RESEARCH LETTER

Photo morphogenesis and photo response of the blue-light receptor gene *Cmwc-1* in different strains of *Cordyceps militaris*

Tao Yang[1,2] & Caihong Dong[1]

[1]State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; and [2]University of Chinese Academy of Sciences, Beijing, China

Correspondence: Caihong Dong, State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, NO.3 1st Beichen West Road, Chaoyang District, Beijing 100101, China.
Tel.: +86-010-64806138;
fax: +86-010-64807468;
e-mail: dongch@im.ac.cn

Received 10 December 2013; revised 7 January 2014; accepted 27 January 2014. Final version published online 14 February 2014.

DOI: 10.1111/1574-6968.12393

Editor: Stefan Olsson

Keywords
white collar-1; *Cordyceps* mushroom; light; albino; degenerate strain.

Abstract

Light is a necessary environmental factor for stroma formation and development of *Cordyceps militaris*, a well-known edible and medicinal fungus. In this study, photo morphogenesis and the blue-light receptor gene were studied using five representative strains of *C. militaris*. The results suggest that light was essential for colony pigmentation and could promote conidia production. *Cmwc-1*, the homologe of the blue-light photoreceptor of *Neurospora crassa*, was cloned from the genome of *C. militaris* by Hi-tail PCR. The protein CmWC-1 was characterized by the presence of the LOV and PAS domains and a GATA-type Znf domain. Genetic variation analysis of *Cmwc-1* in different strains showed that 15-bp deletions occurred in three strains that resulted in 5-Gln deletions in the transcription activation domain. Phylogenetic analysis based on the *Sordariomycetes* WC-1-like proteins suggested that the sequence of WC-1 could be used as a candidate marker for phylogenetic analysis in fungi. *Cmwc-1* mRNA was light inducible and the expression level increased significantly after irradiation in all tested strains. The sequence of CmWC-1 and the relative expressions responding to irradiation in degenerate and albino strains were similar as the cultivated one. This report will help to open the still-unexplored field of stroma development for this fungus.

Introduction

Cordyceps militaris (L.) Link, a well-known edible and medicinal fungus, is the type species of *Cordyceps* that generally parasitizes the larva or pupa of lepidopteron insects. Recent studies have shown its multiple pharmacological effects, including antitumor (Jin *et al.*, 2008), anti-influenza virus (Ohta *et al.*, 2007) and immunomodulatory effects (Kim *et al.*, 2007). The use of this fungal product in medicinal treatment and health foods has become popular in many countries, and it has been listed as a Novel Food by the Ministry of Health of the People's Republic of China.

Currently, the fruit bodies of this fungus have been successfully cultivated and commercialized. Light is the most important environmental factor affecting *C. militaris* stroma production (Sato & Shimazu, 2002). After exposure to light, the color of the colony changes from white to yellow or orange, and then, the primordia begin to develop. No stromata are produced in darkness.

Similar to other mushrooms, *C. militaris* frequently degenerates during continuous maintenance in culture by showing a loss in the ability to reproduce sexually or asexually (Lin *et al.*, 2010). Characteristics of degenerate strains included the loss or reduction of culture pigmentation and fruit body formation after light irradiation. Strain degeneration is the main problem in *C. militaris* cultivation and can lead to great commercial losses. These problems prompted us to study the influence of light on the growth and development of this fungus, as well as its light transduction at the molecular level.

Several fungi sense light as a signal of morphogenesis. The best-characterized model is *Neurospora crassa*, whose blue light receptor, White Collar-1 (NcWC-1), has been identified (Ballario *et al.*, 1996) and characterized (Froehlich *et al.*, 2002). NcWC-1 contains a light sensor domain called LOV (Light, Oxygen, or Voltage) and is a member of the PAS (Per-Arnt-Sim) super family (Ponting & Aravind, 1997; He *et al.*, 2002). As a transcription factor, NcWC-1 is an essential component of all known blue light responses, including mycelia carotenogenesis, phototropism of perithecial beaks and circadian rhythm of conidiation (Ballario *et al.*, 1998). The photoreceptor

FEMS MICROBIOLOGY LETTERS

orthologs BLR1 and BLR2 are known to mediate nearly all known light responses in species of the genus *Trichoderma* (Schmoll *et al.*, 2010). The blue light receptor complex, WC-1/2, is involved in the fruit body formation of *Schizophyllum commune* (Ohm *et al.*, 2012), and similar results were obtained in *Coprinopsis cinerea* (Kamada *et al.*, 2010) and *Lentinula edodes* (Sano *et al.*, 2007).

Increased attention has been paid to *C. militaris*, a valued edible fungus. In this study, we analyzed the influence of light on the growth and conidiation of this mushroom using five representative strains. Then, we cloned the full-length gene of *Cmwc-1* from *C. militaris*. CmWC-1 was possibly involved in the photoreaction and was characterized by the presence of the LOV and PAS domains and a GATA-type Znf domain. The variation of the different strains was subsequently analyzed. Finally, we also briefly described the expression of the *Cmwc-1* transcript after irradiation in different strains.

