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More Than a Repair Enzyme: *Aspergillus nidulans* Photolyase-like CryA Is a Regulator of Sexual Development

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Cryptochromes are blue-light receptors that have presumably evolved from the DNA photolyase protein family, and the genomes of many organisms contain genes for both types of molecules. Both protein structures resemble each other, which suggests that light control and light protection share a common ancient origin. In the genome of the filamentous fungus *Aspergillus nidulans*, however, only one cryptochrome/photolyase-encoding gene, termed *cryA*, was identified. Deletion of the *cryA* gene triggers sexual differentiation under inappropriate culture conditions and results in up-regulation of transcripts encoding regulators of fruiting body formation. CryA is a protein whose N- and C-terminal synthetic green fluorescent protein fusions localize to the nucleus. CryA represses sexual development under $UVA_{350\text{-}370\ nm}$ light both on plates and in submerged culture. Strikingly, CryA exhibits photorepair activity as demonstrated by heterologous complementation of a DNA repair-deficient *Escherichia coli* strain as well as overexpression in an *A. nidulans uvsB*Δ genetic background. This is in contrast to the single deletion *cryA*Δ strain, which does not show increased sensitivity toward UV-induced damage. In *A. nidulans*, *cryA* encodes a novel type of cryptochrome/photolyase that exhibits a regulatory function during light-dependent development and DNA repair activity. This represents a paradigm for the evolutionary transition between photolyases and cryptochromes.

INTRODUCTION

Cryptochromes (CRYs) are blue-light receptors that regulate growth, development, and the circadian clock in higher eukaryotes and that are believed to have evolved from the DNA photolyase protein family (Daiyasu *et al*., 2004; Lin and Todo, 2005). Photolyases, in contrast, repair UV-induced DNA damage by using a mechanism referred to as photorepair. They absorb light in the blue spectrum and transfer an excited electron from the cofactor FAD to an enzyme-bound *c*yclobutane *p*yrimidine *d*imer (CPD), which is thereby cleaved (Sancar, 1990, 1994; Sancar, 1994). Cryptochromes are characterized by an N-terminal *ph*otolyase-*r*elated (PHR) region without significant photorepair activity and by a C-terminal domain of varying length, which is absent in members of the CRY-DASH subfamily (Daiyasu *et al*., 2004). The exact function of the CRY-DASH proteins was elusive; however, recent data imply that they are actually photolyases that specifically repair CPDs in single-stranded DNA (Selby and Sancar, 2006). The PHR domain is the most conserved region of the CRY proteins and contains the cofactor FAD required for electron transfer reactions. In *Xenopus* and *Drosophila*, the PHR domain is physiologically active even in the absence of the C-terminal domain. The C-terminal domain is important for either localization or protein stability, and its expression in *Arabidopsis* results in constitutive growth (Lin and Todo, 2005). Plant CRYs are phosphorylated under illumination, and gene expression control ranges from protein interactions to direct chromatin interactions (Shalitin *et al*., 2002, 2003; Lin and Todo, 2005). In mice, CRYs can affect the activity, interaction, degradation, or nuclear trafficking of circadian clock components in light-independent manner (Griffin *et al*., 1999; Lin and Todo, 2005).

Light-mediated regulation in fungi is primarily conferred by blue-light receptors referred to as WHITE COLLARs (WCs), which are responsible for both light-dependent and -independent processes, such as induction of sporulation, carotenoid biosynthesis, and circadian rhythms (Liu *et al*., 2003; Dunlap and Loros, 2004; Corrochano, 2007). WC proteins have been well characterized in the filamentous ascomycete *Neurospora crassa* (Ballario *et al*., 1996; Linden and Macino, 1997; Froehlich *et al*., 2002; He *et al*., 2002), and homologues have been identified in ascomycetes, basidiomycetes, and zygomycetes (for review, see Corrochano, 2007). For example, *Phycomyces blakesleeanus* mutants defective in blue-light–dependent phototrophism may contain mutations in the *madA* gene, which encodes a protein similar to WC-1 of *N. crassa* (Idnurm *et al*., 2006). Inactivation of a

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wc-1 homologue in the human pathogen *Cryptococcus neoformans* results in a blind phenotype (Idnurm and Heitman, 2005; Lu *et al*., 2005). WHITE COLLAR orthologues are also encoded in the genome of *A. nidulans* (Greene *et al*., 2003), and the corresponding gene products have recently been shown to influence light sensing, sexual development, and mycotoxin production (Purschwitz *et al*., 2008).

In contrast to higher eukaryotes, the exact functions of the photolyase-cryptochrome family proteins in light reception and circadian rhythms are poorly understood in fungi. *N. crassa* exhibits a well-established circadian clock system (Dunlap and Loros, 2006). Although *A. nidulans* also has an oscillator system (Greene *et al*., 2003), it does not show obvious circadian rhythmicity. More than a decade ago, the *N. crassa* photolyase was shown to have photorepair activity in *Escherichia coli*, but its role in other processes of the fungal development remained elusive (Eker *et al*., 1994). It was recently shown that the *Trichoderma atroviride* photolyase PH1 autoregulates its expression, which suggests that fungal photolyases might possess additional regulatory functions (Berrocal-Tito *et al*., 2007). The model ascomycete *A. nidulans* is well suited to study the role of light-dependent processes due to its sensitivity to different light sources (Purschwitz *et al*., 2006). Light promotes its asexual reproduction, which results in conidiophores that form asexual spores (Adams *et al*., 1998). Alternatively, mycelia aggregate and produce a specific tissue of supportive Hülle cells in the sexual cycle, where the primordia differentiate and finally form fruiting bodies (cleistothecia) (Braus *et al*., 2002). This development is repressed by light, aeration, and in submerged culture, whereas it is promoted by the presence of a medium/air interface and the absence of light. To date, the fungal phytochrome FphA is the only analyzed developmental photosensor of *A. nidulans* (Blumenstein *et al*., 2005; Purschwitz *et al*., 2006).

