

# The *dst1* Gene Involved in Mushroom Photomorphogenesis of *Coprinus cinereus* Encodes a Putative Photoreceptor for Blue Light

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## ABSTRACT

The homobasidiomycete *Coprinus cinereus* exhibits remarkable photomorphogenesis during fruiting-body development. Under proper light conditions, fruiting-body primordia proceed to the maturation phase in which basidia in the pileus undergo meiosis, producing sexual spores, followed by stipe elongation and pileus expansion for efficient dispersal of the spores. In the continuous darkness, however, the primordia do not proceed to the maturation phase but are etiolated: the pileus and stipe tissues at the upper part of the primordium remain rudimentary and the basal part of the primordium elongates, producing “dark stipe.” In this study we genetically analyzed five strains that produce dark stipes even if light conditions promoting the maturation are given and then characterized one of them, Uar801 (*dst1-1*). The *dst1* gene was cloned as a DNA fragment that rescues the *dst1-1* mutation. Dst1 is predicted to be a protein of 1175 amino acids that contains two PAS domains, a coiled-coil structure, and a putative, glutamine-rich, transcriptional activation domain (AD). One of the PAS domains exhibits significant similarity to the LOV domains of known blue-light receptors, suggesting that Dst1 is a blue-light receptor of *C. cinereus*. The *dst1-1* mutation is predicted to truncate the putative AD in the C-terminal region.

**B**LUE light is known to be an important signal for many organisms, regulating their developmental and physiological processes. In plants, blue light has effects upon phototropism, chloroplast rearrangement, stomatal opening, hypocotyl elongation, and vegetative-to-floral transition, and several blue-light receptors, including phototropins and cryptochromes, have been identified (for reviews, see LIN 2000; CHRISTIE and BRIGGS 2001; DEVLIN 2002). In fungi, it has been found that the ascomycete *Neurospora crassa* has two blue-light receptors, White Collar-1 (WC-1) (BALLARIO *et al.* 1996; FROELICH *et al.* 2002; HE *et al.* 2002) and Vivid (VVD) (SCHWERDTFEGER and LINDEN 2003). The former is an essential component for all known blue-light responses in this fungus, including circadian rhythms, conidium formation, and carotenoid biosynthesis, while the latter is crucial for responding to daily changes in light intensity. Homologs of WC-1 have recently been identified in two other ascomycetes, *Tuber borchii* (AMBRA *et al.* 2004), *Trichoderma atroviride* (CASAS-FLORES *et al.* 2004), and in a heterobasidiomycete, *Cryptococcus neoformans*

(IDNURM and HEITMAN 2005). Phototropins, WC-1, and VVD contain the light, oxygen, and voltage (LOV) domain, which forms a subgroup of the sensory domain superfamily Per-Arnt-Sim (PAS) (TAYLOR and ZHULIN 1999) and is crucial for binding a flavin chromophore. Cryptochromes exhibit high sequence similarities to microbial DNA photolyases: they do not contain the LOV domain and have pterin in addition to flavin as chromophores (LIN 2000).

Fruiting body (mushroom) formation of the homobasidiomycete *Coprinus cinereus* is a most remarkable example of fungal development regulated by light (TSUSUÉ 1969; KAMADA *et al.* 1978; for a review, see KÜES 2000). The development is initiated as a hyphal knot of 0.2 mm or less in diameter, which rapidly develops into a tiny primordium containing the rudimentary tissues of the future pileus and stipe. The primordium gradually enlarges and then proceeds to the fruiting-body maturation phase under proper light conditions, such as the 12 hr light/12 hr dark cycle (KAMADA *et al.* 1978). In the maturation phase, basidia on the undersurface of the pileus undergo meiosis, resulting in the production of basidiospores, which is followed by stipe elongation and pileus expansion for efficient dispersal of the spores (Figure 1, top; Figure 2A). In the continuous darkness, however, the primordium does not proceed to the maturation phase but is etiolated: the pileus and stipe tissues at the upper part of the primordium remain rudimentary, and the primordial

Sequence data from this article have been deposited with the DDJB/EMBL/GenBank Data Libraries under accession no. AB195817.

