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Note

Cloning and analysis of the *Oswc-1* gene encoding a putative blue light photoreceptor from *Ophiocordyceps sinensis*

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ABSTRACT

Light plays an important role during both sexual and asexual growth of *Ophiocordyceps sinensis*, one of the best-known traditional Chinese medicines. In the present study, we cloned *Oswc-1*, the homologue of the blue light photoreceptor *Ncwc-1* of *Neurospora crassa*, from *O. sinensis* by Hi-tail polymerase chain reaction. The deduced amino acid sequence of *Oswc-1* contains the similar function domains as NcWC-1 including transcriptional activation, LOV (Light, Oxygen, or Voltage), PAS (Per-Arnt-Sim) and Zinc Finger domains. Phylogenetic analysis based on fungal WC-1 supported OsWC-1 was a blue light receptor. The expression of *Oswc-1* messenger RNA was up-regulated upon irradiation significantly.

Ophiocordyceps sinensis (Berk.) G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora (syn. *Cordyceps sinensis* (Berk.) Sacc.) is one of the best-known traditional Chinese medicines and health foods. The fungus parasitizes larvae of moths (Lepidoptera), turning the larvae into sclerotia, from which the fruit bodies grow out. The fungus has been used as a tonic in China for hundreds of years to invigorate lungs and nourish kidneys and has been officially classified as a drug in the Chinese Pharmacopeia (Committee of Pharmacopeia, Chinese Ministry of Health 1964).

Owing to market demand and over-exploitation, the fungus is now an endangered species (State Council of the People's Republic of China 1999). Because it is important ecologically, economically and medicinally, and also acts as a flagship species for its ecosystem, *O. sinensis* has been nominated as the national fungus of China (Zhang et al. 2012). However, the teleomorph, which is the fungal component in traditional Chinese medicine, has not yet been commercially cultivated. The major bottleneck is the low frequency of formation of stromata from artificially infected moth larvae.

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Table 1 – Primers used in this study.

Primers	Sequences (5′–3′)	Notes
Oswc1d-F[a]	GAYATGWSYTGYGCYTTYGT	Partial sequence amplification of *Oswc-1*
Oswc1d-R	SWYTCRAACCARGTRTARCC	
LAD1-1	ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA	Hi-tail PCR of both flanking sequences of *Oswc-1*
LAD1-2	ACGATGGACTCCAGAGCGGCCGCVVNVNNNCCAA	
AC	ACGATGGACTCCAGAG	
Oswc1-1-0L	TGCGATAGTTGATGAGGCTTTGCT	
Oswc1-1-1L	ACGATGGACTCCAGTCCGGCCCGTGGCGATTGTAGCCTGTGAGAT	
Oswc1-1-2L	GACAGTCGTTCATCGTGACATCGC	
Oswc1-1-0R	TGCTACTGGAAAACACGGATGACG	
Oswc1-1-1R	ACGATGGACTCCAGTCCGGCCTTTTCTCTACCTGTCGCCCTCCTG	
Oswc1-1-2R	TCATCCCTCAGACATTGTGCCG	
Oswc1-2-0L	CGCTGCATATCACTCTGGCTGTT	
Oswc1-2-1L	ACGATGGACTCCAGTCCGGCCATGGCGGACTGCGTCTGTAGTGAG	
Oswc1-2-2L	TGGCTGCGAAGTAGACTGTTGATGT	
Oswc1-2-0R	CGGAATAGGCGATAGCGAACTCT	
Oswc1-2-1R	ACGATGGACTCCAGTCCGGCCCCAGGAACTGATGCGTAAAGAGTC	
Oswc1-2-2R	GCAAGCACGAAGTCCAGAACCGA	
Oswc1-F	CTTCGCTACTCCCTTCCCGT	Full length amplification
Oswc1-R	GGGTATCAGTGAATGCCACGTT	
18S-F	CCAGGTCCAGACACAATGAGG	Quantitative real-time PCR
18S-R	GCAGACAAATCACTCCACCAAC	
Qoswc1 F	CTGGTGTCGGACTGGCACAAG	
Qoswc1 R	CGGCACAATGTCTGAGGGATG	

[a] N, R, S, V, W and Y were degenerate nucleotides. N = A or G or C or T; R = A or G; S = G or C; V = A or G or C; W = A or T; Y = C or T.