Inspection of the genome of the homothallic ascomycete *A. nidulans* (Galagan *et al*., 2005) revealed only a single putative CRY/photolyase-like gene, here termed *cryA*. The deduced protein of 567 amino acids includes a PHR domain that contains both DNA photolyase and FAD-binding domains. We made efforts to elucidate the cellular role of this CRY/photolyase-like gene during the *A. nidulans* life cycle. The data obtained from deletion, localization, and expression studies suggest that CryA performs a dual function of regulating gene expression in *A. nidulans* both in a light-dependent and -independent manner as well as exhibiting DNA photorepair activity in a heterologous *E. coli* test system. To our knowledge, this is the first case for the cryptochrome/photolyase-like protein family where one member displays sensory, regulatory, and repair activity. This might be an initial hint as to how cryptochromes evolved from DNA photolyases.

MATERIALS AND METHODS

Sequence Analyses

The sequence of CryA from *A. nidulans* was retrieved from the National Center for Biotechnology Information (gi 40747330). Reference sequences were selected according to Daiyasu *et al*. (2004) and were also retrieved from the National Center for Biotechnology Information Entrez Protein database (see Figure 1B for corresponding gene identifier numbers). Multiple sequence alignments were carried out using T-COFFEE, ClustalW, and MAFFT with default parameters (Thompson *et al*., 1994; Notredame *et al*., 2000; Katoh *et al*., 2005). Phylogenetic trees were estimated by maximum parsimony (PAUP), neighbor-joining (PAUP), and maximum-likelihood (PhyML) methods using standard parameters (Guindon and Gascuel, 2003; Swofford, 2003); bootstrapping was performed with 500 repetitions. Consensus trees were constructed using Clann (Creevey and McInerney, 2005) and visualized with Treeillustrator (Trooskens *et al*., 2005). After orthonormal encoding of the alignments, principal component analysis, k-nearest-neighbor rule and nearest mean classification were computed essentially as described in Duda *et al*. (2001).

Strains, Media, and Growth Conditions

Fungal strains used during this study are listed in Table 1. The FGSC A4 strain served as the wild-type recipient for deletion of *cryA*, and strains AGB152 and AAH14 (*uvsB*Δ) were used for overexpression and subcellular localization experiments (Hofmann and Harris, 2000; Busch *et al*., 2003). Standard laboratory *Escherichia coli* strains DH5α and MACH-1 (Invitrogen) were used for preparation of plasmid DNA.

Recombinant DNA Procedures and Hybridization Techniques

E. coli and A. nidulans transformations were performed as described previously (Bayram *et al*., 2008). Standard protocols were followed to generate recombinant DNA constructs (Sambrook *et al*., 1989). *Taq*, *Pfu* (MBI Fermentas, Hanover, MD), or *Phusion* (New England Biolabs, Ipswich, MA) polymerases were used in polymerase chain reactions (Saiki *et al*., 1986), and crucial cloning steps were confirmed by sequence determination. Fungal genomic DNAs were prepared from ground mycelia (Kolar *et al*., 1988), and Southern blot analyses were performed as described previously (Southern, 1975). Total RNA samples were purified using the TRIzol reagent (Invitrogen, Carlsbad, CA) followed by Northern hybridization according to the protocols cited by (Brown and Mackey, 1997). The Stratagene Prime-It II kit was used to radioactively label hybridization probes in the presence of [α-^{32}P]dATP (Feinberg and Vogelstein, 1983). To produce autoradiographs, washed membranes were exposed to Kodak X-Omat films. Sequence data were analyzed using the Lasergene software package from DNAStar (Thompson *et al*., 1994). cDNAs for reverse transcription-polymerase chain reaction (RT-PCR) were synthesized with the RevertAid first strand cDNA synthesis kit (MBI Fermentas) according to the manufacturer's protocol by using equal amounts of DNA-free RNAs as input that had been isolated from different developmental stages.

Plasmids and Oligonucleotides

Plasmids and oligonucleotides are given in Supplemental Tables 2 and 3, respectively. Construction of the *cryA* replacement cassette in pME3181 was carried out as follows: the 1.8-kb *cryA* 3′ flanking region was amplified (OZG9/10) and inserted into the EcoRV site of pBluescript KS II (Stratagene, La Jolla, CA), followed by the amplification of 1.8-kb *cryA* 5′ flanking sequence (OZG5/6) and cloning into the AatII site of the 3′ *cryA* DS-bearing plasmid; and finally, a *ptrA* pyrithiamine resistance cassette with compatible SfiI ends was inserted into the SfiI sites of the resulting plasmid. A 5.6-kb *cryA*Δ cassette was released from pME3181 by BglII/NotI digestion before *A. nidulans* transformation to yield the *cryA*Δ deletion strain AGB288. A 6.8-kb *cryA* genomic fragment was subcloned into pGEM5 (SpeI/NsiI), creating pME3182. For construction of the complementation cassette, by using primers OZG189/192, *nat*^{R} cassette was amplified from pNV1 (Seiler *et al*., 2006), and 3′ untranslated region (UTR) of *cryA* was amplified from genomic (OZG191/10) DNA, and these two fragments were fused by fusion PCR (Yu *et al*., 2004) and *nat*^{R} + 3′ UTR fusion fragment was cloned in pCR-Blunt II-TOPO (Invitrogen). VspI site of this plasmid served as recipient for OZG199/200 amplified and VspI digested 5′ UTR + *cryA* ORF from pME3182, resulting in the complementation plasmid pME3183 from which 6.5-kb complementation fragment was released with PflMI digest and used for complementation of *cryA*Δ mutant. For localization experiments, N- and C-*sgfp-cryA* fusions were constructed by one-step fusion PCR: *cryA* cDNA was amplified from a cDNA library (OZG7/8 for N- and OZG7/89-noStop for C-terminal fusions, respectively), and *sgfp* was amplified with OZG29/87 and OZG1/88), accordingly. *sgfp* and *cryA* amplicons served as templates in PCRs to produce *sgfp::cryA* and *cryA::sgfp* fusions that were cloned into the PmeI site of pME3160, yielding pME3184 and pME3185 for N- and C-terminal fusions, respectively. For photoreactivation experiments, *cryA* cDNA (OZG44/45) was cloned in SmaI/NotI sites of pGEX-4T-2 giving pME3187; for overexpression driven by the *A. nidulans niiA* promoter, the same amplicon was inserted into PmeI site of pME3160 resulting in pME3186.