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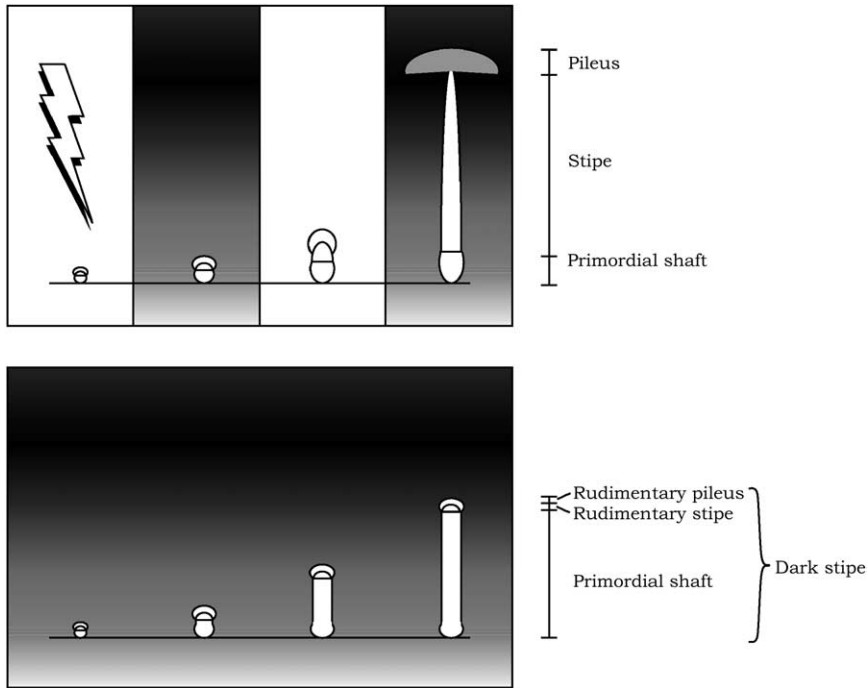


FIGURE 1.—Diagrams illustrating photomorphogenesis during fruiting-body development in *C. cinereus*. When the fungus is cultured under the 12 hr light/12 hr dark cycle, the mature fruiting body is formed. The first 5 min of light (500 lx) on the first day triggers fruiting-body maturation (top) (KAMADA *et al.* 1978). When the fungus was cultured in continuous darkness, the primordium never proceeded to the fruiting-body maturation phase. Instead, the primordial shaft, which is a basal part of the primordium, elongated, producing the dark stipe (bottom).

shaft, which is the basal part of the primordium, elongates, producing “dark stipe” (Figure 1, bottom; Figure 2B) (TSUSUÉ 1969; KAMADA *et al.* 1978). Under continuous light, the maturation starts but stops half-way: meiosis in the basidia is arrested at prophase I, the stipe elongates only slightly, and the pileus does not expand (TSUSUÉ 1969; KAMADA *et al.* 1978; LU 2000). Action spectra have been determined for the initiation of the fruiting-body primordium, for the development of the primordium into the mature fruiting body, and for the inhibition of normal progress of meiosis in *Coprinus congregatus*, a close relative of *C. cinereus*, show-

ing that UV-blue light is effective in all the processes examined (DURAND and JAQUES 1982; DURAND and FURUYA 1985). On the basis of the spectra determined, it has been hypothesized that a flavin-containing blue-light photoreceptor is responsible for mediating photomorphogenesis in *Coprinus* species (DURAND and JAQUES 1982; DURAND and FURUYA 1985). However, a photoreceptor(s) for blue light remains to be identified in homobasidiomycetes.

In this article, we first genetically analyze five photomorphogenetic mutants of *C. cinereus*, which produce dark stipes even if light conditions that normally promote the development of mature fruiting bodies are given (Figure 2C), resulting in identification of two putative gene loci, *dst1* and *dst2*, responsible for the mutations. We then characterize one of the mutations, *dark stipe1* (*dst1-1*). The *dst1* gene is predicted to encode a protein of 1175 amino acid residues, which contains two PAS domains, a coiled-coil structure, and a putative, glutamine-rich, transcriptional activation domain (AD). One of the PAS domains has high similarity to the LOV domains of WC-1, VVD, and phototropins. These results strongly suggest that Dst1 is a blue-light receptor of *C. cinereus*.

