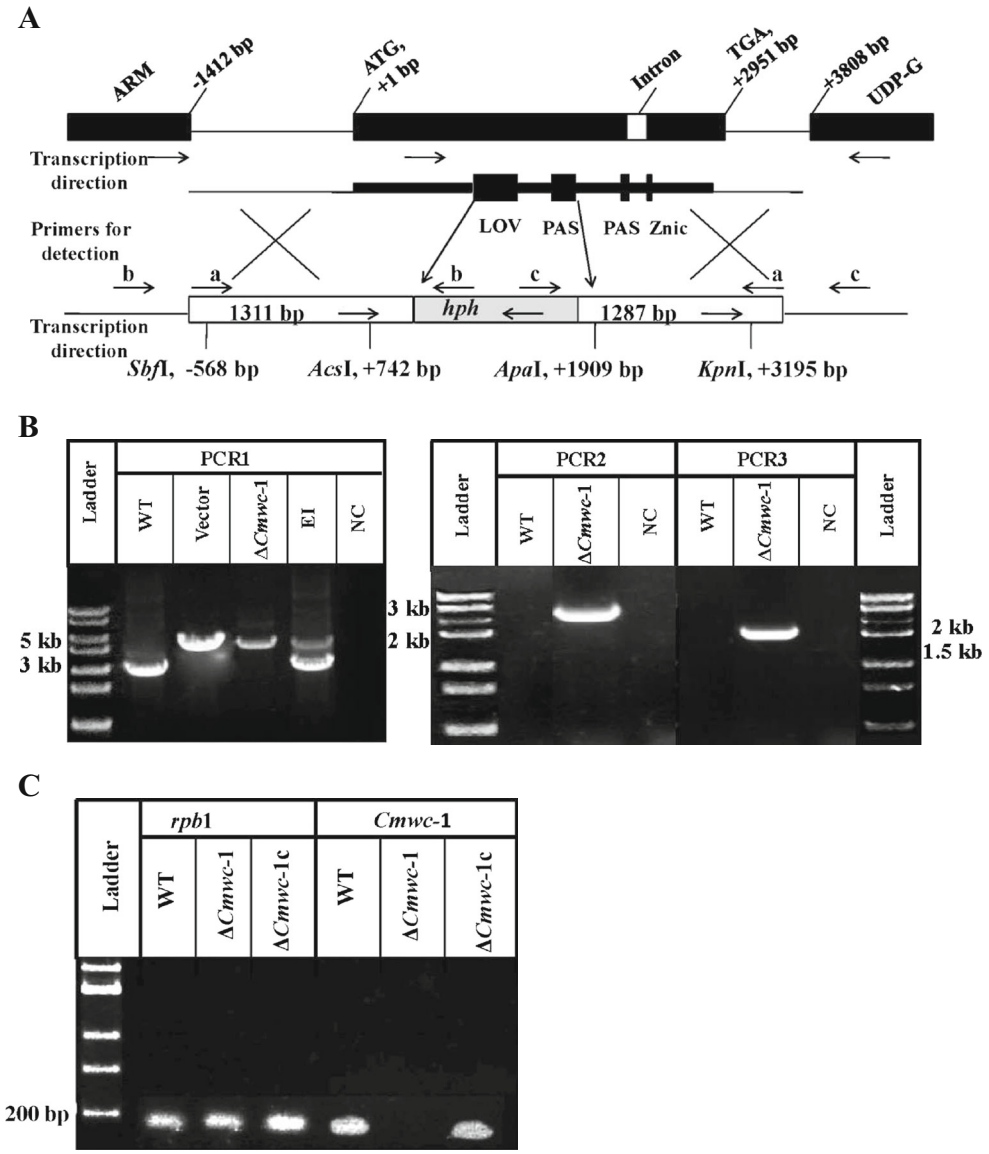

Fig. 1 Construction and confirmation of the *Cmwc-1* disruption mutant. **a** Strategy for the construction of Δ*Cmwc-1* via homologous recombination. Bases are numbered starting with the translational initiation codon for *Cmwc-1*. Light, Oxygen, or Voltage (LOV) chromophore-binding, Per-Arnt-Sim (PAS), and zinc finger domains are the functional domains in CmWC-1. ARM and UDP-G are the up- and down-stream genes of CmWC-1, respectively. *hph* is the hygromycin phosphotransferase gene. Primer sets *Sbf*I–568/*Asc*I+742 and *Apa*I+1909/*Kpn*I+3195 were used to amplify the 1311-bp 5′ region and the 1287-bp 3′ region of *Cmwc-1*, respectively. *a* (Cmwc1-up-*Sb*/Cmwc1-down-*Kp*), *b* (P1985/phph4514), and *c* (Phph6096/P7604R) are the three primer sets used for confirmation of Δ*Cmwc*-1 using PCR. **b** Confirmation of Δ*Cmwc-1* by PCR. PCR1, PCR2, and PCR3 were performed with the primers *a* (Cmwc1-up-*Sb*/Cmwc1-down-*Kp*), *b* (P1985/phph4514) and *c* (Phph6096/P7604R), respectively. *WT* the wild-type strain, *EI* ectopic integration, *NC* negative control. The 3763- and 5105-bp DNA fragments were amplified with primer set *a* from the wild-type and Δ*Cmwc-1* strains, respectively. DNA fragments with lengths of 2530 and 1509 bp were obtained using primer sets *b* and *c* from the Δ*Cmwc-1* strain, respectively, while no fragments were amplified from the wild-type strain. **c** Detection of *Cmwc-1* expression in WT, Δ*Cmwc-1*, and Δ*Cmwc-1c* using RT-PCR. All of the strains were grown in PDA for 10 days at 20 °C. Total RNA extraction and cDNA synthesis were performed as described in the "Materials and methods". Expression of *Cmwc-1* and *rpb1* was detected with primers Qcmwc1-F/Qcmwc1-R and rpb1-F/rpb1-R, respectively

