A DASH-TYPE CRYPTOCHROME GENE FROM *LENTINULA EDODES*

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ABSTRACT

Fruiting body formation of mushrooms, a characteristically morphological differentiation observed in eukaryotic microorganisms, is affected by several environmental factors such as temperature, light and humidity. Especially, light is known to be one of the most important environmental signals and influences morphogenesis. In *Lentinula edodes* (*Shiitake*), blue light is required for initiation of the fruiting body formation. Therefore, photoreceptors, which are capable of light stimuli, are important for adaptation to immediate environments. Blue light photoreceptors are generally divided into two distinct classes, phototropins and cryptochromes. We previously reported that PHRA/PHRB, a blue-light photoreceptor complex in *L. edodes*, could regulate the transcription of the tyrosinase gene in a light-dependent manner. The PHRA belongs to the phototropin family; no cryptochrome, however, has been identified in basidiomycetes.

In this study, we report the isolation of a cryptochrome-encoding gene in *L. edodes*, designated *Le.cry*. Le.CRY, the expression product of *Le.cry*, contained a DNA photolyase domain and a FAD-binding domain, and was homologous to dash-type cryptochrome proteins in the photolyase/cryptochrome family. Gene expressions of *Le.cry*, *phrA* and *phrB* were analyzed by quantitative RT-PCR during fruiting body formation (primordia, immature and mature fruiting bodies) and parts of fruiting body. The transcription levels of *phrA* and *phrB* were similar during fruiting body formation and in parts of fruiting body, whereas *Le.cry* showed the unique expression pattern. The differential expressions of *Le.cry* and *phrA/phrB* imply that Le.CRY might be involved in a light signaling pathway different from that mediated by PHRA/PHRB.

Keywords: *Lentinula edodes*; Shiitake mushroom; blue-light photoreceptor; cryptochrome dash; fruiting body formation

INTRODUCTION

Light is known to be one of the most important environmental signals for various organisms such as plant, fungi and bacteria, and regulates their developmental and physiological processes [1, 2, 3]. In many basidiomycetous mushrooms, light influence morphogenetic events and cytological events such as initiation of fruiting body formation, pileus development, oidia production, meiosis and mycelial growth. Especially, the wavelength range corresponding to blue light is one of the most important light wavelength regions [4, 5, 6]. *Lentinula edodes*, popularly called *Shiitake* mushroom, also required blue light for the initiation of fruiting body formation

and the pileus development, and complete darkness does not induce fruiting body formation and normal maturation of fruiting body.

Blue-light photoreceptors have been first identified in plant and are divided into two general classes, phototropins and cryptochromes (CRY) [7]. The most well-known blue-light photoreceptor in fungi is the phototropin-like protein White Collar-1 (WC-1) in the ascomycete *Neurospora crassa*, which is an essential component for all known blue-light responses in *N. crassa*, such as biosynthesis of carotenoid in mycelia, formation of vegetative spores and resetting of circadian clock [8]. In several basidiomycetes, WC-1 homologs were also identified in *Cryptococcus neoformans* [9], *Coprinopsis cinerea* [10] and *L. edodes* [11]. In *C. cinerea*, it was shown that a blue-light photoreceptor gene, *dst1*, is involved in fruiting body development [10]. Cryptochromes bearing no LOV domain show high similarities to microbial DNA photolyases and are widely distributed in eubacteria, archaea and eukaryotes [12, 13]. Cryptochromes belonging to the member of the photolyase/cryptochrome family exhibit no conventional photorepair activity but ultraviolet A (UV-A)/blue-light photoreceptor activity [12]. The photolyase/cryptochrome family can be classified into five distinct classes by phylogenetic and functional analyses: class I cyclobutane pyrimidine dimer (CPD) photolyase, class II CPD photolyase, plant CRY, animal CRY including (6–4) photolyases, and CRY-DASH family [13]. In ascomycetous fungi, several cryptochrome homologs ((6-4) photolyase and CRY-DASH family protein) were identified, and those functions were analyzed [14, 15, 16, 17]. However, molecular mechanism basics and signaling pathways mediated by cryptochromes are not well understood. Moreover, no cryptochrome in the basidiomycete has been reported.