It was reported that light plays an important role during both the sexual and asexual growth of *O. sinensis*. The formation of the fruit body was induced by faint light (10–30 lx) (Tu et al. 2010). Light can promote the germination of ascospores, and sufficient light is necessary during perithecium formation and ejection of the ascospores (Li et al. 2000). Fieldwork in Kangding, Sichuan Province, has found that *O. sinensis* exhibits strong phototaxis. Light determines the length of the stromata and slows down the shriveling of the sclerotium (when the sclerotium is shriveled, its medicinal value is reduced) (Li et al. 1993).

Furthermore, it was reported that blue light increases fruit body production and carotenoid accumulation in *Cordyceps militaris*, a phylogenetic closely fungus which has been widely used as a substitute for *O. sinensis* in traditional Chinese medicine and health supplements. These results suggest that blue light has a significant impact on the production of stromata. The involvement of blue light in the formation of stromata in *O. sinensis* is supported by the following reports. The blue light receptor complex, WC-1/2 is involved in fruit body formation in the Agaricomycetes fungi *Coprinopsis cinerea* (Kamada et al. 2010) and *Schizophyllum commune* (Ohm et al. 2012). In *Neurospora crassa*, the transcription factor NcWC-1 is an essential component of all known blue light responses, including the carotenogenesis of mycelia, phototropism of perithecial beaks and circadian rhythm of conidiation (He et al. 2002). The studies described above prompted us to investigate a putative blue light photoreceptor in *O. sinensis* in order to study light transduction in this fungus at the molecular level. We succeeded in cloning *Oswc-1*, the homologue of the blue light photoreceptor gene of *N. crassa*, white collar-1 (*Ncwc-1*).

The strains used in this study, CGMCC 3.16318, 3.16319 and 3.16320, were isolated by the authors from the Tibet Plateau and maintained as a stock at 4 °C, on potato dextrose agar (PDA) supplemented with 5% wheat bran and 0.5% peptone (Dong and Yao 2005). The identity of the strains was confirmed using morphological and molecular methods. The Internal Transcribed Spacer (ITS1-5.8S-ITS2) region within nuclear ribosomal RNA (nrRNA) gene was amplified from the cultures and sequenced. These sequences were compared with a data set generated in this laboratory that contained ITS sequences from dried specimens and living strains of *O. sinensis* obtained from various regions of the Tibetan Plateau (Dong and Yao 2011).

Genomic DNA was prepared using the cetyl-, trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). PCR amplification was performed in a PTC-200 thermal cycler (Bio-Rad, Hercules, CA, USA). The resulting PCR products were cloned into the Ultra Power pUM-T vector (Bioteke Corporation, Beijing, China) and used to transform *Escherichia coli* DH5α. cDNA synthesis was performed using the PrimeScript RT-PCR Kit (TakaraBio, Dalian, China). Nucleotide sequencing was performed by the Beijing Genomics Institute (Beijing, China).

Using the degenerate primers, Oswc1d-F and Oswc1d-R (Table 1), a 787 bp product was amplified from the genomic DNA of *O. sinensis*. BLASTX analysis of the obtained sequence revealed that it shared closest homology (94%) with the white collar-1 genes of *Metarhizium anisopliae* (EFY99524.1) and *M. acridum* (EFY92414.1), followed by *Hypocrea jecorina* (AAV80185.1). The partial sequence information was used for genome walking in the 5′ and 3′ directions by the Hi-tail PCR method (Liu and Chen 2007). The nucleotide sequences have

been deposited in GenBank under accession numbers KC510674, KC510675, KC510676 for strains CGMCC 3.16318, 3.16319 and 3.16320, respectively.