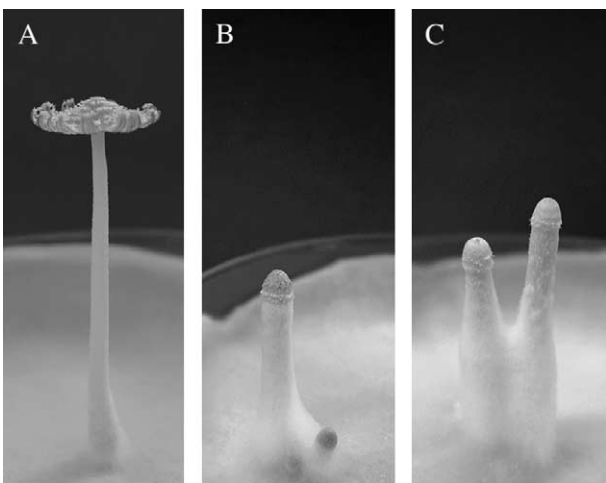


FIGURE 2.—Photographs showing a mature fruiting body developed on a culture of wild-type strain CopD5-12 under the 12 hr light/the 12 hr dark cycle (A), dark stipes formed on a culture of strain CopD5-12 grown in the continuous darkness (B), and dark stipes on a culture of the *dark stipe1* mutant, Uar801, grown under the 12 hr light/12 hr dark cycle (C).

## MATERIALS AND METHODS

**Strains, culture conditions, and genetic techniques:** Strains of *C. cinereus* listed in Table 1 were used. Malt extract/yeast extract/glucose (MY) medium (RAO and NIEDERPRUEM 1969) solidified by 2% agar in 9-cm petri dishes was used for routine mycelial cultures and for fruiting bodies for extraction of RNA. Slants of MY agar medium were used for examination of fruiting phenotypes. MY medium without agar in 9-cm petri dishes was used for mycelial cultures for extraction of DNA and

TABLE 1  
*C. cinereus* strains used in this study

Strain	Genotype/description	Source
5302	A2B2	This laboratory
5337	A8B7	This laboratory
292	A3B1 <i>trp1-1,1-6</i>	P. J. Pukkila
326	<i>AmutBmut</i> /homokaryotic fruiting	P. J. Pukkila
CopD5-12	A12B12/homokaryotic fruiting	This laboratory
Uar801	A12B12 <i>dst1-1</i> /homokaryotic fruiting	This laboratory
T140	A3B1 <i>trp1-1,1-6 dst1-1</i> /a progeny of Uar801 × 292	This study
R1428	<i>AmutBmut dst1-2</i> /a REMI mutant from 326	M. E. Zolan
R1428F <sub>1</sub> #72	A8B7 <i>dst1-2</i> / a progeny of R1428 × 5337	This study
Uar290	A12B12 <i>dst2-3</i> /a UV mutant from CopD5-12	This laboratory
Uar351	A12B12 <i>dst2-2</i> /a UV mutant from CopD5-12	This laboratory
H1-1280	<i>AmutBmut dst2-1</i> /a REMI mutant from 326	M. E. Zolan

RNA. MY medium was supplemented with 100 mg/liter of tryptophan for tryptophan-requiring strains. Cultures were maintained at 28° under a 12 hr light/12 hr dark regime unless otherwise stated. Crosses and isolation of basidiospore germ-lings were performed as described previously (INADA *et al.* 2001).

**Chromosome IX-specific cosmid library screening, the *dst1* gene cloning, and sequencing:** A cosmid library of chromosome IX from the wild-type homokaryon, 5302, was constructed as described by ZOLAN *et al.* (1992). The vector (LLC5200) contains the *trp1* gene of *C. cinereus* as a selectable marker (PUKKILA and CASSELTON 1991). The library was composed of 672 clones (96 clones × 7 plates). Groups of 12 cosmid clones were cultured on a plate of Luria broth/ampicillin solid medium, mixed, and subjected to mini prep. Pooled DNAs were used to transform strain T140 (*A3B1 trp1-1,1-6 dst1-1*) as described by BINNINGER *et al.* (1987). Transformants were cultured on minimal medium to purify the transformed mycelium and then mated to R1428F<sub>1</sub>#72 (*A8B7 dst1-2*). The resulting dikaryons were cultured on slant medium to test for fruiting phenotypes. Of 140 *trp*<sup>+</sup> transformants by a single pool of DNA, 1A, 6 produced wild-type fruiting bodies. Subsequent sib-selection revealed that a single cosmid clone, 1A1, has the rescuing activity.