Complementation of the *Cmwc-1* disruption mutant

The entire *Cmwc-1* gene with a 1412-bp upstream region containing its putative promoter and an 858-bp downstream region was amplified from the *C. militaris* wild-type strain with primers Cmwc1C-F/R and inserted into pUMT to generate pUMT-Cmwc1. A 1.6-kb DNA fragment containing the geneticin resistance gene (kanMX4) from plasmid pBN50 was inserted into the corresponding sites of pAg1-H3, yielding pAg1-G. pUMT-Cmwc1 was digested with *Sbf*I, and the 5221-bp DNA fragment containing the intact *Cmwc-1* was inserted into the corresponding sites of pAg1-G to generate

pAgG-Cmwc1. For complementation, pAgG-Cmwc1 was introduced into the Δ*Cmwc-1* strains by the ATMT method. Transformants were selected on PPD agar plates supplemented with 750 μg of geneticin (G418) at 23 °C. The complemented strain was confirmed by PCR amplification (in the Supplementary Material Table S1).

Determination of carotenoids and cordycepin

All of the wild and mutant strains were incubated in potato dextrose broth (PDB) at 20 °C in the illumination incubator with a light intensity of 500 lux under a static condition over 10 days. Mycelia were collected, dried, and used for carotenoid determination following the previously optimized method (Yang et al. 2014). The cordycepin amount was determined by the high-performance liquid chromatography method as described by Dong and Yao (2010).

Transcriptome analysis

An analysis of whole-genome gene expression levels was performed using the wild-type and Δ*Cmwc-1* strains of 40d8. All of the strains were incubated on a rice substrate within 500-mL glass bottles at 20 °C. After a 7-day incubation in the dark, mycelia were collected from some bottles for RNA extraction. The other bottles were continuously incubated for another 3 days under white light until the mycelia of the wild-type strain turned orange, and then the RNA was extracted from both wild and mutant strains. RNA extracts from three replicate bottles were pooled together for the analysis.

RNA quality and concentrations were evaluated using a NanoPhotometer spectrophotometer (Implen, CA, USA) and an RNA 6000 Nano Assay Kit for the Agilent 2100 Bioanalyzer system (Agilent Technologies, CA, USA). Sequencing libraries were generated using a NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) following the manufacturer's recommendations. The library quality was assessed on an Agilent 2100 Bioanalyzer system. Samples with an RNA integrity number greater than 7.5 were used to construct the cDNA library. The cDNA library was sequenced on an Illumina Hiseq 2000/2500 platform at Novogene Bioinformatics Technology Co., Ltd (Haidian District, Beijing, China).

Clean data (clean reads) were obtained by removing reads containing adapters and poly-Ns and low-quality reads from the raw data. The clean reads were mapped to the *C. militaris* genome (Zheng et al. 2011a) using TopHat v2.0.9. (Trapnell et al. 2009). HTSeq v0.5.4p3 (Anders et al. 2015) was used to count the read numbers mapped to each gene. To identify genes that were differentially expressed between two samples, the number of raw clean tags in each sample was normalized to tags per million. A differential expression analysis was performed using the DEGSeq R package (1.12.0, Anders and Huber 2010). The *P* values were adjusted using the method of Benjamini and Hochberg (1995). A corrected *P* value of 0.005 and log2 (fold change) of 1 were set as the thresholds for significant differential expression levels.

A Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented using the GOseq R package in which the gene length bias was corrected (Young et al. 2010). GO terms with a corrected *P* value less than 0.05 were considered to be significantly enriched by differentially expressed genes. KOBAS software was used to test the statistical enrichment of differentially expressed genes in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Mao et al. 2005). The raw Illumina sequencing data of *C. militaris* was submitted to NCBI as BioProjectPRJNA278309.

Quantitative reverse-transcription (RT)-PCR

Total RNA was isolated from 100 mg of frozen mycelia using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and was then treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd, Osaka, Japan), and quantitative real-time PCR (qPCR) was performed using the Mastercycler ep realplex (Eppendorf, Hamburg, Germany) real-time PCR system. The 25-μL qPCR reactions contained 5 ng of cDNA, 0.1 μM primers, and 12.5 μL of QPCR SYBR Green Mix (Toyobo Co., Ltd, Osaka, Japan). The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The obtained data represent three biological replicates, with two technical replicates each.

Results

Disruption of *Cmwc-1*

To study *Cmwc-1* functions, homologous recombination was employed to disrupt *Cmwc-1* (Fig. 1a). Plasmid pAg1-H3-Cmwc1 was constructed and transformed into the wild-type strains 40d8 and 40d26 through ATMT. Putative Δ*Cmwc-1* strains (five from 40d8 and three from 40d26) were obtained and confirmed by PCR analysis (Fig. 1b). The 3763- and 5105-bp DNA fragments were amplified with the primer set Cmwc1-up-*Sb*/Cmwc1-down-*Kp* from the wild-type and Δ*Cmwc-1* strains, respectively. When using the P1985/phph4514 and Phph6096/P7604R primer sets, 2530- and 1509-bp DNA fragments were amplified from Δ*Cmwc-1*, respectively, while no fragments were amplified from the wild-type strain. These results indicated that the native *Cmwc-1* was partially replaced by the hygromycin phosphotransferase gene (*hph*) in Δ*Cmwc-1*. The RT-PCR analysis indicated that Δ*Cmwc-1* abolished its expression and that its expression

was restored in the complemented strain (Δ*Cmwc-1c*) (Fig. 1c).