The gene *Oswc-1* from *O. sinensis* extends 3391 bp from the predicted translational start site to the stop codon and includes a single 73 bp intron, whose presence was deduced by comparison of genomic and cDNA sequences. The open reading frame encodes a predicted polypeptide that is 1105 amino acids in length, which is 62 aa shorter than NcWC-1, the blue light receptor of *N. crassa*. The putative molecular weights and pI values of OsWC-1 calculated using the ProtParam tool (http://web.expasy.org/protparam) were 120 KDa and 6.84, respectively. The deduced amino acid sequences have been deposited in GenBank, under accession numbers AGK44467, AGK44468 and AGK44469 for strains CGMCC 3.16318, 3.16319 and 3.16320, respectively.

Database searches using BLAST analysis revealed that the predicted OsWC-1 protein had high similarity (54% identity) to NcWC-1 (Froehlich et al. 2002). Motif scan analysis using the program Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) showed that white collar-1 proteins from the Ascomycetes fungi *O. sinensis*, *N. crassa*, *M. anisopliae*, *Fusarium fujikuroi* and *Tuber borchii* had similar functional regions of highly conserved colinearity (Fig. 1). As with the other white collar-1 proteins, OsWC-1 had the N-terminal glutamine-rich activation region (aa 21–85), the PAS A domain (aa 415–484), the PAS B domain (aa 610–676), the PAS C domain (aa 724–795), a nuclear localization signal (NLS, aa 940–947) and a single putative GATA-type zinc finger (Znf) domain (aa 950–1001). However, unlike WC-1 from *N. crassa*, OsWC-1 lacked the C-terminal glutamine-rich region and a coiled-coil structure (Fig. 1). The PAS A, B and C motifs of OsWC-1 were 87%, 89% and 78% identical, respectively, to the corresponding PAS motifs in *N. crassa* WC-1 (XP959777).

The PAS A motif contained a light-oxygen-voltage (LOV) domain characterized by eight conserved amino acids

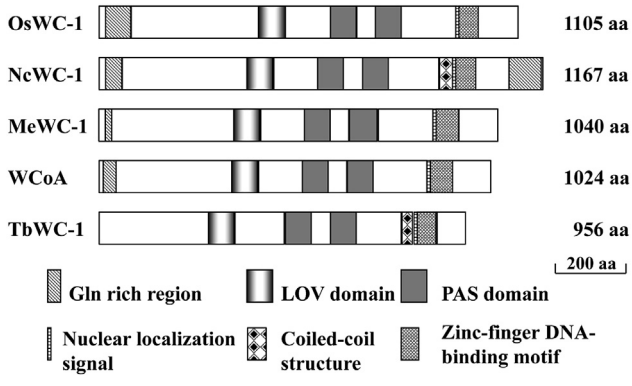

Fig. 1 – Schematic representation of members of the fungal photoreceptor families of Ascomycetes (OsWC-1, NcWC-1, MeWC-1, WCoA and TbWC-1 represent white collar-1 from *Ophiocordyceps sinensis*, *Neurospora crassa*, *Metarhizium anisopliae*, *Fusarium fujikuroi* and *Tuber borchii*, respectively). The size of each protein is shown on the right. The regions corresponding to the Gln-rich region, LOV, PAS domains, nuclear localization signal, coiled-coil structure and zinc finger DNA-binding motif are shown.

(GQNCRFLQ, aa 460–467), which included a reactive cysteine (C) that forms a covalent bond with the C (4a) carbon of a flavin upon photo excitation (Ballario et al. 1998). The Znf domain, which recognizes and binds to the consensus DNA sequence (A/T)GATA(A/G) in other genes for transcriptional activation, was well conserved and belonged to the zinc finger type IVb family (C-x2-C-x18-C-x2-C) (Teakle and Gilmartin 1998).