Photoreactivation Experiments

The *E. coli* strain SY2 (*uvrA*$^-$, *recA*$^-$, *phr*$^-$) was transformed with pMS969, pME3187 (*cryA* cDNA in pGEX-4T-2) and pGEX-4T-2 as an empty control plasmid. Transformants were propagated in LB medium supplemented with 150 μg/ml ampicillin at 37°C overnight; expression from pMS969, pME3187, and pGEX-4T-2 was induced by adding isopropyl β-D-thiogalactoside (0.1 mM final concentration) to the LB medium, and cultures were further incubated for 1 h with shaking before being spread on LB plates. Plates were irradiated with far-UV at fluences of 0.3 and 0.6 Jm^{-2} (SYLVANIA, G8T5 U.S.A., GERMICIDAL 8W) followed by irradiation with 366 nm UV-A (0306 μmol m^{-2}) (SYLVANIA, F8T5/BLB U.S.A., GERMICIDAL 8W). Plates were kept overnight in darkness at 37°C, and surviving colonies were counted the following day. To test for photoreactivation in *A. nidulans*, all experiments were performed in a dark room under red safelight. Freshly harvested *A.*

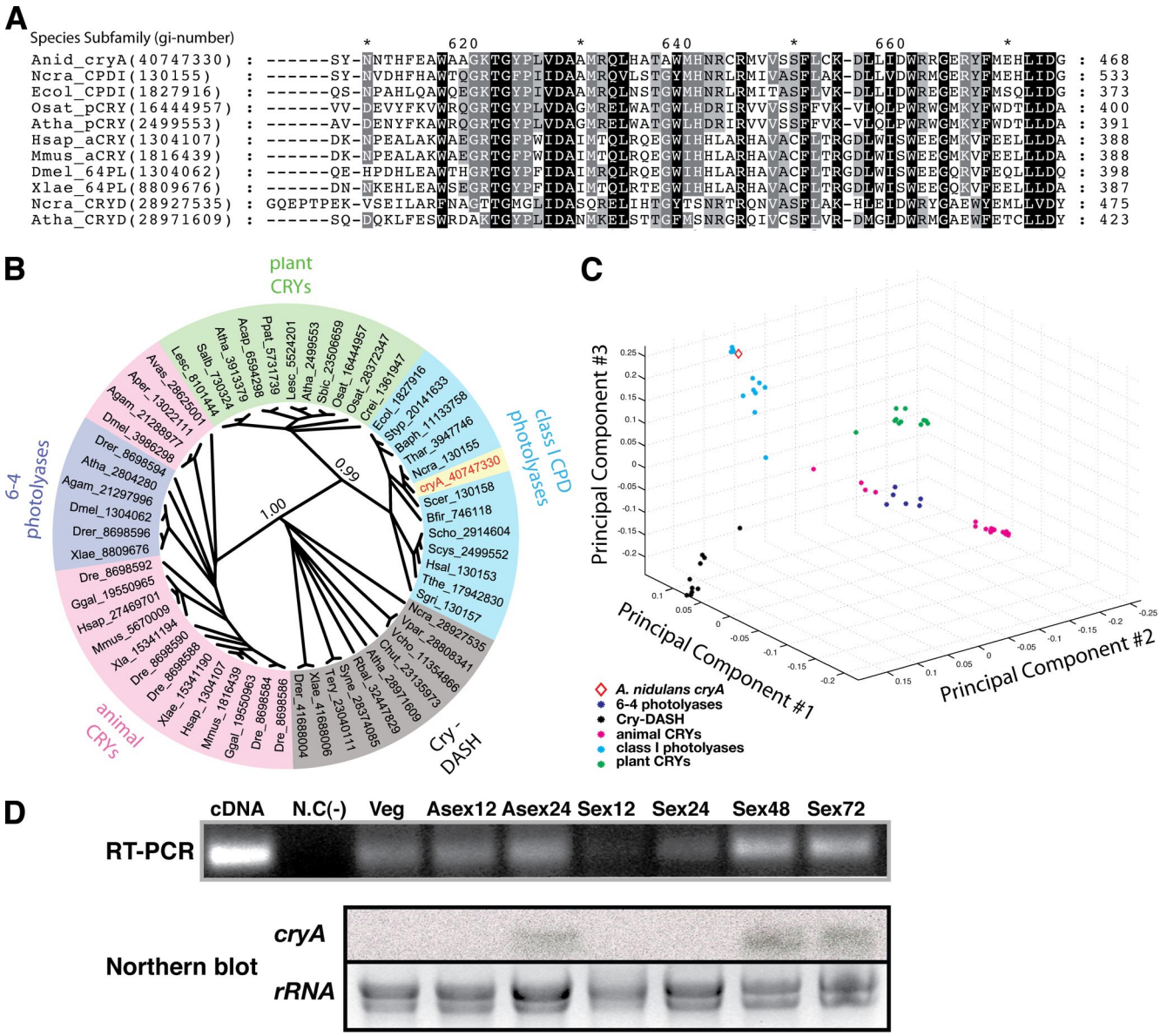

Figure 1. CryA classification. CryA amino acid sequence was aligned to six (6-4)-photolyases, 17 animal cryptochromes, 10 CRY-DASH proteins, 12 class I CPD-photolyases, and 11 plant cryptochromes (Daiyasu *et al.*, 2004). (A) Multiple sequence alignment (T-COFFEE; position 601–675) illustrates sequence conservation of CryA and two sequences from each cryptochrome/photolyase subfamily in FAD binding domain. Degree of sequence conservation: black (100%), dark gray (80%), light gray (60%), and white (<60%); amino acids of the same similarity group were treated as identical. (B) Radial cladogram of phylogenetic relationships of cryptochromes/photolyases including CryA. The cladogram represents a consensus tree combining maximum parsimony, neighbor-joining, and maximum-likelihood calculations, based on the multiple-sequence alignment generated with T-COFFEE. Bootsrap probabilities are given for the nodes separating animal CRYs/6-4 photolyases from plant CRYs/class I CPD photolyases (1.00) and plant CRYs from class I CPD photolyases (0.99). (C) Pseudo three-dimensional representation of the PCA from the ClustalW alignment. Shown are the first three principal components of a vector space, which represent the subspace with greatest variance. (D) *cryA* expression was monitored in an *A. nidulans wild-type* strain by RT-PCR (top) and Northern hybridization experiments (bottom). Strain FGSC A4 was vegetatively grown in submerged culture for 24 h (Veg), transferred onto plates, and kept in the light to induce the asexual sporulation (Asex) or incubated in the darkness to induce sexual development (Sex). Samples were prepared at the given time points of 12 and 24 h of asexual and 12, 24, 48, and 72 h of sexual development. Ethidium bromide-stained rRNA was used as loading control in Northern blots. Signals from RT-PCRs and Northern hybridizations are compatible with each other, indicating that *cryA* expression is low during vegetative growth and early asexual and sexual development.

nidulans spores were counted and adjusted to a density of 1.5×10^4 per plate, and spores from strains FGSC A4, *cryA*Δ, AGB152-*cryA*OE, AAH14, and AAH14-*cryA*OE were spread on Petri dishes (Ø 7 cm) and grown for 6–7 h in darkness to initiate germination. Germinating spores were exposed to far-UV at fluences of 448, 897, and 1795 Jm^{-2} and subsequently irradiated for 1 h with UV-A (366 nm; 0.306 μmol m^{-2}). Treated plates were kept at 30°C in darkness for 3 d, and surviving *A. nidulans* colonies were counted. Photoreactivation experiments were the mean of the four different experimental setups.

Light Sources and Irradiation Measurements

The light source for generating photon fluence rate–response curves was a slide projector (Prado Universal 31047; Ernst Leitz, Wetzlar, Germany) in combination with a heat-absorbing filter (KG1, 5 mm; Schott Glaswerke, Mainz, Germany). Monochromatic light was obtained with interference filters (type IL, 10- to 12-nm half bandwidth; Schott Glaswerke). Fluence rates were controlled by a resistor attached to the slide projector and/or by neutral