This prompted us to isolate a cryptochrome gene from the basidiomycete *L. edodes* (named *Le.cry*) and to elucidate the relationship between cryptochrome and fruiting body formation by analyzing its detailed expressions in vegetatively growing mycelia and developmental tissues in the course of fruiting body formation. In this study, we demonstrate that the *Le.cry* gene play a role for mycelial development and might be involved in other light-signaling pathway during morphological differentiations, which is different from that mediated by both *phrA* and *phrB*, other blue-light photoreceptor genes in *L. edodes.*

MATERIALS AND METHODS

Fungal strains, media and culture conditions. A commercial dikaryotic (binucleate-celled) strain *L. edodes* A567 (Akiyama-Shukin Corporation, Japan) was used in the experiments. The vegetatively growing mycelia were cultured in MYG agar medium (0.4% glucose, 1% malt extract, 0.4% yeast extract and 1.5% agarose, pH 5.6) at 25°C for 40 days. Primordia, immature fruiting bodies and mature fruiting bodies were grown using on sawdust–rice bran medium. Small ball-like and hard clumps (less than 1 cm in diameter) that had not developed stipes and pilei were defined as primordia. Fruiting bodies containing no obvious pileus were defined as immature fruiting body, whereas those harboring completely developed pileus with gills were defined as mature fruiting body. After harvest, all samples were immediately frozen by liquid nitrogen and were subjected to the subsequent analyses.

Cloning of genomic DNA fragments containing the *Le.cry* gene. Genomic DNA was prepared from the *L. edodes* A567 strain according to the previous method [18]. Based on the conserved amino acid (aa) sequences of the DNA photolyase domains and the flavin adenine dinucleotide (FAD) binding domains in fungal cryptochromes, *N. crassa* CRY and *Sclerotinia sclerotiorum*

CRY1, degenerated primer DNAs were designed: 5'- GTIGGIGCIGTITGGATGACI-3' (primer A (forward)) and 5'- ICKCATRTARTCICKCCAIARIARYTC-3' (primer B (reverse)). PCR amplification was done using Blend Taq® (TOYOBO Co., Ltd.) under the standard condition. To obtain the genomic DNA fragment including the complete sequence of the *Le.cry* gene, subsequent inverse PCR was performed using TaKaRa LA Taq® (TAKARA Bio. Inc.). Two primers were designed based on the nucleotide sequences of the amplified PCR fragment: ATCCCAGACCTTCCACTCAATCTTTCCGTCGC (primer 1) and TGTCACCGCGAGACAG GCCAATGCATACATGG (primer 2). Then, the amplified genomic fragment was cloned into pTA2 vector and arbitrarily selected three clones were sequenced by ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

RNA extraction from *L. edodes.* Total RNAs were isolated from vegetatively growing mycelia, primordia, immature fruiting bodies and mature fruiting bodies using RNeasy midi kit (QIAGEN), according to the manufacturer's instructions.

Cloning of full-length cDNA of the *Le.cry* gene. To clarify the structure of the *Le.cry* gene, its cDNA was isolated by reverse transcriptase-PCR (RT-PCR), 5' rapid amplification of cDNA ends (RACE) and 3' RACE. One set of primers was designed based on the genomic sequences of the *Le.cry* gene: CTTCCTCCAAACCAAATCGAAGTATCAGG (primer 3) and TCGTGCGCG CTTGTCTCACC (primer 4). 3' RACE was carried out using Roche Diagnostics GmbH kit (Roche, Basel, Switzerland). Reverse transcription was done using primer C: GACCACGCGTATCGATGTCGAC$(T)_{\sim 20}$. Then, PCR with this reverse transcribed template was done using the following primers: GACCACGCGTATCGATGTCGAC (primer C') and GGATTGAGCGGGCCGTGAAGAAAGAGGGCG (primer 5). 5' RACE was carried out using the 5' RACE System (Invitrogen) according to the manufacturer's protocol. Specific primers of the *Le.cry* gene for 5'RACE were as follows: primer 4 and TGTCCCGAAGTGGTTCAAAGGA TTTTCGGAAGGAAG (primer 6). The 5' RACE and 3' RACE products were subcloned into pTA2 vector and were sequenced.