Nucleotide differences among the three strains were infrequent, with only 22 base changes in 3391 nucleotides (0.65%). Sixteen of these nucleotide variations resulted in amino acid changes. The translated *Oswc-1* ORF sequence was aligned with the sequences of WC-1 proteins of 34 ascomycetous fungi, retrieved from GenBank (Fig. 2). This alignment was deposited in TreeBASE (S14492, with the username wuweibuzhi2010 and password 48618121) and analyzed using a Bayesian approach (MrBayes version 3.2.1 http://mrbayes.sourceforge.net/download.php) (Huelsenbeck and Ronquist 2001). Models of sequence evolution were chosen using ProtTest (Darriba et al. 2011). A 50% majority-rule consensus tree from a Bayesian analysis of amino acids is shown (Fig. 2).

Some major groups of Ascomycota were recovered as monophyletic using WC-1 protein with significant posterior probabilities. These include the Sordariomycetes, Leotiomycetes, Eurotiomycetes, Dothideomycetes and Pezizomycetes classes. Using this analysis, 22 WC-1 proteins were identified in the Sordariomycetes class in the orders Hypocreales, Glomerellales, Sordariales and Ophiostomatales. The relationship among these four orders revealed by the WC-1 protein was consistent with the results of Réblová et al. (2011) based on multi-gene analysis. These results supported the hypothesis that OsWC-1 was a blue light receptor and suggested that the amino acid sequence of WC-1 might be a candidate marker for phylogenetic analysis of fungi at the order level.

To understand the regulation of the *Oswc-1* gene by light, levels of the corresponding mRNAs were ascertained by real-time PCR. The total RNA of *Oswc-1* was isolated in 40-day-old *O. sinensis* mycelia cultures that were grown in darkness and then irradiated for 15, 30, 45 and 60 min with an intensity of 5000 lx in an illumination incubator (PRX-450C-30, Ningbo, Zhejiang, China) using white fluorescent lamps (Philip, Eindhoven, Netherlands). The total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). Quantitative real-time PCR (qPCR) was performed with QPCR SYBR Green Mix (Toyobo CO., LTD, Osaka, Japan) using the Master cycler ep realplex (Eppendorf, Hamburg, Germany) real-time PCR System. The RNA of 18S ribosomal RNA gene (Table 1) from *O. sinensis* was used as an internal control for constitutive expression. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method. The expression level of gene *Oswc-1* increased by 4–5 fold after irradiation for 15 min (Fig. 3). However, the expression level decreased as the irradiation time increased from 30 min to 45 min. These results suggested that the *Oswc-1* mRNA was light-inducible, similar to genes encoding other WC-1-like proteins, such as *Tbwc-1* from *T. borchii* (Ambra et al. 2004), *Mcwc-1a* from *Mucor circinelloides* (Silva et al. 2006) and *wc-1* from *N. crassa*.

In conclusion, the functional domain analysis of OsWC-1 and phylogenetic analysis based on the fungal WC-1