Clone 1A1 was digested with *EcoRI*, *EcoRV*, *ClaI*, *PstI*, and *BglII*, and the digests were used to transform strain T140. We found that *PstI* and *ClaI* did not destroy the *dst1* activity. Each fragment of the *PstI* digest was ligated into the *PstI* site of pGEM-7zf+ (Promega, Madison, WI) and examined for the *dst1* activity by cotransformation of strain T140 with plasmid pCc1003 carrying the *C. cinereus trp1* gene (SKRZY尼亚 *et al.* 1989). We found that a 10-kb fragment had the *dst1* activity. Further digestion of the 10-kb *PstI* fragment with *XbaI*, followed by examination of the *dst1* activity, revealed that a 6-kb *PstI-XbaI* fragment contained the whole *dst1* gene. This 6-kb fragment was ligated into pBluescript II SK(+) and sequenced from both ends.

**5'- and 3'-RACE experiments:** *dst1* cDNA was amplified using an existing cDNA library synthesized with the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA) by MURAGUCHI and KAMADA (1998) as a template. The gene-specific primers for the 5' and 3' RACE experiments are listed in Table 2 and their sites in the *dst1* gene are shown in Figure 4A. PCR was performed with the Advantage cDNA PCR kit (CLONTECH) and the PCR products were cloned into pCR 2.1 vector with the TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced.

**Southern analysis:** DNA was transferred to Hybond N (Amersham, Arlington Heights, IL) according to SAMBROOK *et al.* (1989). The Gene Images system (Amersham) was used for probe labeling and detection.

**Northern analysis:** Total RNAs were prepared from vegetative mycelia, fruiting-body tissues at various developmental stages, and dark stipes. Vegetative mycelia in MY liquid medium were harvested on a nylon filter, washed thoroughly with distilled water, and squashed between sheets of filter paper. Fruiting body tissues were harvested from cultures grown under a 12 hr light/12 hr dark regime. Dark stipes were harvested from cultures grown in the continuous darkness for 2 days after preincubation for 8 days under a 12 hr light/12 hr dark regime. Samples harvested were frozen in liquid nitrogen and ground into fine powder with a mortar and pestle. RNA was extracted from the powder using Isogen (Nippon Gene, Toyama, Japan). About 15 µg of total RNA was fractionated by electrophoresis in 1.0% agarose formaldehyde gel, transferred to a Hybond-N<sup>+</sup> membrane (Amersham) according to SAMBROOK *et al.* (1989), and then fixed by UV crosslinking using a UV Stratilinker 1800 (Stratagene, La Jolla, CA). A part (2640 bp) of the *dst1* ORF was amplified using the 6-kb *PstI/XbaI* fragment as a template and a pair of primers, A1F1 and A5R2 (see Table 2; Figure 4A), labeled using the Gene Images system (Amersham), and used as the probe.

TABLE 2  
PCR primers used in this study

Primer name	Sequence
a1	GATGATGAACGCTGTTCTGG
a2	ACCACCCCATCTCCCAT
b1	ACGCAACAACGCCAGTTT
b2	GGGTCGTAGATCTGCGTATGA
c1	GGTGGAGCCCTTTCTACCC
c2	TTGGACCCCACTACTACC
d1	GAAGGAGCGATGGGACTCTC
d2	CTGCTCTTCCCGCAGCTACC
e1	CGCCTACACAGCAGCATT
e2	GCAGCAGCAGATGTATGGAG
A1F1	GATTACTACACGTCGACGCAGCAG
A5R2	ACCCACGGCCTCTTCAT
A4F3	GAAGGAGCGATGGGACTCTC
3DR3-2	AATCGGGAAGGAGAGAGAGC

TABLE 3

## Complementation tests between dark-stipe mutant strains

Strain	Uar801		R1428		H1-1280	
	F <sub>1</sub> #140	F <sub>1</sub> #52	Uad290	Uad351	F <sub>1</sub> #5	
Uar801F <sub>1</sub> #140		DS	WT	WT	WT	
R1428F <sub>1</sub> #52			WT	WT	WT	
Uad290				NT	DS	
Uad351					DS	
H1-1280F <sub>1</sub> #5						

WT, wild type; DS, dark stipe; NT, not tested.