Disruption of *Cmwc-1* affects the conidial production of *C. militaris*

The growth of the wild-type and Δ*Cmwc-1* strains (five from 40d8 and three from 40d26) were tested on PDA plates under 12 h:12 h white light/dark (W/D) cycles, 12 h:12 h blue light/dark (B/D) cycles or under 24 h dark conditions. The aerial hyphae in the Δ*Cmwc-1* strains under both incubation conditions were much thicker than those of the wild-type under W/D and B/D cycles, but were similar to those of the wild-type under 24 h dark conditions (Fig. 2a). The growth rates of Δ*Cmwc-1* strains from both mating types were not significantly different from those of their corresponding wild-type strains (Fig. 2b). However, conidial production was significantly inhibited in all of the Δ*Cmwc-1* strains (Fig. 2c). Similar phenotypes were observed for all of the disruption strains. The conidial production in the complemented strains (*Cmwc-1c*, Fig. 2c) was restored almost to the same levels as the wild-type strains.

CmWC-1 regulates carotenoid and cordycepin production in *C. militaris*

The colors of the two wild-type strains and their Δ*Cmwc-1* strains were distinctly different. The aerial mycelia on agar plates of the Δ*Cmwc-1* strains for both mating types was snow white (1A1; color based on Kornerup and Wanscher 1978) growing under the W/D and B/D cycles, which was the same as in the wild-type strain cultured under dark conditions (Yang and Dong 2014). The reverse sides of the plates containing the two Δ*Cmwc-1* strains were very slightly colored under both W/D and B/D cycles (Fig. 2a). However, the mycelial color of the wild-type strains was deeper when grown under the B/D cycle than when grown under the W/D cycle. The reverse sides of the 40d8 and 40d26 plates were pale yellow (1A3) and yellowish white (1A2), respectively, when grown under the W/D cycle and deep yellow (4A8) and light yellow (4A4), respectively, when grown under the B/D cycle. The mycelial color of the Δ*Cmwc-1c* strain was similar to that of the wild-type (Fig. 2a). These results indicate that *Cmwc-1* plays an important role in pigment production.

We determined the carotenoid content after the strains were incubated in PDB under static conditions. It was nearly eight times lower in the selected 40d8 Δ*Cmwc-1* strain and three times lower in the 40d26 Δ*Cmwc-1* strain compared with their respective wild-type strains (Fig. 2d). The cordycepin content also decreased significantly in both Δ*Cmwc-1* strains (Fig. 2e). However, there was no statistical difference in the contents of carotenoids and cordycepin between the complemented and wild type strains, suggesting that the complemented strains restored the carotenoids and cordycepin production (Fig. 2d, e).

Gene *Cmwc-1* is involved in fruit body formation

A wild-type mating 1-1 or mating 1-2 strain can form fruit bodies without mature perithecia and ascospores after 40 days of cultivation. In contrast, both the Δ*Cmwc-1* strains formed aerial hyphae but no fruit bodies, and they retained a snow white color under W/D cycles for 40 days (Fig. 3a), resembling that of wild-types grown under dark conditions.

When mating 1-1 strain 40d8 is crossed with mating 1-2 strain 40d26, fruit bodies can develop along with ascospore production. The same results occurred when the Δ*Cmwc-1* strain was crossed with the wild-type of the opposite mating type. However, if the two Δ*Cmwc-1* strains with different mating types were crossed, there were no fruit bodies, primordia, or ascospores formed, but white mycelia developed (Fig. 3b).

Genome-wide transcriptional responses to the *Cmwc-1* deletion

To understand how *Cmwc-1* influences the growth of *C. militaris*, we examined genome-wide transcriptional responses to the *Cmwc-1* deletion under dark and light conditions (Fig. 4a) by high-throughput Illumina sequencing.

After quality filtering, we obtained 6,640,791 and 8,310,754 clean reads from the wild-type strain and 8,011,029 and 10,420,870 from the Δ*Cmwc-1*strain under dark and light conditions, respectively. Over 80 % of the reads for each sample could be mapped to the *C. militaris* genome (Zheng et al. 2011a).

To verify the transcriptome analysis results, selected gene expression levels were analyzed using the quantitative RT-PCR method. In general, transcriptional changes in most of the selected genes analyzed by qRT-PCR correlated well with the Digital Gene Expression (DGE) profiling data (in the Supplementary Material Fig. S1), despite the fold change discrepancy.

An analysis revealed that 1042 and 458 genes were significantly differentially expressed ($P<0.05$; FDR<0.001) for the wild-type and Δ*Cmwc-1* strains, respectively (Fig. 4b, in the Supplementary Material Data S1), covering 10.7 and 4.7 % of the *C. militaris* genes (in the Supplementary Material Data S1), respectively. There were 166 genes in common that were differentially expressed in response to light in the wild-type and Δ*Cmwc*-1 strains (Fig. 4c). Compared with the wild-type strain, 617 and 851 genes differed significantly under dark and light growth conditions, respectively, in the Δ*Cmwc-1* mutants (Fig. 4b, in the Supplementary Material Data S1), and there were 224 genes differentially expressed under both conditions (Fig. 4c).