Table 1. Strains used in this study

Strain	Genotype	Reference
A. nidulans		
FGSCA4	Glasgow wild-type	FGSC
AAH14	*pabaA1 yA2; argB2; uvsB*Δ*::argB*	(Hofmann and Harris, 2000)
KHH62	*niiA*(p)::*nsdD*, *veA*+	(Han *et al.*, 2001)
AGB152	*pyroA4, pyrG89, veA*+	(Busch *et al.*, 2003)
AGB288	*cryA*Δ*::ptrA*	This study
AGB289	*cryA* <*nat*^{R}>	This study
AGB290	^{p}*niiA::sgfp::cryA::niiA*^{T}, *A.f. pyrG;* ^{p}*gpdA::mrfp::h2A,* ^{p}*gpdA::nat*^{R}*; pyroA4, pyrG89*	This study
AGB291	^{p}*niiA::cryA::sgfp::niiA*^{T}, *A.f. pyrG;* ^{p}*gpdA::mrfp::h2A,* ^{p}*gpdA::nat*^{R}*; pyroA4, pyrG89*	This study
E. coli		
SY2	*uvrA*$^-$, *recA*$^-$, *phr*$^-$	(Hitomi *et al.*, 2000)

FGSC: Fungal Genetics Stock Center, Kansas City, MO, USA.

density filters (type NG; Schott Glaswerke). Fluence rates were determined with a UV-enhanced photodiode (Meßkopf BN-9102-4; Gigahertz-Optik, Puchheim, Germany) and a calibrated readout instrument (Optometer P-9201; Gigahertz-Optik). Photon fluence rate–response curves were generated with a threshold box that contained 12 adjacent compartments (125 × 125 × 200 mm height) (Thielman *et al.*, 1991) that provided overhead irradiation for one Petri dish with mycelia. The light from the light source was partially reflected by beamsplitters (60% transmittance, 40% reflectance; Pörschke, Hoechst, Germany), which were centered above the Petri dishes at a 45° angle relative to the horizontally incident light. For the densitometric quantification of cleistothecia formation; a 1-cm^2 area was cut out from 10 different plates and the number of fruiting bodies was counted under binocular microscope.

Fluorescence Microscopy

A. nidulans spores (0.6×10^6) were inoculated on 18- × 18-mm coverslips submerged in appropriately supplemented liquid medium and incubated under white light or in darkness at 37°C overnight. Coverslips were mounted on microscope slides by using spore storage solution (0.002% Tween and 0.5% NaCl) and fixed with wax. Images of fluorescence were taken with an Axiovert S100 microscope (Carl Zeiss, Jena, Germany) supported with an OCRA-ER digital camera (Hamamatsu, Bridgewater, NJ) by using the OpenLab tmV5.0.1 software package (Improvision, Coventry, United Kingdom).

RESULTS

A. nidulans CryA Is Closely Related to Class I CPD Photolyases

The cryptochrome/photolyase protein family is composed of six subgroups, which are distinguished according to phylogenetic relationships and functions: class I CPD photolyases; class II CPD photolyases; plant cryptochromes; animal cryptochromes, which include (6-4) photolyases; and CRY-DASH proteins. All subfamilies, except class II CPDs, share significant sequence similarities. The *A. nidulans* genome reveals only a single putative cryptochrome/photolyase gene (AN0387.2; gi 40747330), which we termed *cryA* due to the functional analysis described in this study. To assign CryA to one of the subfamilies, several analyses based on multiple sequence alignments with 56 members of the cryptochrome/photolyase family as classified by Daiyasu *et al*. (2004) were performed (Figure 1). Due to their divergence from the other subfamilies, the class II CPD photolyases were not included. In the multiple sequence alignments performed with T-COFFEE, ClustalW, and MAFFT, the deduced CryA sequence showed a high degree of conservation with all other groups (Figure 1A). Phylogenetic trees were estimated by maximum parsimony, neighbor-joining, and maximum likelihood methods to result in the expected classification of the reference sequences into the appropriate cryptochrome/photolyase subfamilies. As a general result for all three alignments, the CryA sequence clustered with the class I CPD photolyases (Figure 1B).