Materials and methods

Fungal strains

The strains CGMCC 5.699, 3.16321, 3.16322, 3.16323 and 3.16324 that were used in this study were maintained on potato dextrose agar at 4 °C as stocks. The identification of the strains was confirmed by morphological and molecular methods.

Genomic DNA was prepared using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987). Primers MAT1-1-1F/R, MAT1-1-2F/R and MAT1-2-1F/R (Supporting information, Table S1) were used to identify the mating type by PCR.

Fruit body characteristics of different strains

The fruit bodies of the five strains were cultivated according to the method of Zhan *et al.* (2006). The colors were observed and the lengths of fruit bodies were measured after 60 days of cultivation. Six biological replicates were used for the determination of the height of fruit bodies. Microscopic observations were carried out and photographed using a Zeiss Axioscope microscope (Zeiss, Welwyn Garden City, UK).

Influence of light on the growth and conidiation of different strains

Vegetative mycelia of *C. militaris* on solid medium were exposed to alternating, 12-h intervals of dark and light (including white and blue light, defined here as WL and BL) or kept in the dark (D). The blue light was produced by Samvol power 12-W LEDs (Light Emitting Diodes, Zhongshan, China). The distance between the LEDs and the agar plates was 50 cm. The growth of each colony was measured after 2 weeks of culture. Three biological replicates were used.

Conidiation was assessed using cultures grown for 14 days on PDA medium. Mycelia were scraped from the plate, resuspended in 10 mL of Tween 80 solution (20% w/v) and filtered. The conidia were counted using a Thoma chamber following appropriate dilution. The presented data represent six biological replicates with two technical replicates each.

Amplification of the *Cmwc-1* gene using degenerate primers and genome walking with Hi-tail PCR

The degenerate primer set Cmwc1d-F and Cmwc1d-R (Table S1) was designed based on the conserved regions of the known fungal blue light receptors. The PCR mixture contained 12.5 μL 2 × Es Taq Master Mix (CWBIO, Beijing, China), 2 μL of each degenerate primer (10 mmol L^{-1}), 1 μL of genomic DNA and 7.5 μL ddH_2O in a total volume of 25 μL. PCR amplification was performed using a thermo cycler (Bio-rad) as follows: initial denaturing at 94 °C for 3 min; followed by 30 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min.

Hi-tail PCR was used to characterize the sequences flanking the 3′ and 5′ ends of the partial *Cmwc-1* gene. The cycling conditions were followed according to the protocol of Liu & Chen (2007).

Cloning, sequencing and analysis of PCR products

The resulting PCR products were cloned into vector Ultra Power pUM-T following recommendations of the supplier (Bioteke Corporation, China) and transformed into *Escherichia coli* DH5α cells. The nucleotide sequences were sequenced by Beijing Genomics Institute (Beijing, China) and were assembled using the ContigExpress program (Invitrogen, Carlsbad, CA). The nucleotide sequences were translated into protein by EXPASY. Conserved domain analysis was performed by SMART (http://smart.embl-heidelberg.de/). The molecular weights and isoelectric points (pI) were calculated using the PROTPARAM tool (http://web.expasy.org/protparam/).

Phylogenetic analysis

Deduced amino acid sequences were aligned using CLUSTALW and manually refined using BIOEDIT 7.0. Models of sequence evolution were chosen using PROTTEST (Darriba *et al.*, 2012),

and maximum likelihood analyses were performed using PHYML (Guindon *et al.*, 2010) by running 500 bootstrap replicates. Bayesian inference was performed using MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001) for 1000 000 generations with running four chains, sampling every 100th tree and a burnin value of 2500. The phylogenetic tree was drawn using FIGTREE v.1.4.0. The blue light receptors of *Sclerotinia sclerotiorum* (XP001586924.1) and *Botryotinia fuckeliana* (XP001547999.1) were used as the out groups. The protein sequences used are listed in Table S2.

Photo response of *Cmwc-1* in *Cordyceps militaris*

Strain CGMCC 3.16222 was grown on PDA medium in the dark for 14 days, and irradiation was conducted for 15, 30, 45 and 60 min with an intensity of 500 lux. The mycelia were collected and kept at −80 °C before RNA extraction.

Total RNA was isolated from 100 mg of frozen mycelia using the TRIzol reagent (Invitrogen) and was then treated with RQ1 RNase-free DNase (Promega). cDNA synthesis was achieved using the ReverTra Ace qPCR RT Master Mix (Toyobo CO., LTD, Japan), and quantitative real-time PCR (qPCR) was performed using the Master cycler ep realplex (Eppendorf, Germany) real-time PCR System. The 25 μL qPCR reactions contained 5 ng cDNA, 0.1 μM primers and 12.5 μL QPCR SYBR Green Mix (Toyobo CO., LTD, Japan). The thermal cycling conditions were as follows: 95 °C for 1 min; followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C and 45 s at 72 °C; and 58 °C for 1 min, 58 °C to 95 °C over 20 min, and 15 s at 95 °C for the dissociation curve analyses. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

The expression levels of the different strains were compared under dark conditions and irradiation for 15 min. The presented data represent three biological replicates with two technical replicates each.