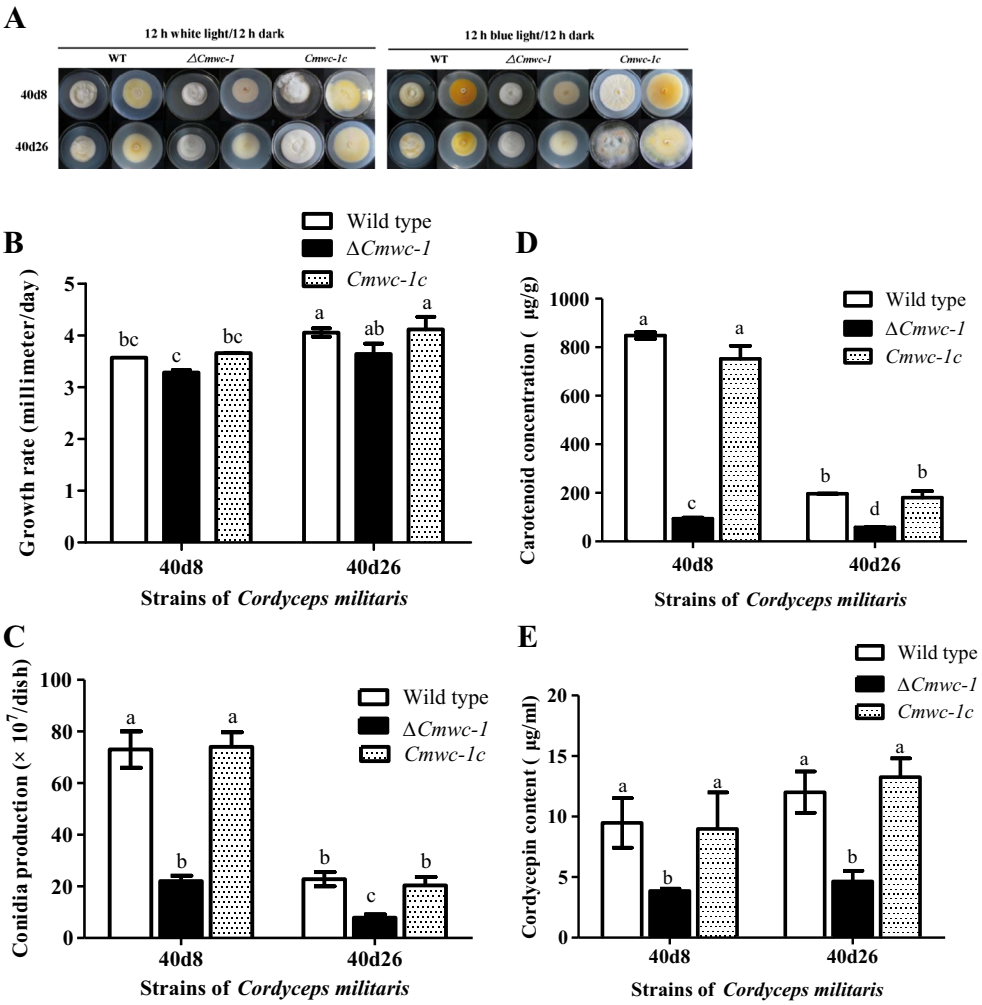

Fig. 2 Phenotypes and metabolite production in the wild-type and Δ*Cmwc-1* strains. **a** Phenotypes of the wild-type, Δ*Cmwc-1*, and complemented (*Cmwc-1c*) strains exposed to 12 h:12 h white light/dark and blue light/dark cycles. Growth was observed after incubation for 10 days at 20 °C on PDA plates. The upper and reverse sides of the plates are shown. **b** Growth rates of the wild-type and Δ*Cmwc-1* strains after the blue-light period. **c** Conidial production of the wild-type and Δ*Cmwc*-1 strains after the blue-light period. **d** Carotenoid contents of the wild-type and Δ*Cmwc-1* strains of *Cordyceps militaris*. **e** Cordycepin contents of the wild-type and Δ*Cmwc-1* strains of *C. militaris*

In addition to WC-1 and WC-2, Vivid (VVD), phytochromes (PHYs), cryptochromes (CRYs), and rhodopsins are four additional classes of fungal photoreceptors (Corrochano 2007). Genes encoding homologs of VVD, PHY, and CRY have been identified in the *C. militaris* genome (in the Supplementary Material Fig. S2). No differential expression levels of photoreceptors WC-2, VVD, CRY-1, and cyclobutane pyrimidine dimer (CPD) photolyase were detected for Δ*Cmwc-1* under dark and light conditions, whereas significant up- or down-regulation occurred for the wild-type strain (Table 2). The expression levels of WC-2, VVD, CRY-1, and CPD photolyase may be regulated by CmWC-1, but PHY and CRY-2 were not regulated by CmWC-1.

Of the TF genes identified as WCC targets in *N. crassa* (Smith et al. 2010), six orthologous genes (CCM_01467, CCM_04014, CCM_02196, CCM_07587, CCM_04849, and CCM_05610) were light-regulated in the wild-type strain (in the Supplementary Material Data S2). This response was abolished in the Δ*Cmwc-1* strain, suggesting that these six TF genes were regulated by CmWC-1 in *C. militaris*. Among these six TF genes, CCM_02196 and 07587 were Zn_2Cys_6-type TFs.