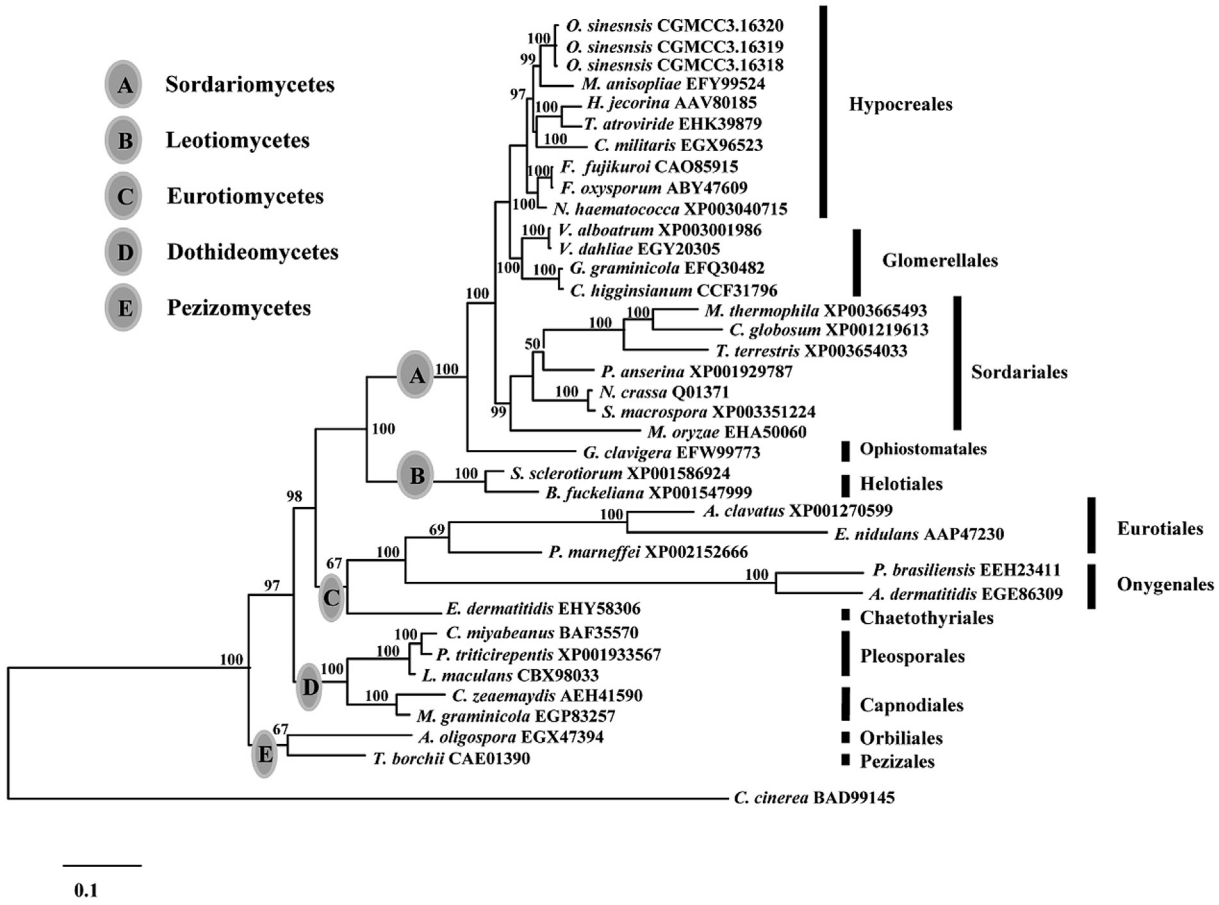

Fig. 2 – Phylogenetic analysis of the WC-1. Bayesian phylogenetic tree of WC-1 from 34 ascomycetous fungi under the JTT + I + G model (1 MrBayes run of 300,000 generations). Numbers indicate posterior probabilities.

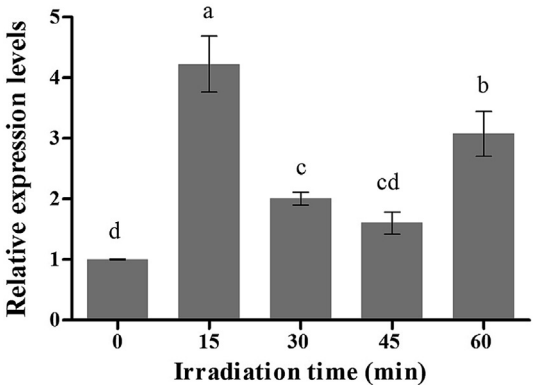

Fig. 3 – Light-regulated gene expression of *Oswc-1* revealed by quantitative real-time PCR. Mycelia were grown for 40 d in the dark and then irradiated for 15, 30, 45 and 60 min before harvesting and subjecting to RNA preparation. Relative expression was referred to the value of the mycelium in the dark. The bars represent standard deviations for four determinations from two independent experiments. Different lower-case letters above the error bars indicate significant differences at the 0.05 level (ANOVA and Tukey's HSD test).

supported the hypothesis that OsWC-1 is a blue light receptor in *O. sinensis*. *Oswc-1* mRNA was up-regulated upon irradiation. Identification of the putative blue light photoreceptor gene of *O. sinensis* should provide a basis for molecular analyses of light responses in this fungus. Identification of target genes whose expression is regulated by the complex of the OsWC-1 and its collaborator protein is the ongoing study for the future.

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