Phylogenetic analysis. The aa sequences of photolyase/cryptochrome family proteins for phylogenetic analysis were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were carried out using the ClustalW. Phylogenetic tree was constructed using the Neighbor-Joining algorithm in Molecular Evolutionary Genetics Analysis (MEGA) version 4 system.

Table 1. Primers and TaqMan Probes for quantitative RT-PCR

Target gene	Primer/probe	Sequence of oligonucleotide (5'-3')	Amplicon length (bp)	Amplification efficiency (%)
Le.cry	Forward primer	GCCTCCAATACCACCCAAAGT	117	99.9
	Reverse primer	GGTGGTCCGAGTCCACAATC		
	TaqMan MGB probe	FAM-CCTACCCAAGCTGC-MGB		
phrA	Forward primer	TTGTCTGTTCTGGCCCGAGTA	104	93.0
	Reverse primer	TCGTAGGGCCGAAGGTCTAT		
	TaqMan MGB probe	FAM-CTGCTCGACCAGACC-MGB		
phrB	Forward primer	ATCCAAGTCCACCTCCACAAC	127	97.9
	Reverse primer	GCTGGCGTTGTGGTTGAGT		
	TaqMan MGB probe	FAM-CACTTGTTGGGGTTTG-MGB		
β-actin	Forward primer	GTGCTCCTGAGGCCCTCTT	57	97.3
	Reverse primer	GGATTCCGGCACCTTCTAAA		
	TaqMan MGB probe	FAM-CAGCCTGCCTTCCT-MGB		

Analysis of gene expression by real-time PCR. Quality of total RNA was checked by Experion (Bio-Rad Laboratories, Inc.) according to the manufacture's instruction. First-strand cDNA synthesis as templates for real-time PCR was as follows. The same amounts (1 μg each) of total RNA samples were mixed with dNTP mix (each 500 μM) and Oligo $(dT)_{20}$ (500 nM), and volumes of the mixtures were up to 12 μl with distilled RNase-free water. After heated at 65°C for 5 min and quickly chilled on ice, dithiothreitol (10 μM), first-strand synthesis buffer for ReverTra Ace® (TOYOBO Co., Ltd.) and ribonuclease inhibitor (20 units, Wako) were added into the mixtures, and then the mixtures were incubated at 42°C for 2 min. After adding of ReverTra Ace® (100 units), the reaction mixtures (20 μl each) were incubated at 42°C for 60 min and the reactions were inactivated by heating at 98°C for 5 min. Real-time PCR was carried out using TaqMan® assay Fast PCR by StepOnePlus™ Real-Time PCR System (Applied Biosystems). The real-time PCR reaction was conducted in 20 μl consisting of 10 μl TaqMan® universal PCR master mix (Applied Biosystems), 1 μl of above first-strand cDNA mixture, 1 μl of gene-specific primer sets (each 10 μM), 0.5 μl of gene-specific TaqMan probes (each 5 μM) (these sequence was shown in table 1) and 7.5 μl of distilled water. The reactions were performed under the following condition: the pre-denaturing at 95°C for 20 sec (hold stage), the denaturing at 95°C for 1 sec, annealing and extension at 60°C for 20 sec (cycle stage). Expressions of genes were measured in triplicate. Furthermore, the amplified fragments were confirmed by 2.0% agarose gel electrophoresis and were detected as a single band in the predicted size (data not shown).