Statistical analysis

All the data obtained from this investigation were analyzed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Tukey's HSD tests ($P = 0.05$) using SPSS 10.0 (SPSS Inc.).

Results

Characterization of strains used in the study

Five strains were chosen for the study. *Cordyceps militaris* is heterothallic (Zheng *et al.*, 2011) with two mating types, i.e., Mating 1-1 and 1-2. Among the five strains, CGMCC 3.16321 and 3.16324 contained only Mating 1-1, while the others contained MAT1-1 and MAT1-2 loci (Fig. S1).

The characterizations of fruit bodies from different strains were summarized in Table 1. CGMCC 5.699 was the degenerate strain that could not produce fruit bodies, and CGMCC 3.16324 was the albino mutant with white fruit bodies. The morphology of the fruit bodies varied distinctly among the other three strains. No perithecia formed in the stroma of strain CGMCC 3.16321, whereas distinct perithecia formed in those of strains CGMCC 3.16322 and 3.16323 (Fig. S2). The fruit body of strain CGMCC 3.16322 was significantly longer than that of CGMCC 3.16323.

Influence of light on the growth and conidiation of *Cordyceps militaris*

As shown in Fig. 1a, changes in the morphology of the colonies occurred in different light conditions and strains. CGMCC 3.16324, an albino, was white in all light conditions. The upper and reverse of the plates of each strain were nearly white in the dark. However, in white or blue light conditions, five strains showed different colors, especially in the reverse of the plates. The color of the colony was much heavier in the blue-light condition than in white light except the albino. Under white- and blue-light conditions, CGMCC 5.699, a degenerate strain that

Table 1. Characterization of strains used in the study

		Fruit bodies		
Strains (CGMCC)	Mating type	Color*	Height (cm)†	Perithecium
5.699	Both MAT1-1 and MAT1-2	—‡	—	—
3.16321	MAT1-1	Orange	7.13 ± 1.01 a	No perithecium
3.16322	Both MAT1-1 and MAT1-2	Light yellow	6.25 ± 0.89 a	Distinct perithecium, heads swell slightly
3.16323	Both MAT1-1 and MAT1-2	Light yellow	3.06 ± 0.46 b	Distinct perithecium, heads swell significantly
3.16324	MAT1-1	White	3.12 ± 0.47 b	No perithecium

*Color based on Kornerup & Wanscher (1978): light yellow (3A4, 5), orange (5A6, 7) and white (1A1).

†Values are presented as the mean ± SD ($n = 6$), and values followed by the same letters are not significantly different by the ANOVA and Tukey's HSD tests ($P < 0.05$).

‡No fruit body formation (degenerate strain)