**DNA isolations:** Cosmid and plasmid DNAs were isolated with the Plasmid Miniprep kit (Bio-Rad, Hercules, CA). Genomic DNA from *C. cinereus* was prepared as described by ZOLAN and PUKKILA (1986).

## RESULTS

**Genetic analysis of the dark stipe mutations:** We have obtained five *dark stipe* mutants, which produce the dark-stipe structure under the light conditions that promote the development of mature fruiting bodies in *C. cinereus* wild-type strains. Three of the mutants, Uar801, Uad290, and Uad351, were isolated from the homokaryotic fruiting strain, CopD5-12, after UV mutagenesis (MURAGUCHI *et al.* 1999), and the remaining two strains, R1428 and H1-1280, were found among hygromycin-resistant transformants of the *AmutBmut* strain after restriction enzyme-mediated integration (REMI) mutagenesis (CUMMINGS *et al.* 1999). We first carried out dominance tests on these mutants by mating them with wild-type homokaryons and leaving the resulting heterozygous dikaryons to the conditions that promote the development of mature fruiting bodies. The dikaryons all produced mature fruiting bodies, showing that the five mutations are all recessive. We then performed complementation tests in dikaryons among the five mutations, which revealed that the mutants compose two complementation groups (Table 3). The presumptive gene responsible for the mutations in one group containing strains Uar801 and R1428 was designated *dst1* (*dark stipe1*), and that in the other group containing strains Uad290, Uad351, and H1-1280 was designated *dst2* (*dark stipe2*).

**Cloning of the *dst1* gene:** If the *dst1* gene responsible for the mutation in REMI strain R1428 were tagged by the plasmid, it would be possible to perform a plasmid rescue for cloning the *dst1* gene. However, F<sub>1</sub> analysis of a cross between R1428 and the wild-type homokaryon, 5302, revealed that the *dst1* gene in R1428 was not tagged: the *dst1* mutation segregated independently from the hygromycin-resistance in the progeny (data not shown). Therefore, we chose to first map the *dst1* gene to a chromosome and then screen a cosmid library from the chromosome for a clone that complements the

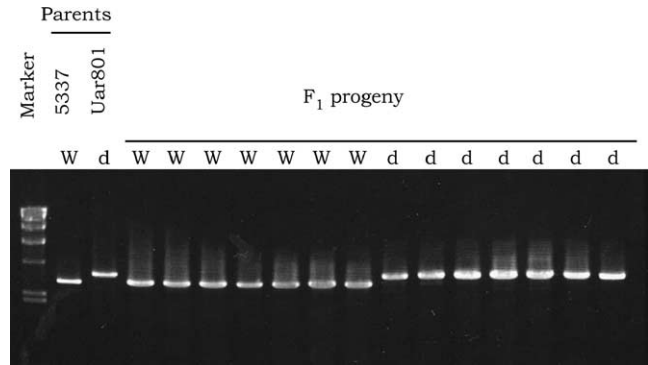


FIGURE 3.—Cosegregation between length polymorphism of a 3-kb region in 1A1 and the dark-stipe phenotype in F<sub>1</sub> progeny from the cross Uar801 × 5337. The progeny were scored for fruiting phenotypes. A selection of the dark-stipe mutant and wild-type progeny were then scored for length polymorphism of a 3-kb genomic region in 1A1 amplified using primers A4F3 and 3DR3-2 (see Figure 4A). W, wild type; d, dark stipe. Marker, the *Hind*III digest of λDNA.

mutation. We crossed another *dst1* mutant strain, Uar801 (*A12B12 dst1-1*), with the wild-type homokaryon, 5302 (*A2B2*), and analyzed its F<sub>1</sub> progeny. We found that the *dst1* gene is linked to Hiroshima VII-1#58, an RFLP marker on chromosome IX (ARIMA *et al.* 1996), at a map distance of 25% (4/16). We also confirmed that the dark-stipe phenotype was caused by mutation in a single chromosomal gene: of 186 progeny analyzed, 86 exhibited the wild-type phenotype and 100 the dark-stipe phenotype (*P* for 1:1 = 0.50–0.30).