RESULTS AND DISCUSSION

Cloning of a cryptochrome-encoding gene in *L. edodes*. In the basidomycetous mushrooms, light affects morphological changes such as initiation of fruiting body formation, oidia production, maturation of fruiting body and hyphal aggregation [19, 20, 21, 22, 23, 24, 25, 26]. WC-1 homolog genes, *dst1* in *C. cinerea* and *phrA* in *L. edodes*, were identified as a blue-light photoreceptor-encoding gene in basidiomycetous mushrooms [10, 11], and the involvement of the *dst1* gene in fruiting body development is evidenced by genetical analyses [10]. However, the other photoreceptors such as cryptochromes, phytochromes and opsins have not yet been reported in basidiomycetous fungi. Recently, blue-light photoreceptor genes of the photolyase/cryptochrome family have been reported in several ascomycetous fungi such as *N. crassa* [15], *Aspergillus nidulans* (17), *Cercospora zeae-maydis* [14] and *S. sclerotiorum* [16]. This prompted us to investigate whether *L. edodes* has a cryptochrome-type photoreceptor and to analyze its expression in the course of fruiting-body formation and in parts of fruiting body.

Firstly, we carried out PCR using the degenerate primers and isolated the 740-bp genomic DNA fragment corresponding to the *Le.cry* gene, which encoded characteristic conserved aa sequences similar to the DNA photolyase domains and to the FAD-binding domains of DNA photolyase found in fungal cryptochromes. Based on the nucleotide sequence of the 740-bp DNA fragment, we carried out inverse PCR and identified 5.8-kbp *Bam*HI-*Bam*HI genomic fragment containing the complete structural gene of *Le.cry*. Then, its cDNA was isolated by RT-PCR and 5'/3' RACE. The *Le.cry* gene was found to encode 606 aa and to be interrupted by only one small intron (50 bp).

Comparison of Le.CRY and other fungal cryptochromes. Several domains contained in the expression product of *Le.cry* (Le.CRY) were characterized by Conserved Domain Database of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The deduced aa sequence of the Le.CRY protein contained characteristic domains for DNA photolyase (4-189 aa) and FAD-binding (260-526 aa), which were commonly found in DNA photolyases [27]. Le.CRY was homologous to the cryptochrome dash family proteins in other fungi and plant, e.g. *N. crassa* CRY (Nc.CRY) [15], *S. sclerotiorum* CRY1 (Ss.CRY1) [16] and *Arabidopsis thaliana* (At.CRY3) [28]. As described above, the photolyase/cryptochrome family constitutes five classes, and cryptochromes are further classified into three general groups on the basis of phylogenetic analysis: (1) plant cryptochrome, (2) animal cryptochrome/6-4 photolyase and (3) cryptochrome DASH [12, 27]. Plant and animal cryptochromes including 6-4 photolyase have both C-terminal photolyase-related region and carboxyl-terminal domain, whereas Le.CRY had no obvious carboxyl-terminal domain as well as other DASH-type cryptochromes [27].

To examine the evolutionary divergence between Le.CRY and other photolyase/cryptochrome family proteins, phylogenetic analysis was performed. The resulting phylogram revealed that DASH-type cryptochromes formed a monophyletic clade, which included Le.CRY (Fig. 1). Identities of the entire protein sequences between Le.CRY and Nc.CRY/Ss.CRY1 were 43.9%/48.3%. In the cases of comparing of their respective domains of