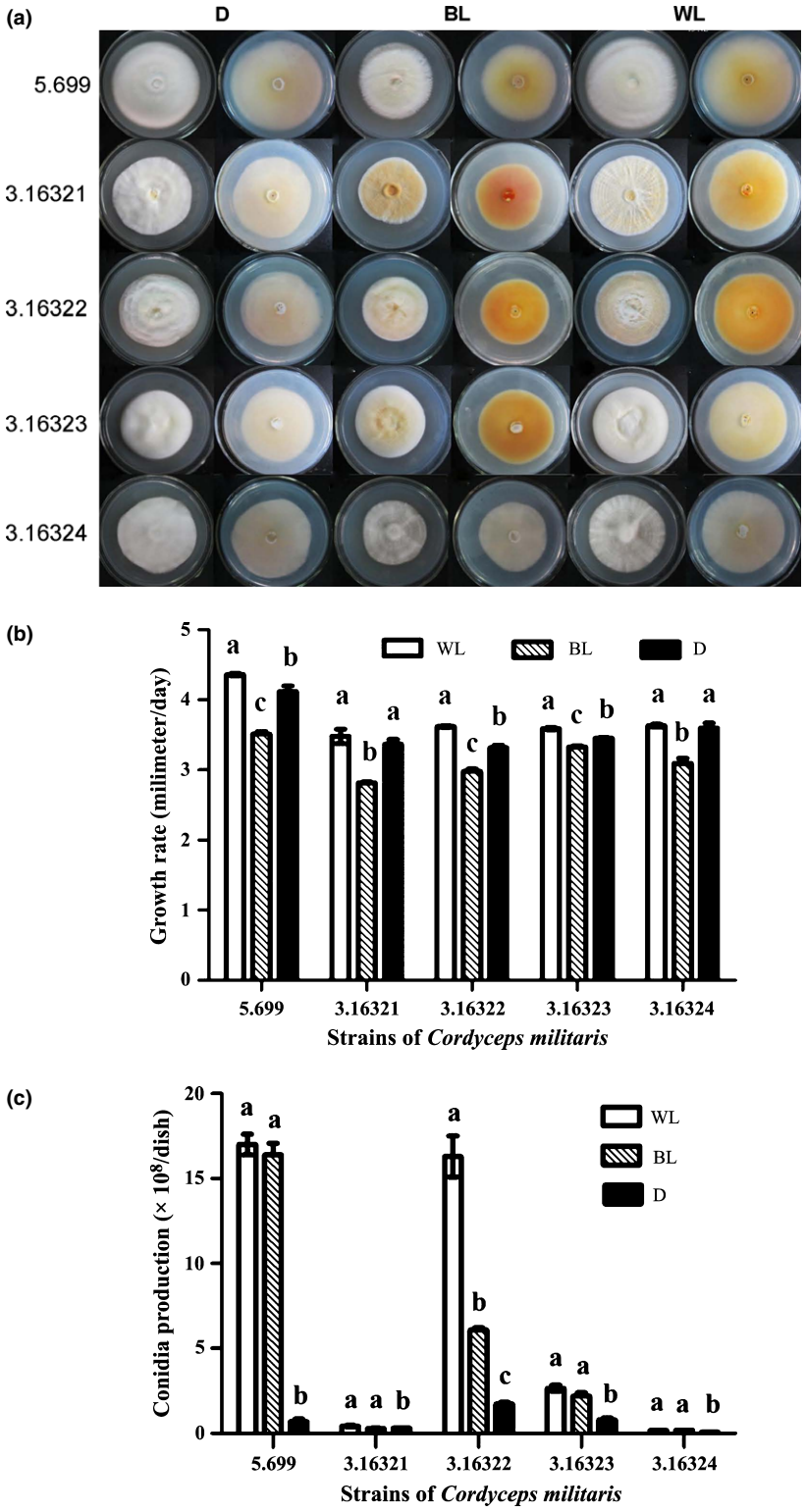

Fig. 1. Photo morphogenesis of the five strains of *Cordyceps militaris* that were used in this study. (a) Effect of light on morphology. (b) Effects of light on growth rates (c) Effects of light on conidiation. 5.699, 3.16321, 3.16322, 3.16323 and 3.16324 are the CGMCC number of the strains. Error bars in b and c indicate the standard deviation of three independent experiments. Different letters above the bars for the same strain indicate significant differences (ANOVA followed by Tukey's HSD tests, $P < 0.05$). WL, white light; BL, blue light; D, dark.

could not form a fruit body, showed the lightest color. The aerial hyphae of all strains in the dark condition were much denser than those under light conditions.

The growth rates of the five strains were determined by the diameters of their colonies (Fig. 1b). All strains presented the same trend under the three light

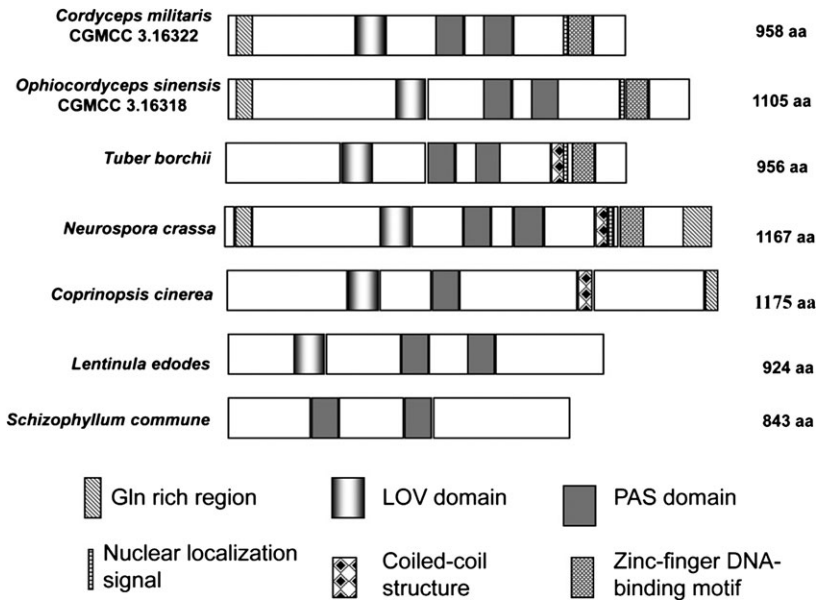

Fig. 2. Schematic representation of WC-1 from several mushrooms and *Neurospora crassa*. Conserved domains of all putative photoreceptors were determined using Pfam (http://pfam.sanger.ac.uk/search) and SMART (http://smart.embl-heidelberg.de/). The size of each protein is indicated on the right. The regions corresponding to the AD (Gln-rich region), LOV (Light-Oxygen-Voltage) and PAS (Per-Arnt-Sim) domains and the NLS (nuclear localization signal), CC (coiled-coil structure) and ZnF (zinc-finger DNA-binding motif) are shown.

conditions, white light > dark > blue light. When comparing the growth rates of the different strains under the same light conditions, CGMCC 5.699 exhibited the fastest rate.