In addition to the WCC targets in *N. crassa*, the other 20 TF genes were studied. Five genes (CCM_01809, 01638, 02196, 03011, and 08260) were regulated by CmWC-1. They were expressed significantly differently after light exposure in the wild-type strain, but this was abolished in the *Cmwc-1* deletion strain (in the Supplementary Material Data S2). All five TF genes were shown to be Zn_2Cys_6-type TFs, indicating that these TFs were predominantly involved in the light reaction of *C. militaris*.

Among the enzymes involved in the cordycepin metabolism pathway (Yin et al. 2012), the expression of pyruvate kinase (CCM_05734, 07110), adenylate kinase (CCM_02335), adenosine nucleosidase (CCM_09682), and adenine deaminase (CCM_07169) were regulated by light in the wild-type strain, but there was no light effect in the deletion strains (in the Supplementary Material Data S3). As the key enzyme for the de novo synthesis of adenosine, adenylosuccinate synthase (CCM_07353) was up-regulated fourfold under light conditions in the wild-type strain but down-regulated threefold in the deletion strain. These enzymes, which are involved in cordycepin metabolism (Zheng

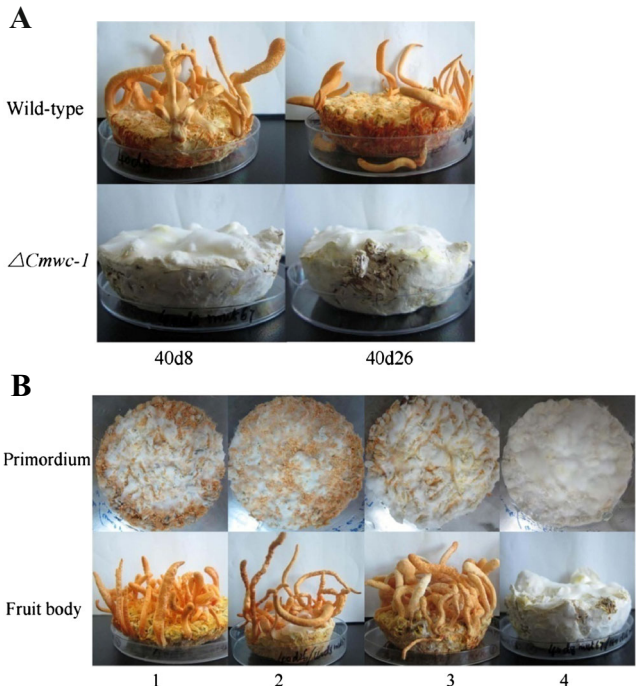

Fig. 3 Primordium formation and fruit body development from the wild-type, Δ*Cmwc-1*, and the crossing strains. **a** Fruit body development for the wild-type and Δ*Cmwc-1* strains. The wild-type strain 40d8 with *MAT1-1* and 40d26 with *MAT1-2* can form fruit bodies without mature ascospores after 40 days of cultivation, but both the Δ*Cmwc-1* strains only formed white aerial hyphae without fruit body. **b** Primordium formation and fruit body development from crossing the wild-type and Δ*Cmwc-1* strains. *1* 40d8×40d26; *2* 40d8Δ*Cmwc-1*×40d26; *3* 40d8×40d26Δ*Cmwc-1*; and *4* 40d8Δ*Cmwc-1*×40d26Δ*Cmwc-1*. Wild-type strain 40d8 is crossed with 40d26, and fruit bodies can develop along with perithecia and ascospore production (40d8×40d26). The same results occurred when the Δ*Cmwc-1* strain was crossed with the wild type of the opposite mating type (40d8Δ*Cmwc-1*×40d26; 40d8×40d26Δ*Cmwc-1*). There were no fruit bodies, primordia, or ascospores formed when the two Δ*Cmwc-1* strains with different mating types were crossed (40d8Δ*Cmwc-1*×40d26Δ*Cmwc-1*)

et al. 2011a), may also be regulated by CmWC-1 directly or indirectly.

A putative pheromone receptor (CCM_01499), a Pth11-like G-protein-coupled receptor (GPCR) (CCM_03015), and an STM1-like GPCR (CCM_07359) were significantly up-regulated ($P<0.05$, FDR<0.001) in the wild-type strain after illumination, but no change or down-regulation occurred in the mutated strain. Neither adenylate cyclase (CCM_06928) nor protein kinase A (PKA; CCM_03352, 01778) gene transcription was affected in wild-type and deletion strains after light irradiation.

GO function predictions and KEGG pathway analyses were performed. All of the DEGs were mapped to the GO terms in the three main categories (biological process, cellular component, and molecular function) in the GO database. In the *Cmwc-1* deletion strain, there were 33 terms with *P* values <0.05 (in the Supplementary Material Fig. S3) compared with the wild-type strain (40d8) under light conditions. To identify the major pathways affected by the deletions of specific WC-1 genes, KEGG orthologs (KOs) were identified for all of the differentially expressed transcripts in the KOBAS database (http://kobas.cbi.pku.edu.cn). There were eight and nine pathways that were significantly enriched in the *Cmwc-1* mutant under light and dark conditions, respectively (in the Supplementary Material Data S4). The steroid biosynthesis pathway was up-regulated in the *Cmwc-1* mutant under light/dark cycle conditions with the lowest *P* value (Fig. 5). Eight genes in the pathway showed significantly increased expression levels in the deletion strain compared with the wild-type under the light/dark cycle conditions. These genes included those for lanosterol synthase (CCM_09526), sterol 14-α-demethylase (CCM_03617, CCM_05535), Δ(24(24(1)))-sterol reductase (CCM_00528), sterol 24-C-methyltransferase (CCM_04684, CCM_08656), lathosterol oxidase (CCM_08632), and Δ(14)-sterol reductase (CCM_08633). Among these genes, the lanosterol synthase gene was the most up-regulated in the pathway, which should result in the production of lanosterol with a basic steroid structure.