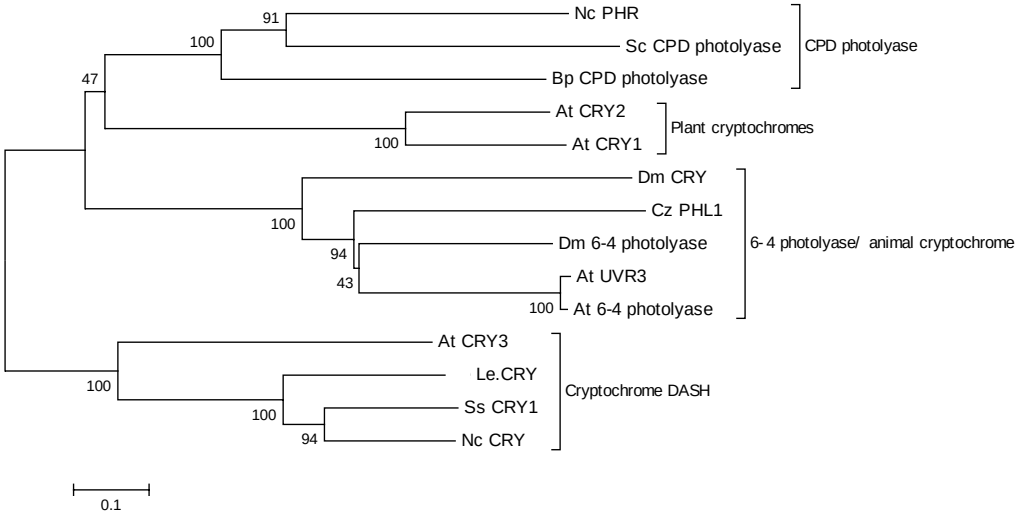

Figure 1: Phylogenetic analysis of Le.CRY and photolyase/cryptochrome families.

Protein sequences of the members in photolyase/cryptochrome family were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Organisms and accession number of proteins are as follows: Nc PHR, *Neurospora crassa*, P27526; Sc CPD photolyase, *Saccharomyces cerevisiae*, P05066; Bp CPD photolyase, *Bacillus pseudofirmus*, Q04449; At CRY1, *Arabidopsis thaliana*, S66907; At CRY2, U43397; Dm CRY, *Drosophila melanogaster*, NM_169852; Cs PHL1, *Cercospora zeae-maydis*, ACB38886; Dm 6-4, *D. melanogaster*, AY089584; At UVR3, *A. thaliana*, NM_001035626; At 6-4, *A. thaliana*, AB003687; At CRY3, *A. thaliana*, NM_122394; Ss CRY1, *Sclerotinia sclerotiorum*, XP_001593735; Nc CRY, *N. crassa*, XP_965722. A multiple sequence alignment was performed with the ClustalW. The phylogenetic tree was constructed using the Neighbor-Joining algorithm in Molecular Evolutionary Genetics Analysis (MEGA) version 4 system. Neighbor-Joining consensus tree used 1000 bootstrap replicates. The number represents the percentage of bootstrap values.

Le.CRY and Nc.CRY/Ss.CRY1, the identities of aa sequences of the DNA photolyase domain and the FAD-binding domain were 45.7%/49.7% and 55.9%/59.1%, respectively. The characteristic aa residues bound to chromophores (essential for FAD- and methenyltetrahydrofolate (MTHF)-interaction) were highly conserved [29, 30] (data not shown), implying that Le.CRY likely binds to FAD and MTHF and responds to UV-A as well as other DASH-type cryptochromes [15, 31]. In addition, it was reported that the DASH-type cryptochrome in *A. thaliana* interacts with DNA through specific six aa residues [32, 33]. Also in Le.CRY, the corresponding aa residues were completely conserved, strongly suggesting the possibility that Le.CRY has DNA-binding ability. Although Nc.CRY and Ss. CRY1, DASH-type cryptochromes in ascomycetous fungi, have glycine-rich region in the C-termini, Le.CRY lacked the corresponding region. The glycine-rich region has been found to function as protein-protein interaction domain [34, 35], protein-RNA interaction domain [35, 36] and processing signal [37]. These data suggest that the light signal transduction mechanism mediated by Le.CRY might differ from those by Nc.CRY and Ss.CRY1.