All strains produced the fewest conidia under the dark condition and the most under white light, suggesting light could stimulate conidiation in *C. militaris*. Conidia were remarkably abundant in CGMCC 5.699 under white- and blue-light conditions, and the numbers were 100-times greater than those of the strains CGMCC 3.16321 and 3.16324 (Fig. 1c).

Amplification of the *Cmwc-1* gene using degenerate primers and genome walking with Hi-tail PCR

Using degenerate primers, a 787-bp product was amplified from *C. militaris* genomic DNA. TBLASTX analysis showed the closest homology (97%) to WC-1 of *Beauveria bassiana* (EJP60778.1) followed by that of *Hypocrea jecorina* (AAV80185.1).

The partial sequence was used for genome walking in the 5′ and 3′ directions by Hi-tail PCR. In all, we obtained

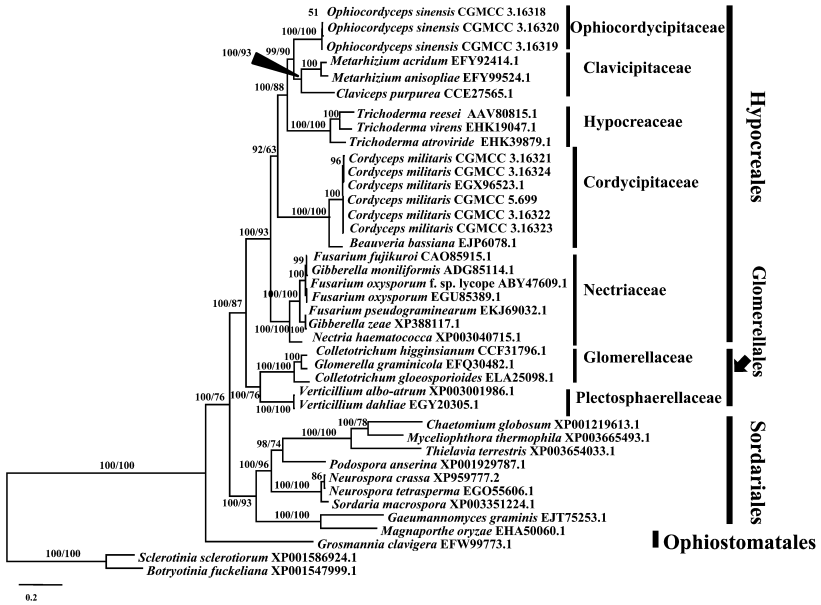

Fig. 3. Phylogenetic tree of WC -1 from *Sordariomycetes* fungi. The topology of this tree was generated using MRBAYES. The Bayesian posterior probability values as obtained by MRBAYES 3.1.1 (1000 000 generations) are shown first and are followed by the ML bootstrap values. When only one value is shown, that value represents the Bayesian posterior probability value. ML bootstrap values were obtained using PHYML under the JTT + I + G + F model with 500 bootstrap replicates. The scale bar represents 0.2 substitutions per site.

4322 bp of *Cmwc-1* sequence information. The nucleotide sequences have been deposited in GenBank under accession numbers KC702390, KC702391, KC790039, KC790040 and KC790041 for strains CGMCC 5.699, 3.16321, 3.16322, 3.16323 and 3.16324, respectively.

Analysis of the deduced amino acid sequences

The gene *Cmwc-1* of strain CGMCC 3.16322 extended for 2936 bp from its start to stop codons and included a single, 59-bp intron that was deduced from genomic and cDNA sequence comparison. The open reading frame coded for a predicted, 958-aa polypeptide, which implied that this sequence was 209 aa shorter than that of the NcWC-1. The putative molecular weights and pI values of CmWC-1 were 104.53 KD and 8.56, respectively.

Database searches using BLAST revealed that the predicted CmWC-1 protein was highly similar (68%) to NcWC-1. CmWC-1 had the N-terminal Gln-rich activation region (6–40 aa), the PAS A domain (LOV domain 310–379 aa), PAS B domain (505–571 aa), PAS C domain (619–690 aa), a nuclear localization signal (NLS 819–827 aa) and a single, putative GATA-type Znf domain (830–882 aa, Fig. 2).