To further verify this classification of CryA, the multiple alignments were analyzed with nearest-mean and k-nearest-neighbor-rule classification methods, as well as by principal component analysis (PCA). By means of orthonormal encoding, each position in the alignment was encoded as a 20-dimensional vector where each dimension represents one amino acid. In this way, each sequence in the alignment is represented as a vector in a 22.520-dimensional vector space. To visualize the positioning of the sequences in the resulting vector space, a dimension reduction by PCA was carried out. Color coding of the sequences from different subfamilies shows that the subfamilies form separate clusters within this high-dimensional feature space (Figure 1C); the CryA sequence is located within the group of class I CPD photolyases. For nearest-mean classification, the mean of the vectors of each subfamily was calculated, and the distance to the CryA sequence was analyzed. Here, the nearest mean belonged to the group of class I CPD photolyases with a distance of 0.6982 (CRY-DASH 1.0611, plant CRYs 1.1599, 6-4 photolyases 1.1994, animal CRYs 1.2195). For the k-nearest-neighbor-rule classification the distance of the CryA sequence with respect to all other sequences from the alignment was determined. For $k = 7$, all sequences in the vector space neighborhood belong to the class I CPD photolyase family. Therefore, all analyses carried out in the present study indicate that in terms of the primary amino acid sequence CryA is most closely related to class I CPD photolyases.

cryA Is Expressed in A. nidulans Late during Development

Temporal and spatial expression patterns of developmental regulators determine morphogenesis and many other developmental processes that are vital for any organism. Therefore, we wanted to see how and during which stages the *cryA* gene is expressed in *A. nidulans*. To this end, first hints were obtained by amplification of the *cryA* cDNA from an *A. nidulans* cDNA plasmid library. The *cryA* cDNA amplicon was sequenced and found to lack the intronic region that separates the two exons that encode the DNA photolyase and FAD binding domains on the genomic locus (Supplemental Figure S1A). Thus, we concluded that the *cryA* gene is indeed expressed and that the corresponding pre-mRNA is spliced to yield the mature transcript. Interestingly, the *cryA* cDNA could be reproducibly amplified from a sexual cDNA library (Krappmann *et al*., 2006) but not from a vegetative cDNA library (Krappmann *et al*., 1999). Therefore, *cryA* mRNA expression was followed in a time course experiment growing the fungus up to 24 h vegetatively, 12 and 24 h asexually, and different time periods sexually. Samples of total RNA, which served both as template for the synthesis of PCR-compatible cDNA and RNA for Northern analysis, were prepared from each stage. Results from RT-PCRs and Northern hybridizations (Figure 1D) were in good agreement to show increased mRNA levels at 24 h of asexual, as well as at 48 and 72 h of sexual development. From RT-PCR signals, basal expression still can be observed during vegetative growth and early asexual development. This basal expression increases during late asexual sporulation. Similarly, expression levels remain low during early stages of cleistothecia formation, and after 48- to 72 h after induction of sexual development, steady-state levels of the *cryA* transcript increase. Together, this expression pattern hints at

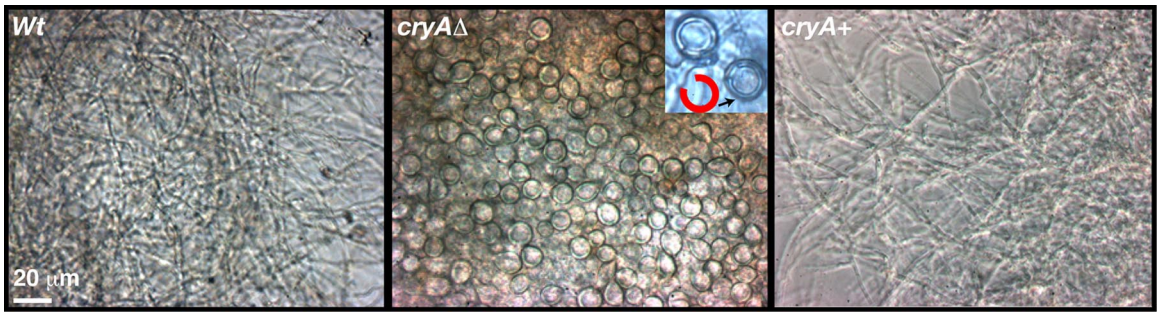

Figure 2. Deletion of fungal *cryA* results in Hülle cell formation in submerged culture. Phenotypical characterization of the *cryA*Δ strain. Light microscopy illustrates wild-type growth resulting in hyphal mats (left), whereas a *cryA*Δ strain produces Hülle cells with circular, banana-like cell wall shape (middle); the deficiency is complemented by *cryA* homologous gene replacement (right).

a potential role of the *cryA* gene product during late asexual and sexual differentiation.

Deletion of cryA Results in Hülle Cell Formation in Liquid Culture Associated with Pigment Secretion

To address the cellular function of the *A. nidulans* cryptochrome-photolyase–encoding gene, we deleted the *cryA* locus (Supplemental Figure S1A). Asexual or sexual development on solid media is normal, whereas the *cryA*Δ strain displays a distinct phenotype when cultivated submerged in liquid minimal medium. Although the wild-type strain grows vegetatively and forms long hyphal structures after 48 h of growth (Figure 2, left), the mutant forms large numbers of Hülle cells that cover the mycelial balls and that normally support sexual fruit body formation in the wild type (Figure 2, middle). The formation of cleistothecia was not observed and sexual development remained in this incomplete state. Moreover, the *cryA*Δ mutant excretes a pink–purple pigment after prolonged incubation in submerged culture, which might be the result of already committed sexual development (Supplemental Figure S1B). The knockout phenotypes could be rescued by integrating a complementation fragment at the original locus (Figure 2, right).