Transcription analysis of *Le.cry*, *phrA* and *phrB* during fruiting body formation. In basidiomycetous mushrooms, near UV-A wavelength or blue-light positively contributes to the initiation of fruiting body formation and the development of fruiting body [20, 21, 38]. In addition, it has been reported that the formation of sclerotia, hyphal knots and chlamydospores is inhibited in *C. cinerea* cultivated under continuous UV-A or blue light [22]. Both the blind mutant of *dst1* (the WC-1 homolog gene in *C. cinerea)* and the WC-2 homolog disruptant of *C. cinerea* revealed the aberrant development of fruiting body and the abolishment of the repression of oidia production by light [10, 20]. However, genes responsible for other photomorphogenesis such as formation of sclerotia, hyphal knots, and chlamydospores have not been identified. In addition, the action spectrum study revealed that hyphal aggregation in the basidiomycetous mushroom *Pleurotus ostreatus* is likely to be regulated by a cryptochrome-like photoreceptor [24]. These reports suggest the possible existence of a light signaling pathway different from that mediated by WC-1 and WC-2 homologs. In *L. edodes*, *phrA* and *phrB* have been isolated as the WC-1 and the WC-2 homologous genes [11, 39]. It was revealed that PHRB has sequence-specific DNA-binding activity and forms a complex with PHRA [39]. In this study, the Le.CRY protein was identified as another blue-light photoreceptor in *L. edodes*. To compare the detailed

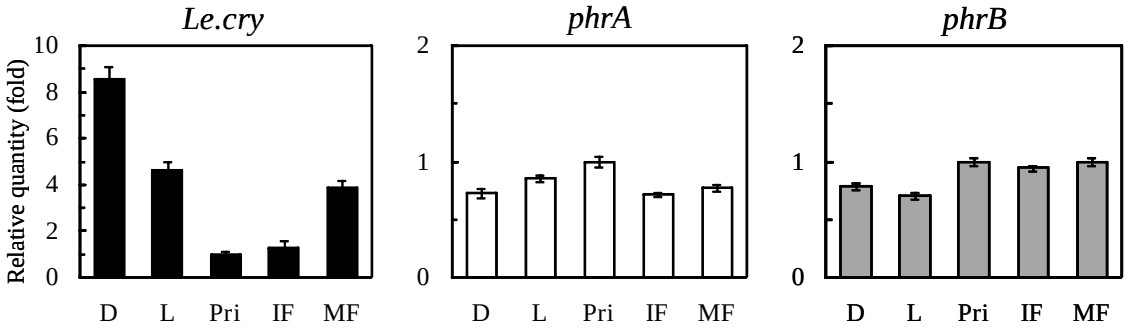

Figure 2: Quantitative RT-PCR of *Le.cry*, *phrA* and *phrB* during fruiting body formation.
D, vegetatively growing mycelia grown under continuous darkness; L, vegetatively growing mycelia grown under continuous light; Pri, primordia; IF, immature fruiting bodies; MF, mature fruiting bodies. Error bars represent standard deviations. The *actin* gene was used as an internal control.

difference of the expression of the *Le.cry*, *phrA* and *phrB* in *L. edodes* during fruiting body formation, we performed quantitative RT-PCR. The analysis was performed using a variety of *L. edodes* tissue samples, vegetatively growing mycelia cultivated under continuous light (L) or continuous darkness (D), primordia (P), immature fruiting bodies (IF) and mature fruiting bodies (MF). The *actin* gene was used as an internal control. The results revealed that the *phrA* gene and the *phrB* gene were constitutively expressed in all developmental stages. By contrast, the exceedingly highest expression of *Le.cry* was detected in vegetatively growing mycelia cultivated under continuous darkness (Fig. 2). The expression of *Le.cry* was significantly weakened during fruiting body formation, but gradually increased along with the developmental process of fruiting body. These results suggest that Le.CRY might be involved in a light signaling pathway different from that mediated by PHRA/PHRB and play a role mainly in vegetatively growing mycelium cells of *L. edodes*. In addition, it has been reported that the transcription level of the *Ss.cry1* gene in *S. sclerotiorum* is weakened in sclerotial stage, but is strengthened in vegetatively growing mycelia and the late stage of apothecia development [16]. Similarly, the *cryA* gene in *A. nidulans* (*An.cryA*) is highly expressed before sexual development and at the late stage of sexual development, and the transcription level of it decreases in the early stage of sexual development [17]. These data imply that Le.CRY might have a similar role to An.CryA and Ss.Cry1, in *L. edodes*.