Discussion

Light is an essential factor for fruit body development in most macrofungi; however, there are few detailed studies on their photoreceptors and photoreactions, except those using model fungi such as *N. crassa*, *Aspergillus* spp., *Fusarium* spp., *Trichoderma reesei*, and *Phycomyces blakesleeanus*. Despite the basic mechanisms involved in light perception being similar, differences in details have been observed when characterizing photoresponses in these fungal models and other fungi, especially macrofungi. The light-signaling mechanisms in unexplored fungal systems need to be investigated (Canessa et al. 2013; Fuller et al. 2013).

Many photo responses, such as photocarotenogenesis, photo induction of protoperithecia formation, phototropism of perithecial beaks, and circadian rhythmicity, depend on the light-regulated activity of the WCC formed by WC-1 and WC-2 in *N. crassa* (Chen et al. 2010). Studies on other fungi also indicate various functions of the WCC in fungal life. The WCC was found to affect the differentiation and virulence of the plant pathogen *Botrytis cinerea* (Canessa et al. 2013). The WC protein WcoA of *Fusarium fujikuroi* was not essential for photocarotenogenesis, but was involved in the regulation of secondary metabolism and conidiation (Estrada and Avalos 2008). The WC-1/2 of *S. commune* is involved in fruit body formation and protection against photo toxicity (Ohm et al. 2013). In the present study, the *Cmwc-1* deletion not only resulted in disordered fruit body development but also affected conidial production and led to a significant reduction in pigmentation as well as cordycepin production. Spore formation and secondary metabolite production were also affected

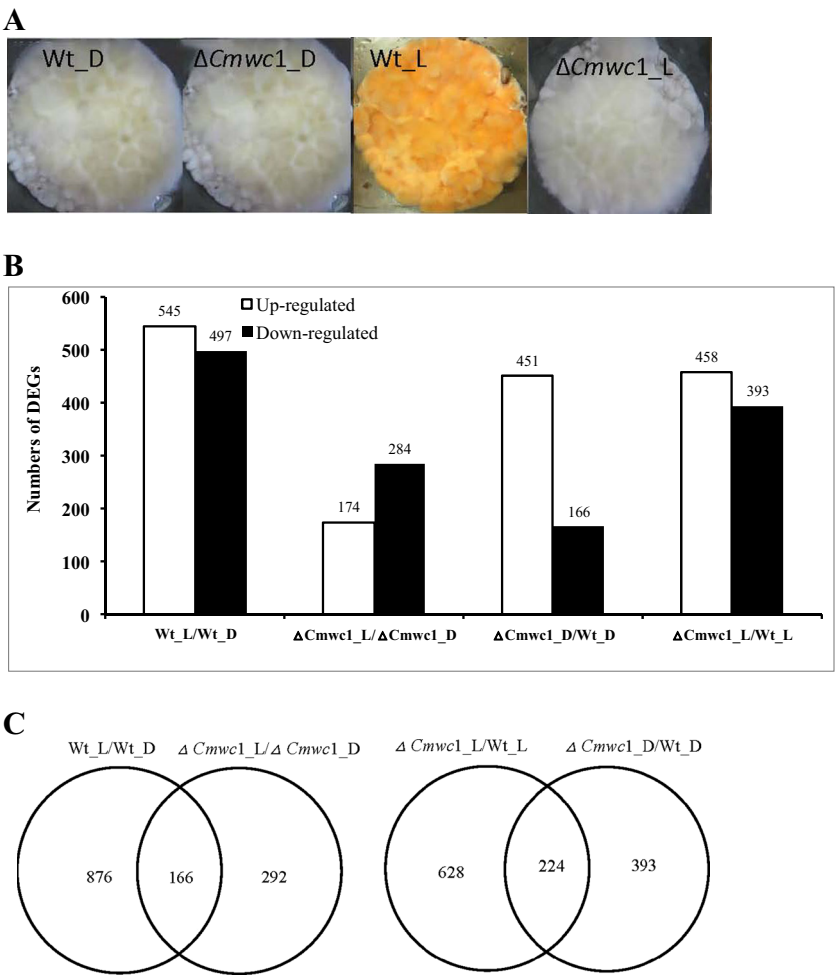

Fig. 4 Transcriptome analysis of *Cordyceps militaris* mycelia. **a** Mycelia of *C. militaris* used for transcriptome analysis. **b** Differentially expressed genes in the wild-type and Δ*Cmwc-1* strains under dark and light conditions. The number of genes differentially expressed is indicated on top of the histograms. **c** A Venn diagram illustrates the number of differentially expressed genes. Wt_D and Δ*Cmwc*_D are mycelia from the wild-type and Δ*Cmwc-1* strains, respectively, cultured in rice medium in the dark for 7 days. Wt_L and Δ*Cmwc*_L are the mycelia of wild-type and Δ*Cmwc-1* strains, respectively, cultured in rice medium under light irradiation for 3 days after 7 days in the dark

by LreA (WC-1) in *A. alternata* (Pruß et al. 2014). Those results suggested that CmWC-1 regulates growth, fruit body development, and metabolite production in fungi.