CryA Is a Nuclear Protein and Represses Transcription of Regulators of Sexual Development

To monitor subcellular localization of CryA, functional *cryA* cDNA fusions to the green fluorescent protein were created and expressed from the nitrate-inducible promoter *niiA* (Muro-Pastor *et al.*, 1999). Both N- and C-synthetic green fluorescent protein (sGFP)::CryA fusion proteins showed constitutive localization in fungal nuclei in the absence or presence of illumination (Figure 3A). These results suggest that CryA represses sexual development of *A. nidulans* in the nucleus. Whereas CRYs are involved in transcriptional regulation, photolyases mostly have DNA repair functions and do not operate at the level of transcription. The formation of Hülle cells in the *cryA* mutant under inappropriate conditions is reminiscent of an imbalance of fungal developmental regulators. When overexpressed, the light-dependent sexual regulator *veA* or sexual transcription factor *nsdD* cause Hülle cell formation in submerged culture (Han *et al.*, 2001; Kim *et al.*, 2002). The *veA* gene is responsible for light-dependent fruit body formation in *A. nidulans* (Mooney and Yager, 1990; Kim *et al.*, 2002), and *n*ever in sexual *d*evelopment (NsdD) is a putative GATA-type transcription factor that activates sexual development (Han *et al.*, 2001). Deletion of the *rosA* gene of *A. nidulans* results in the same phenotype characterized by Hülle cell formation in liquid medium (Vienken *et al.*, 2005). *rosA* (*r*epressor *o*f *s*exual development A) of *A. nidulans* encodes a $Zn(II)_2Cys_6$ transcription factor that inhibits sexual development under low-carbon conditions and submerged culture (Vienken *et al.*, 2005). Analysis of expression of these regulators in a *cryA*-null mutant (Figure 3B) revealed that transcripts for the light-dependent activator VeA increase during 48 h of vegetative growth in the *cryA*Δ strain, which further increases during 72 h of growth, whereas there is only weak expression in wild-type background. Expression of *nsdD* activator transcripts also escalates during 48 h of growth, correlating to the observation of Hülle cells, but decreased sharply after this time point. mRNA encoding RosA, which is responsible for repressing sexual development under inappropriate conditions, is undetectable during vegetative growth and also accumulates exactly at the time when Hülle cells come into being, which imitates the *nsdD* expression pattern. These increased transcript levels suggest coregulation of the developmental regulators by *A. nidulans* CryA. A *rosA*Δ strain, which produces Hülle cells in submerged culture, showed up-regulated transcripts of *nsdD* and *veA* (Vienken *et al.*, 2005). Because we observed a similar expression pattern for *nsdD* and *rosA*, we analyzed *rosA* mRNA levels in an *nsdD*-overexpression strain (Figure 3C). The *rosA* transcript levels were only up-regulated in this strain when grown under conditions that forced *nsdD* expression, suggesting that *nsdD* activates *rosA* transcription. We propose a hierarchical negative feedback loop between activator and repressor elements (Figure 3D) where *cryA* normally regulates *veA* expression negatively, whereas *veA* positively regulates *nsdD* expression. NsdD activates expression of its own negative regulator *rosA* that does not affect *veA* expression but represses the transcription of *nsdD* by preventing sexual commitment at the level of Hülle cells. The *rosA*/*nsdD* feedback loop would explain why *nsdD* expression is primarily important for the development of Hülle cells, one of the early stages of the sexual life cycle, whereas *veA* is also essential for the development of other subsequently formed sexual organs.

CryA-mediated Photoinhibition of A. nidulans Sexual Development

Light reception is the major function of a typical CRY, which senses blue light to regulate gene expression. In contrast, DNA photolyases are able to absorb UVA and blue light for photoreactivation and do not influence gene expression. CryA resembles a DNA photolyase whose DNA repair feature is activated in *E. coli* by $UVA_{366\ nm}$ light (Sancar *et al.*, 1984; Sancar, 1990, 1994). The formation of cleistothecia