On the basis of the above result suggesting that the *dst1* gene is located on chromosome IX, we constructed a cosmid library from chromosome IX and screened it for a clone that complements the *dst1* mutation. We found that the *dst1* mutation was rescued in 32% (112/346) of *trp*<sup>+</sup> transformants by DNA from cosmid clone 1A1. As a negative control, we transformed strain T140 (*A3B1 trp1-1, 1-6 dst1-1*) with plasmid pCc1003 carrying the wild-type *trp1* gene: all 598 *trp*<sup>+</sup> transformants isolated showed the dark-stipe phenotype. We scored 16 progeny from a cross between Uar801 and the wild-type homokaryon, 5337, for length polymorphism of a 3-kb fragment in clone 1A1, showing that the 1A1 region is closely linked to the *dst1* locus (Figure 3). We then defined the *dst1* region within a 6-kb *Pst*I-*Xba*I fragment by digestion of clone 1A1 with various restriction enzymes, followed by examination of each digest for the *dst1* activity.

**The *dst1* gene encodes a putative blue-light receptor:** Genomic DNA sequencing, together with 5'- and 3'-RACE experiments, identified an ORF interrupted by a single intron of 68 nucleotides (nt), which encodes 1175 amino acids (Figure 4A). The 5' splice site, GTACGT, agrees with the consensus sequence GTRNGT found for filamentous fungi and the 3' splice site, CAG, with the consensus sequence YAG (GURR *et al.* 1987). 5'-RACE

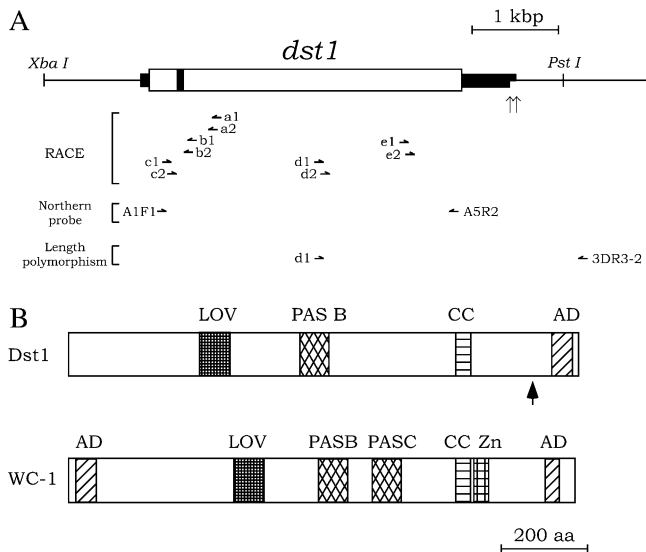


FIGURE 4.—(A) Structure of the *dst1* gene. The thick lines, open box, and solid box indicate the regions transcribed, a deduced ORF, and the intron, respectively. Two *dst1* transcripts with distinct poly(A) binding sites were identified by 3'-RACE experiments. Vertical arrows indicate the 3'-ends of the *dst1* transcripts. The primers for 5'- and 3'-RACE experiments, for the Northern probe, and for length polymorphism are indicated by horizontal half-arrows. (B) Schematic alignment of domains in Dst1 and WC-1. WC-1 is a blue-light receptor in *N. crassa* (BALLARIO *et al.* 1996). The vertical arrow beneath the Dst1 protein indicates the site of the *dst1-1* mutation, which truncates AD in the C-terminal region.

experiments showed that the *dst1* mRNA has a 113-nt 5'-untranslated region, while 3'-RACE experiments identified two kinds of transcripts with distinct poly(A) sites, 3'-untranslated regions of which are predicted to be 564 and 629 nt, respectively (Figure 4A). Database searches using the BLAST procedure (ALTSCHUL *et al.* 1990) revealed that the predicted Dst1 protein has a high similarity to WC-1 (BALLARIO *et al.* 1996), a blue-light receptor of *N. crassa* (FROEHLICH *et al.* 2002; HE *et al.* 2002). Motif Scan analysis using the program Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>) showed that, like WC-1, Dst1 has the PAS A domain (LOV domain), the PAS B domain, a coiled-coil structure, and the C-terminal glutamine-rich region, but unlike WC-1, it lacks the PAS C domain, a zinc-finger DNA-binding motif, and the N-terminal glutamine-rich activation region (Figure 4B). It was noted that the LOV and PAS B domain of Dst1 are highly similar to those of WC-1, respectively (Figure 5). Although SMART did not identify the PAS C domain in Dst1, manual comparison revealed a low similarity (11.9% identify) between the PAS C region in WC1 and the corresponding region in Dst1.