Genetic diversity of *Cmwc-1* among different strains and phylogenetic analysis

The five *Cmwc-1* gene sequences from the tested strains were aligned. The complete sequences of the *Cmwc-1* genes were 2951 bp in length for strains CGMCC 3.16321 and 3.16324 but was 2936 bp for the other three strains because 15-bp nucleotides were deleted from their 5′ ends. It was interesting that the deleted nucleotides were 'GCAGCAGCAGCA' and resulted in a 5-Gln deletion in the Amino-terminus of the amino acid sequence. The CmWC-1 protein sequence was blasted against the genome of *C. militaris* CM01 (Zheng *et al.*, 2011) and it was found to exhibit 99% similarity with the GATA-type Zinc finger domain-containing protein (EGX96523.1). No evident variation in the *Cmwc-1* sequences of the degenerate and albino strains, compared with the other strains, was observed.

A phylogenetic tree was constructed using the identified protein sequences and the *Sordariomycetes* WC-1-like proteins that were retrieved from GenBank. The Jones-Taylor-Thornton (JTT) model was chosen. The two methods of Maximum Likelihood (ML) and Bayesian inference generated phylogenetic trees with the same topologies. The tree (Fig. 3) showed four major clades with relatively strong supports, each with proteins from *Hypocreales*, *Sordariales*, *Glomerellales* and *Ophiostomatales*. In the *Hypocreales* clade, five families were involved, and every family formed a sub-clade. Specifically, *Ophiocordycipitaceae* and *Cordycipitaceae* were two independent branches with 100/100 of posterior probabilities/ML bootstrap value, which was consistent with the result of multi-gene phylogenetic analysis of *Cordyceps* and the clavicipitaceous fungi by Sung *et al.* (2007).

Cmwc-1 mRNA is light inducible

Levels of the *Cmwc-1* mRNA were ascertained by real-time PCR using CGMCC 3.16322. After the selection of reference genes using the BESTKEEPER algorithms (Pfaffl *et al.*, 2004), the *tef1* gene was used as an internal control for constitutive expression (Fig. S3). A single amplification product was formed from each primer (Fig. S4). The expression level of the *Cmwc-1* gene increased significantly after irradiation for 15 min (Fig. 4a) and the same occurred in the other strains (CGMCC 5.699 and 3.16324, Fig. 4b). However, the expression level decreased as the irradiation time increased. When the irradiation time lasted for 45 min, the expression level was nearly the same as that in the dark (Fig. 4a).

Discussion

Light is essential for the development of stromata of *C. militaris*. In the study, the influence of light on the growth and conidiation of this mushroom are analyzed and the gene of *Cmwc-1*, the homologe of the blue-light photoreceptor of *Neurospora*, is cloned and analyzed.

Five strains are used according to their mating types and characterization of the fruit bodies. There are degenerate, albino and normal strains. The stromata are with distinct or without perithecium. The strains with single or double mating types are also included. Therefore the strains used in the study are representative.

Light is the essential factor for the pigment production (Fig. 1A) and can also stimulate conidiation (Fig. 1C). Blue light can inhibit the growth rate (Fig. 1C). So light is not only essential for the stroma development but also important for the asexual growth of this fungus. In fact, light can regulate the development of many ascomycete fungi such as *Paecilomyces fumosoroseus* (Sánchez-Murillo *et al.*, 2004), *Tuber borchii* (Ambra *et al.*, 2004) and *Trichoderma atroviride* (Casas-Flores *et al.*, 2004).

Motif Scan analysis shows that the WC-1 proteins from the Ascomycetes *C. militaris*, *Ophiocordyceps sinensis*, *T. borchii* and *N. crassa* have similar functional regions of highly conserved co linearity. CmWC-1 lacks the C-terminal glutamine-rich domain and a coiled-coil structure compared to NcWC-1. However, the WC-1 proteins from the Basidiomycetous mushrooms *C. cinerea*, *L. edodes* and *S. commune* are quite different. The Znf and NLS