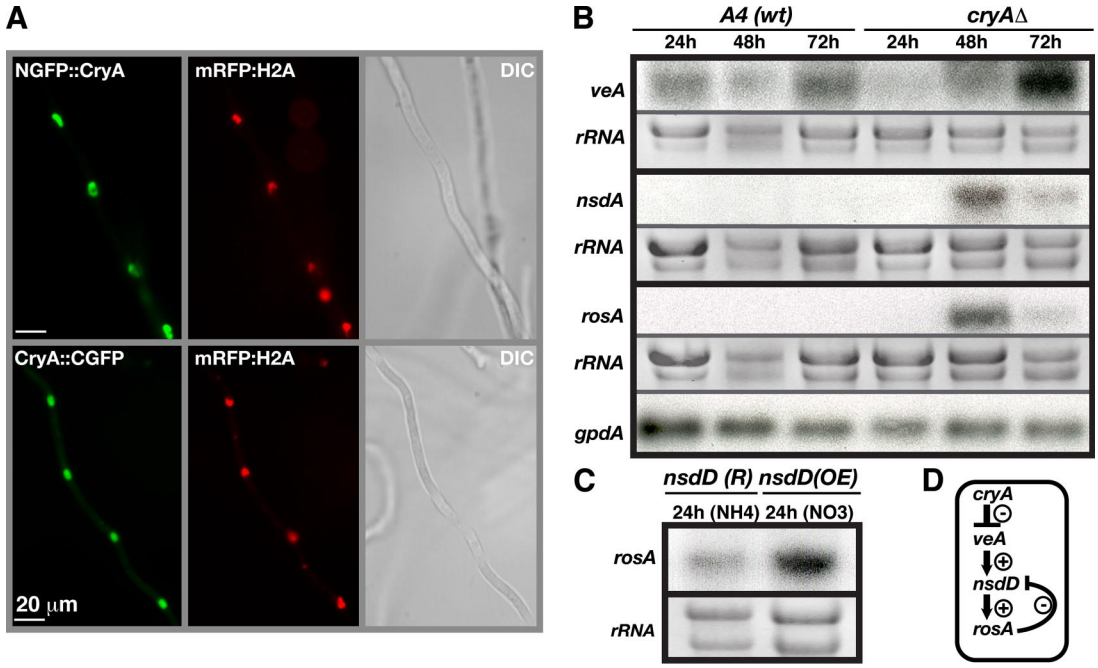

Figure 3. Nuclear CryA represses the transcripts of regulatory factors of sexual development to reveal a negative feedback loop. (A) N- and C-terminal fusions of CryA to sGFP were expressed to monitor subcellular location of the fusion proteins; to visualize the nuclei in vivo, a red fluorescence mRFP::H2A fusion was used. (B) Comparative Northern hybridization of sexual development regulators in the wild-type strain A4 and the *cryA*Δ strain: RNA levels for the light-dependent regulator *veA* increase in *cryA*Δ after 48 h after induction, corresponding to the appearance of Hülle cells, and increase further up to 72 h. *nsdD* and *rosA* regulator mRNAs are elevated in *cryA*Δ, but almost undetectable in A4. *gpdA* (glycolytic gene) served as internal control. (C) *rosA* expression is dependent on expression of the *nsdD* gene as demonstrated by overexpression of *nsdD*. (D) Model of *cryA*-regulated negative feedback loop of fungal development. Northern blot experiments were repeated three times.

(fruiting bodies) is suppressed by light, a response that is at least in part mediated by a phytochrome (Blumenstein *et al*., 2005). Therefore, we tested whether a fungal *cryA*Δ mutant is impaired in any light response.

To study the potential contribution of other photoreceptors to this photoresponse, we generated photon fluence-rate response curves for the photoinhibition of cleistothecia in the near-UV, blue-, and red-light spectra (Figure 4A). The resulting dose–response curves were rather complex because they displayed a low-irradiance component (10^{-4}–10^{-2} μmol m^{-2} s^{-1}) and a high-irradiance component ($>10^{-1}$ μmol m^{-2} s^{-1}) component. At 350, 370, and 454 nm, the wild type of *A. nidulans* and strain comp$^+$ (complemented with *cryA* locus) displayed thresholds near 8×10^{-5} μmol m^{-2} s^{-1}; in red light (680 nm); however, the threshold of all three strains were greatly elevated and near 8×10^{-3} μmol m^{-2} s^{-1}. In mutant *cryA*, the photoinhibition was substantially affected at 350, 370, and 454 nm, but not in red light. At 370 and 454 nm, the photon-fluence rates required to inhibit 50–60% cleistothecia were ~20-fold higher in the *cryA* mutant compared with those of the wild-type and strain comp+ (Figure 4A). The effect of the *cryA* deletion is thus 1) restricted to the low-irradiance component and 2) specific for the near-UV and blue spectral region. In the high-irradiance region, i.e., $>10^{-1}$ μmol m^{-2} s^{-1}, *cryA* did not affect photoinhibition (Figure 4A, 370, 454, and 680 nm). This finding implies that the CryA repressive function on sexual development depends on the UVA and blue-light spectrum, corroborating that CryA is involved in light signaling. Moreover, this finding indicates that UVA light, which is usually absorbed by DNA photolyases, regulates the number of cleistothecia via the CryA regulatory protein.

CryA-mediated Photoreactivation in E. coli *and* A. nidulans

The high degree of similarity between CRYs and photolyases makes a distinction based on the primary sequence difficult. A valuable functional discrimination test is to demonstrate whether the studied protein has near-UV–inducible DNA repair activity in a defective *E. coli* strain (Hitomi *et al*., 2000). We found that strains expressing the endogenous bacterial photolyase or fungal CryA were able to survive high irradiances of far-UV to a greater extent upon near-UV irradiation than strains that did not express any photolyase activity. Surprisingly, the CryA gene conferred substantial photoreactivation activity to the *E. coli* strain lacking DNA photolyase (Figure 5A). Therefore, the *A. nidulans cryA* gene includes a DNA photolyase activity that complements the phenotype of DNA repair-defective *E. coli* cells.

The photoreactivation activity of CryA was further analyzed in the fungus. Spores from a wild-type strain of *A. nidulans*, the *cryA*Δ strain, and the overexpression strain, and a *uvsB*Δ strain deficient for the DNA damage response (Hofmann and Harris, 2000), were exposed to $UV_{254\ nm}$ light (Figure 5B). Subsequently, plates were illuminated with $UVA_{366\ nm}$ light for photoreactivation, and eventually plates were incubated either in the dark or in white light for 3–4 d to determine the number of surviving fungal colonies. Whereas no UV-induced sensitivity was observed for the *cryA*Δ mutant, the *uvsB*Δ strain seemed to be highly sensitive to UV irradiation. Strikingly, this phenotype was partially rescued when the *cryA* gene dose was increased by forced overexpression, whereas in a wild-type background, *cryA* overexpression did not result in elevated UV-light re-