A genome-wide transcriptional analysis showed that there were 166 common genes differentially expressed in response to light in the wild-type and *Cmwc-1* deletion mutant, suggesting that additional photoreceptors could perceive a light signal other than CmWC-1. Homologs of the photoreceptors CmWC-2, VVD, PHY, and CRY (CRY-1, CRY-2 and CPD photolyase) were present in *C. militaris* (in the Supplementary Material Fig. S2). VVD, CRY-1, and CPD photolyase were strongly induced by light in a *Cmwc1*-dependent manner (Fig. 6), as in *N. crassa* (Cheng et al. 2003; Froehlich et al. 2010), while the expression levels of PHY and CRY-2 were not regulated by CmWC-1. In fact, some potential roles for other photoreceptors in the absence of WC-1 in *Trichoderma atroviride* and *Bipolaris oryzae* have been reported. The expression of two light-induced genes (*blu-4* and *blu-15*) and

Table 2 Differential expression of genes putative as photo-acceptor in the genome of *Cordyceps militaris*

Gene	Annotation	TPM_wild type		Ratio[a]	Up/down	TPM_Δ*Cmwc1*		Ratio[a]	Up/down
		Dark	Light			Dark	Light		
CCM_00072	White Collar-2	269.1	86.0	−1.6	Down	191.9	243.3	0.3	–
CCM_04514	VVD	9.1	296.3	5.0	Up	0.3	0.2	−0.5	–
CCM_00774	CRY-1	41.4	158.8	1.9	Up	21.2	16.3	−0.4	–
CCM_02392	CRY-2	18.0	30.2	0.7	–	16.3	18.5	0.2	–
CCM_00151	CPD photolyase	18.1	127.8	2.8	Up	17.35	11.2	−0.6	–
CCM_04461	PHY	31.6	25.0	−0.3	–	28.2	33.6	0.3	–

[a] Ratio was log2 (TPM_Wt_Light/TPM_Wt_Dark) and log2 (TPM_ΔCmwc1_Light/TPM_ΔCmwc1_Dark)

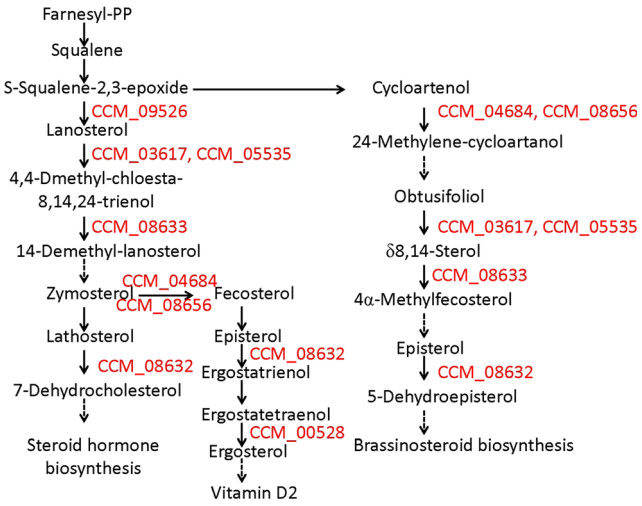

Fig. 5 Differentially expressed genes associated with a putative steroid biosynthesis pathway in Δ*Cmwc-1* mutants after light irradiation. *Red boxes* represent up-regulated genes

one light-repressed gene (*bld*-5) were independent of *blr-1* and *blr-2* in *T. atroviride* (Rosales-Saavedra et al. 2006). The *blr*-1 mutant of *B. oryzae* showed a near UV-dependent activation of the expression of certain genes (Kihara et al. 2007). The blue-light receptor LreA and red-light receptor FphA play unique and overlapping roles in regulating the photoresponsive behaviors of *A. fumigatus* (Fuller et al. 2013). Besides the WCC, which is the main fungal photoreceptor for blue light, other photoreceptors may also be involved in fungal photobiology.

Although photolyase and flavin adenine dinucleotide-binding domains occur among the three types of CRY (CRY-1, CRY-2, and CPD photolyase), their protein identities were less than 30 %. Considering the different responses to light, the three CRY proteins should have different functions. One cryptochrome gene, *cry*, was found in the *N. crassa* genome and was strongly induced by blue light in a WC-1-dependent manner (Froehlich et al. 2010). In *A. nidulans*, the CryA gene was not required for the inhibition of conidial germination (Röhrig et al. 2013) but exhibited a regulatory function during light-dependent development and DNA repair activity (Bayram et al. 2008). Three *cry* genes in *C. militaris* were targeted for our future investigations. The protein rhodopsin, composed of a retinal chromophore bound to an opsin apoprotein, was not found in the genome of this fungus.

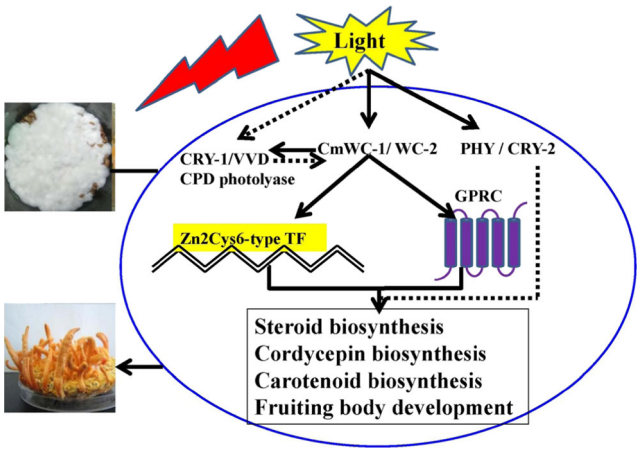

Fig. 6 Putative model for light-induced fruit body formation in *Cordyceps militaris* based on a transcriptome analysis