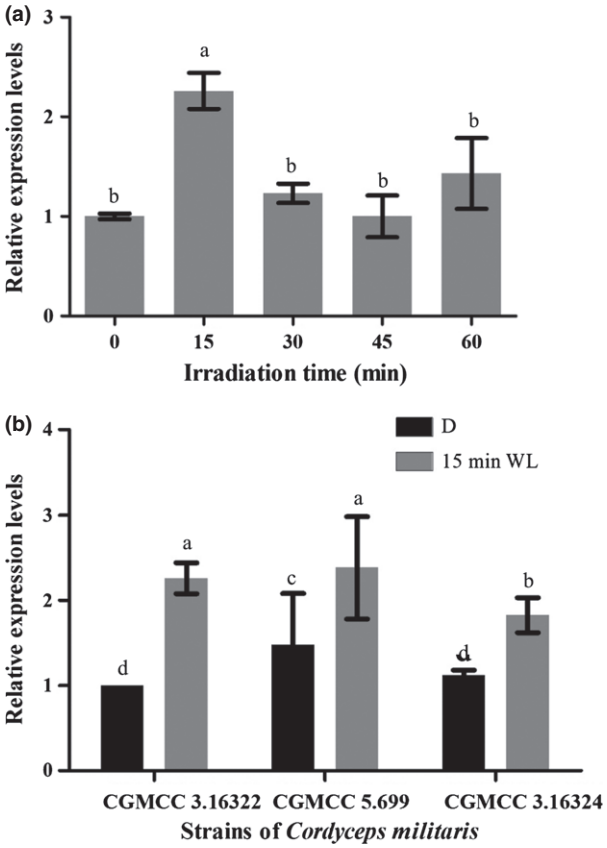

Fig. 4. Light-regulated gene expression of *Cmwc-1* revealed by quantitative real-time PCR. (a) *Cmwc-1* gene expression of strain CGMCC3.16322 after being irradiated for different time. (b) Comparison of *Cmwc-1* gene expression among different strains under dark and white light irradiation for 15 min. Error bars indicate the standard deviation of three independent experiments. Different letters above the bars for the same strain indicate significant differences (ANOVA followed by Tukey's HSD tests, $P < 0.05$).

domains are deficient. The PAS C domain is also missing in *C. cinerea* and *S. commune*. It seems that some of the domains of WC-1 have been lost during evolution.

The 5-Gln deletions in CmWC-1 proteins of three strains occur in the N-terminal Gln-rich region, which might serve as an activation domain for a transcription factor. For Gln-rich transcription activation domains, the proportion of Gln residues seems to be more important than the overall structure (McLennan *et al.*, 2012). It is unclear whether the reduction in the amount of polyglutamine repeats of CGMCC 5.699, 3.1322 and 3.16323 affects the activity of *Cmwc-1*. Phylogenetic analysis based on the *Sordariomycetes* WC-1-like proteins suggests that the sequence of WC-1 can be used as a candidate marker for phylogenetic analysis in fungi.

There is no relevant effect of light on the amounts of *wcoA* (white collar protein) mRNA in *Fusarium fujikuroi* (Estrada & Avalos, 2008), *bwc1* in the basidiomycete *Cryptococcus neoformans* (Idnurm & Heitman, 2005) and *madA* in the zygomycete *Phycomyces blakesleeanus* (Idnurm *et al.*, 2006). However, the results in this study suggest that the *Cmwc-1* mRNA is light inducible, as are the genes for other WC-1-like proteins, such as *Tbwc-1* from *T. borchii* (Ambra *et al.*, 2004) and *Oswc-1* from *O. sinensis* (Yang *et al.*, 2013). The importance of this finding is that it demonstrates a light-dependent response at the molecular level.

Compared to the cultivated strain, the color of the colony changes slowly for the degenerate strain and no change occur for the albino one after exposure to light (Fig. 1). It's unexpected that the sequences and the relative expressions respond to irradiation of *Cmwc-1* in degenerate and albino strains are similar as those of the cultivated one. It is suggested that the phenotype changes are not caused by light acceptor *Cmwc-1* directly.

The proteins of the WC family govern many light-controlled processes in fungi. We believe this study will help to open the still-unexplored field of stroma development of this important edible fungus.

Acknowledgements

This work is supported by National Natural Science Foundation of China (31100014) and the project of State key laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences.

References

Ambra R, Grimaldi B, Zamboni S, Filetici P, Macino G & Ballario P (2004) Photomorphogenesis in the hypogeous fungus *Tuber borchii*: isolation and characterization of *Tbwc-1*, the homologue of the blue-light photoreceptor of *Neurospora crassa*. *Fungal Genet Biol* **41**: 688–697.

Ballario P, Vittorioso P, Magrelli A, Talora C, Cabibbo A & Macino G (1996) White collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. *EMBO J* **15**: 1650–1657.

Ballario P, Talora C, Galli D, Linden H & Macino G (1998) Roles in dimerization and blue light photoresponse of the PAS and LOV domains of *Neurospora crassa* white collar proteins. *Mol Microbiol* **29**: 719–729.

Casas-Flores S, Ríos-Momberg M, Bibbins M, Ponce-Noyola P & Herrera-Estrella A (2004) BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*. *Microbiology* **150**: 3561–3569.

Darriba D, Taboada GL, Doallo R & Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* **9**: 772.

Doyle JJ & Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* **19**: 11–15.

Estrada AF & Avalos J (2008) The White Collar protein WcoA of *Fusarium fujikuroi* is not essential for photocarotenogenesis, but is involved in the regulation of secondary metabolism and conidiation. *Fungal Genet Biol* **45**: 705–718.

Froehlich AC, Liu Y, Loros JJ & Dunlap JC (2002) White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. *Science* **297**: 815–819.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W & Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PHYML 3.0. *Syst Biol* **59**: 307–321.

He Q, Cheng P, Yang Y, Wang L, Gardner KH & Liu Y (2002) White collar-1, a DNA binding transcription factor and a light sensor. *Science* **297**: 840–843.