***dst1-1* mutant allele:** We performed PCR amplifications on the genomic DNA of the *dst1-1* mutant using primers that were designed on the basis of the wild-type *dst1* sequence and sequenced the PCR products directly.

Comparison of the *dst1-1* mutant allele with the wild-type *dst1* gene of the parental CopD5-12 strain as well as with that of strain 5302, from which the cosmid library was constructed, revealed a one-nucleotide substitution (T to G), which yields a stop codon (TGA) at 3200–3202 bp downstream from the translational start site, truncating the C-terminal 109 amino acids.

**Developmental regulation of *dst1* transcription:** Total RNAs from various development phases of mycelia and tissues were subjected to Northern analysis for examination of *dst1* transcription. As expected from cDNA analysis, we identified a transcript at ~4.3 kb in all mycelia and tissues examined (Figure 6). The transcription of *dst1* was higher in fruiting-body primordia than in vegetative mycelia (Figure 6, lanes 1 and 2). The transcription level was also higher in young fruiting bodies, and the level was higher in young stipes as compared with that in young pileus (Figure 6; lanes 3–5). The level of *dst1* transcription was also high in the dark stipe (Figure 6, lane 6). The results that the primordia and dark stipes exhibited high levels of *dst1* transcription are explainable, because both have to regulate their development in response to faint light (KAMADA *et al.* 1978). However, we cannot give any explanation for a high level of *dst1* transcription in young fruiting bodies at present, because young fruiting bodies in this developmental stage undergo stipe elongation and pileus development irrespective of light conditions (KAMADA *et al.* 1978).

## DISCUSSION

In this study, we characterized a photomorphogenetic mutant, *dst1-1*, of *C. cinereus*. The mutant produces dark stipes under light conditions that normally promote the development of fruiting-body primordia into mature fruiting bodies. Because the dark stipe is the figure that the primordium forms when left in the dark, it seems that the mutant is defective in perception of light. *dst1*, the gene responsible for the mutation, was found to encode a protein (Dst1) containing a LOV domain, which has strong similarities to those of the blue-light receptors in *N. crassa*, WC-1 and VVD, and of the blue-light receptors in plants, phototropins ZTL and FKF (Figure 5A). It has been demonstrated in the fern *Adiantum capillus-veneris* that the 11-amino-acid residues in the LOV2 domain of PHY3 interact with its chromophore, flavin mononucleotide (FMN) (CROSSON and MOFFAT 2001). Also, it has been shown in *N. crassa* that 7 of the 11 residues are conserved in WC-1 and that amino acid replacement at any of 4 residues (429, 430, 432, and 471) from among the 7 residues completely eliminates blue-light responses in this fungus (CHENG *et al.* 2003b). In the LOV domain of Dst1, 6 of the 11 residues, including all of those corresponding to the 4 residues, are conserved (Figure 5A). These findings strongly suggest that Dst1 is a blue-light photoreceptor in *C. cinereus*.



In addition, we failed to identify a WC-2 homolog in the draft sequence of the *C. cinereus* genome ([http://www.broad.mit.edu/annotation/fungi/coprinus\\_cinereus](http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus)). A presumptive, interacting partner of Dst1, therefore, seems to be much different in amino acid sequence from WC-2. A candidate for the interacting partner of Dst1 might be a protein encoded by another gene, *dst2*, a mutation of which causes the dark-stipe phenotype, as described above. Cloning of *dst2* is now in progress.

In the development of the fruiting-body primordium, light plays two roles. One is the inhibition of etiolation of the primordium and the other is the triggering of development of the primordium into the mature fruiting body (KAMADA *et al.* 1978). The *dst1-1* mutant is defective in the inhibition of etiolation, producing the dark stipe (Figure 2). Because the mutant Dst1 is predicted to lack the C-terminal AD, retaining other regions including LOV and PAS B domains and the coiled-coil structure, as described above, it is clear that AD is a prerequisite for the inhibition of etiolation. The *dst1-1* mutant regularly developed mature fruiting bodies at the top of dark stipes after a prolonged cultivation. It may be that the mutant Dst1 without AD still functions in the photocontrol of fruiting-body maturation. Alternatively, there may be another photoreceptor in *C. cinereus*. A future challenge would be analysis of a null *dst1* mutant as well as of *dst1* mutants in which each of the domains was disrupted.

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