Both Zn_2Cys_6-type and GATA-type TFs are important for fruiting in both *A. nidulans* and *N. crassa* (Masloff et al. 2002; Pöggeler et al. 2006; Vienken and Fischer 2006), but it was found that the Zn_2Cys_6-type TFs were highly transcribed during fruiting in *C. militaris* (Zheng et al. 2011a). In the current study, 10 TF genes were significantly up- or down-regulated by CmWC-1 after irradiation of the wild-type strain but the regulation was abolished in the *Cmwc-1* deletion strain. Six were Zn_2Cys_6-type TFs, and no GATA-type TFs were found, indicating that Zn_2Cys_6-type TFs were predominantly involved in the light reaction in *C. militaris*.

Smith et al. (2010) reported that the WCC directly regulated the expression of 24 TF genes that have the potential to control downstream target genes at a second hierarchical level. Among these 24 TF genes, only six orthologous genes were regulated by CmWC-1 in *C. militaris*. These results were also verified by qPCR (data not shown). Other TF genes were not light-induced in the wild-type strain. Differences in light signal transduction between *N. crassa* and *C. militaris* might be due to their different life cycles. *N. crassa* is an obligate saprophyte that lives on dead organic material, produces conidia, and cannot attack a living host, while *C. militaris* is facultative saprophyte that requires specialized functions to infect and obtain nutrients from living insects.

A significant reduction in carotenoids and cordycepin production occurred in the Δ*Cmwc-1* strain. Six genes in the cordycepin synthetic pathway were regulated by CmWC-1, including the key enzyme for adenosine de novo synthesis, adenylosuccinate synthase (CCM_07353). Although carotenoids, including lutein, zeaxanthin, and four cordyxanthins, have been isolated from *C. militaris* fruit bodies (Yan et al. 2010; Chen et al. 2013; Dong et al. 2013), the pathway has not been studied. The blue-light induction of carotenogenesis requires the transcriptional activation of the albino genes coding for geranylgeranyl pyrophosphate synthetase, phytoene synthetase, and phytoene dehydrogenase in *N. crassa*. We have reported three types of geranylgeranyl diphosphate synthases in this fungus (Lian et al. 2014b); however, the expression of these synthases (CCM_03697, 03059, and 06355) were nearly the same as in the wild-type and Δ*Cmwc-1* strains under dark and light conditions. The orthologous gene products of two other enzymes, carotenoid oxygenase 2 (CCM_06728) and aldehyde dehydrogenase (CCM_09155), which catalyzed toluene to neurosporaxanthin in *N. crassa*, were also shown to be the same as geranylgeranyl diphosphate synthases. These

results were verified by qPCR (data not shown). However, orthologous genes of the other two key enzymes (phytoene synthetase and phytoene dehydrogenase) were not found in the genome of this fungus, indicating that the light induction of carotenoids is totally different between *N. crassa* and *C. militaris*. A challenge for future studies is to characterize the carotenoid synthetic pathway in *C. militaris*.

The involvement of the cAMP pathway in blue-light signal transduction was also reported in *N. crassa* (Belozerskaya et al. 2012) and *T. atroviride* (Casas-Flores et al. 2006). However, transcription of adenylate cyclase and PKA were not affected by light irradiation in wild-type and deletion strains, indicating that the cAMP pathway was not critical for blue-light signal transduction in *C. militaris*. Zheng et al. (2011a) also reported that *C. militaris* fruiting in the absence of a partner was more dependent on the MAPK pathway than on the cAMP-dependent PKA pathway. In addition, GPCRs that controlled fungal fruit body formation and sexual cycles, but not vegetative growth, in *Sordaria macrospora* (Pöggeler et al. 2006) were also regulated by CmWC-1 in *C. militaris*.

Steroid biosynthesis was up-regulated in the *Cmwc-1* mutant compared with the wild-type strain in light conditions and had the smallest *P* value in the dataset. Fungi are a rich source of sterol, which provides characteristic functions necessary for vegetative growth (Yuan et al. 2008). The active steroid biosynthesis in the deletion strain appeared to be required for mycelial growth. These eight genes were down-regulated in the wild-type strain under light conditions compared with dark conditions, but were up-regulated in the deletion strain after light irradiation. This suggested that CmWC-1 may suppress related gene expression and can, therefore, switch from vegetative growth to primordia differentiation.

Because of its wide distribution and economic impact, the *C. militaris* genome has been de novo sequenced to better understand its biology (Zheng et al. 2011a). The ATMT method used to obtain insertion mutants in this fungus was also established (Zheng et al. 2011b), and an antioxidant glutathione peroxidase gene from *A. nidulans* has been engineered in *C. militaris* strains (Xiong et al. 2013). However, no gene has been analyzed by disruption in this fungus until now. We successfully constructed a gene deletion mutant by homologous replacement and the ATMT method, using mating 1-1 or 1-2 strains that were isolated from a single ascospore as host strains.

We hypothesize that photoreceptors CmWC-1/2, PHY, and CRY-2 perceive the light signal in *C. militaris* after illumination (Fig. 6). The CmWC-1/2 complex activated the transcription of photoreceptors VVD and CRY-1 gene cascades. Additionally, Zn_2Cys_6-type TFs and GPRCs functioned downstream of the CmWC-1/2 complex to regulate the biosynthesis of steroids, carotenoids, and cordycepin, as well as fruit body development.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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