Huelsenbeck JP & Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

Idnurm A & Heitman J (2005) Light controls growth and development via a conserved pathway in the fungal kingdom. *PLoS Biol* **3**: e95.

Idnurm A, Rodriguez-Romero J, Corrochano LM, Sanz C, Iturriaga EA, Eslava AP & Heitman J (2006) The Phycomyces *madA* gene encodes a blue-light photoreceptor for phototropism and other light responses. *PNAS* **103**: 4546–4551.

Jin CY, Kim GY & Choi YH (2008) Induction of apoptosis by aqueous extract of *Cordyceps militaris* through activation of caspases and inactivation of Akt in human breast cancer MDA-MB-231 Cells. *J Microbiol Biotechnol* **18**: 1997–2003.

Kamada T, Sano H, Nakazawa T & Nakahori K (2010) Regulation of fruiting body photomorphogenesis in *Coprinopsis cinerea*. *Fungal Genet Biol* **47**: 917–921.

Kim K, Kim H, Han S, Kim K, Kwon J & Lee S (2007) Immunomodulatory effects of CMDB, the component of *Cordyceps militaris* mycelium, by the enhanced production of cytokines in macrophages. *J Immunol* **178**: B64.

Kornerup A & Wanscher JH (1978) *Methuen Handbook of Colour*. Eyre Methuen, London.

Lin QQ, Qiu XH, Zheng ZL, Xie CH, Xu ZF & Han RC (2010) Characteristics of the degenerate strains of *Cordyceps militaris*. *Mycosystema* **29**: 670–677.

Liu YG & Chen Y (2007) High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *Biotechniques* **43**: 649–656.

Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and $2^{-\Delta\Delta C(T)}$ method. *Methods* **25**: 402–408.

McLennan A, Bates A, Turner P & White M (2012) *BIOS Instant Notes in Molecular Biology*. Taylor & Francis, New York.

Ohm RA, Aerts D, Wosten HA & Lugones LG (2012) The blue light receptor complex WC-1/2 of *Schizophyllum commune* is involved in mushroom formation and protection against phototoxicity. *Environ Microbiol* **15**: 943–955.

Ohta Y, Lee JB, Hayashi K, Fujita A, Park DK & Hayashi T (2007) *In vivo* anti-influenza virus activity of an immunomodulatory acidic polysaccharide isolated from *Cordyceps militaris* grown on germinated soybeans. *J Agric Food Chem* **55**: 10194–10199.

Pfaffl MW, Tichopad A, Prgomet C & Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BESTKEEPER – Excel-based tool using pair-wise correlations. *Biotechnol Lett* **26**: 509–515.

Ponting CP & Aravind L (1997) PAS: a multifunctional domain family comes to light. *Curr Biol* **7**: 11.

Sánchez-Murillo RI, Torre-Martínez M, Aguirre-Linares J & Herrera-Estrella A (2004) Light-regulated asexual reproduction in *Paecilomyces fumosoroseus*. *Microbiology* **150**: 311–319.

Sano H, Narikiyo T, Kaneko S, Yamazaki T & Shishido K (2007) Sequence analysis and expression of a blue-light photoreceptor gene, *Le.phrA* from the Basidiomycetous mushroom *Lentinula edodes*. *Biosci Biotechnol Biochem* **71**: 2206–2213.

Sato H & Shimazu M (2002) Stromata production for *Cordyceps militaris* (Clavicipitales: Clavicipitaceae) by injection of hyphal bodies to alternative host insects. *Appl Entomol Zool* **37**: 85–92.

Schmoll M, Esquivel-Naranjo EU & Herrera-Estrella A (2010) *Trichoderma* in the light of day-Physiology and development. *Fungal Genet Biol* **47**: 909–916.

Sung GH, Hywel-Jones NL, Sung JM, Luangsa-Ard JJ, Shrestha B & Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud Mycol* **57**: 5–59.

Yang T, Xiong WP & Dong CH (2013) Cloning and analysis of the *Oswc-1* gene encoding a putative blue light photoreceptor from *Ophiocordyceps sinensis*. *Mycoscience*. doi. 10.1016/j.myc.2013.09.003. (in press).

Zhan Y, Dong CH & Yao YJ (2006) Antioxidant activities of aqueous extract from cultivated fruit-bodies of *Cordyceps militaris in vitro*. *J Integr Plant Biol* **48**: 1365–1370.

Zheng P, Xia YL, Xiao GH *et al.* (2011) Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. *Genome Biol* **12**: R116.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used in this study.
Table S2. Protein sequences used in the phylogenetic analysis.
Fig. S1. Mating type of the five strains used in this study.
Fig. S2. The perithecium of *Cordyceps militaris.*
Fig. S3. Candidate reference genes for real time PCR using BESTKEEPER.
Fig. S4. Amplification efficiency of the primer pairs under real-time PCR conditions.