Gene expressions of *Le.cry*, *phrA* and *phrB* in parts of fruiting body. The *Le.cry* gene was most abundantly expressed in mycelial stage, whereas mature fruiting bodies also contained relatively large amounts of the *Le.cry* transcript. Next, we performed quantitative RT-PCR to investigate whether the expression of *Le.cry* is different in parts of fruiting body. The fruiting body was separated into stipe (S), pileus without hymenophore (gills) (P) and hymenophore (H) (Fig. 3). Despite the fact that hymenophores contained slightly low amounts of the *phrB* transcipts, no significant difference of the expressions of *phrA* and *phrB* was detected in the investigated tissues. In contrast with *phrA* and *phrB*, the transcript of the *Le.cry* gene was more abundantly accumulated in pilei than in stipes and hymenophores. The result implies that *Le.cry* is likely to work predominantly in pilei.

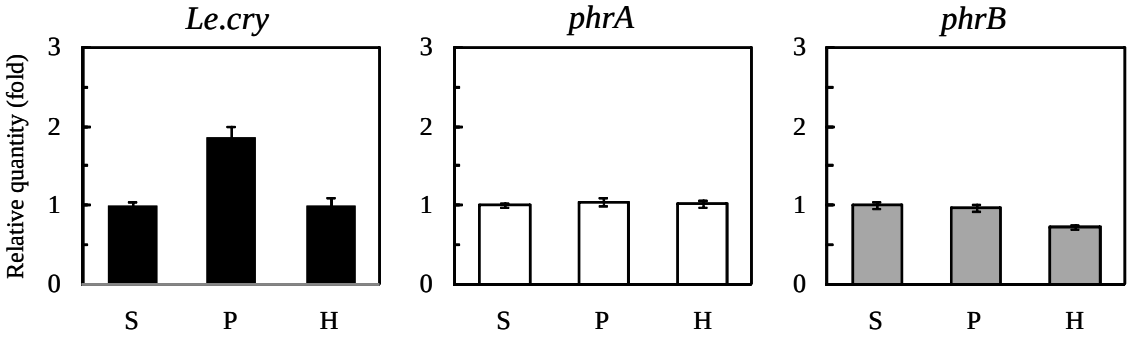

Figure 3: Transcript accumulation of the *Le.cry* gene, the *phrA* gene and the *phrB* gene in parts of fruiting body. S, stipes; P, pilei without hymenophores (gills); H, hymenophores. Error bars represent standard deviations. The *actin* gene was used as an internal control.

CONCLUSIONS

In conclusion, we identified a new blue-light receptor gene, which encodes a DASH-type cryptochrome, and analyzed transcripts of the *Le.cry* gene during fruiting body formation and in

parts of fruiting body. The mycelial stages, especially vegetatively growing mycelia cultivated under continuous darkness, contained large amounts of the transcript of the *Le.cry* gene. In addition, it was revealed that the *Le.cry* transcription appears to differ obviously from the transcriptions of *phrA* and *phrB* during fruiting body formation and in parts of fruiting body. The detail of the molecular mechanisms of light signaling pathway mediated by Le.CRY may enhance our understanding of the mechanism underlying *L. edodes* photomorphogenesis.

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