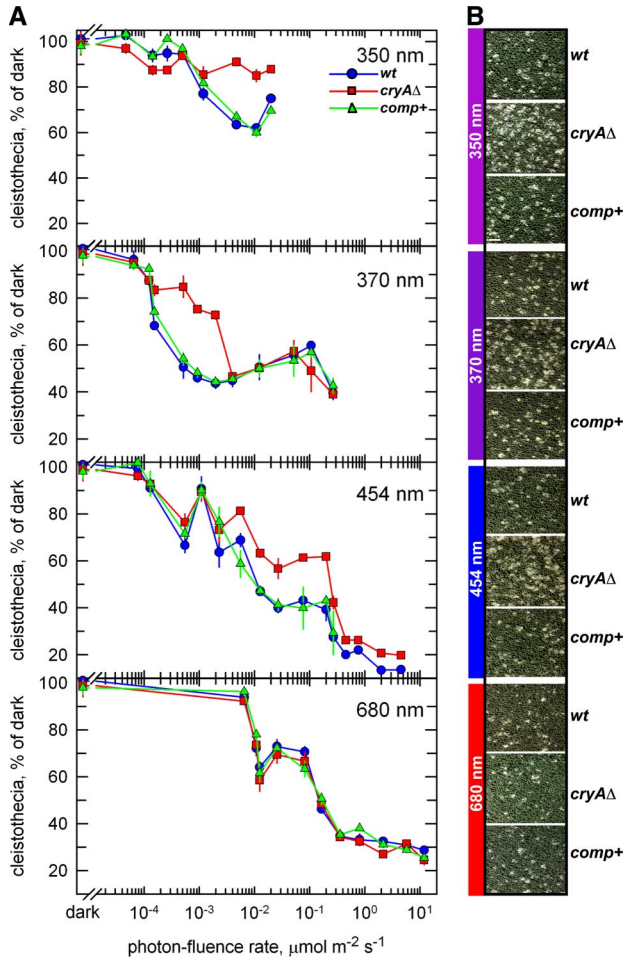

Figure 4. Photon fluence-rate response curves for the photoinhibition of cleistothecia formation. (A) After inoculating Petri dishes with 10^4 spores per plate, the materials were irradiated for 100 h with monochromatic overhead light at the indicated photon-fluence rates. Blue circles, wild-type; red squares, *cryA* mutant; green triangles, complementation strain (comp+). Vertical bars indicate the standard deviations. (B) Formation of cleistothecia (spherical fruiting bodies) of *A. nidulans* growing on solid medium. The photographs show the densities of the cleistothecia of the wild-type, mutant, and complementation strains that were raised under 350-, 370-, 454-, and 680-nm light sources. White bar, 200 μm.

sistance (Figure 5B). This suggests that in the wild-type strain, mechanisms such as excision repair mask the DNA photolyase activity of CryA. In the *uvsB*Δ background, where such repair mechanisms are inactivated, *cryA* overexpression increases the resistance to UV irradiation. Together, these results from photoreactivation experiments clearly indicate that CryA confers a pronounced DNA photolyase activity.

DISCUSSION

The *cryA* locus of the filamentous ascomycete *A. nidulans* encodes a protein with functions typical for a cryptochrome combined with photolyase activity. Our phylogenetic analysis strongly indicates that the fungal CryA sequence is most closely related to class I CPD photolyases. The cryptochrome functions of the CryA protein, namely, regulation of gene expression and a blue-light reception, are corroborated by several findings: the fungal *cryA*Δ mutant strain develops sexual organs—Hülle cells—in submerged culture conditions, which usually repress sexual and asexual differentiation of in *A. nidulans* by maintaining vegetative growth. This phenotype has also been described for a mutant strain of *rosA*, as well as for strains overexpressing sexual regulators *nsdD* and *veA* (Han *et al.*, 2001; Kim *et al.*, 2002; Vienken *et al.*, 2005). A common feature of these genes is that they encode transcriptional regulatory proteins for sexual development. VeA is a light-dependent regulator of sexual development without any obvious DNA binding domain (Kim *et al.*, 2002), NsdD is a putative GATA type transcription factor necessary for sexual development (Han *et al.*, 2001), and RosA is a $Zn(II)_2Cys_6$ transcription factor required for the repression of sexual development (Vienken *et al.*, 2005). Our detailed analysis of the transcription data revealed a hierarchical feedback loop between activator and repressor elements. Formation of Hülle cells in submerged culture in a light-independent manner suggests that CryA governs the negative feedback loop between transcriptional regulators by which many more downstream genes are controlled.

The light-independent nuclear localization of CryA indicates that it might regulate gene expression by interacting with other nuclear proteins, such as transcription factor, and activators or by directly binding to DNA, thereby repressing gene expression crucial for sexual development. This is substantiated by *cryA*Δ mutant, which is less sensitive to UVA

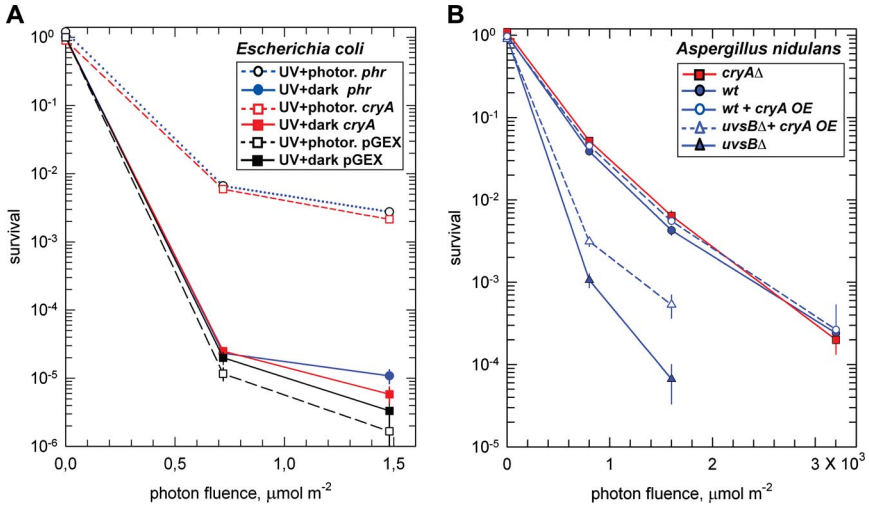

Figure 5. Dose–response curves for photokilling and photoreactivation in *E. coli* and *Aspergillus nidulans* strains. (A) Photoreactivation experiments in the repair-defective *E. coli* strain SY2 (*uvrA*$^-$, *recA*$^-$, *phr*$^-$) transformed with plasmid pGEX-4T-2 as control, pGEX-*cryA*, or pMS969 as positive control. UV-irradiated *E. coli* cells were either kept in darkness (black symbols) or illuminated with $UVA_{366\ nm}$ (light symbols). *E. coli* Phr and *A. nidulans* CryA show photoreversal activity after $UVA_{366\ nm}$ treatment. (B) *A. nidulans* UV sensitivity experiment; spores of *A. nidulans* strains A4 (wild-type), *cryA*Δ, *uvsB*Δ, and *cryA*-OE were exposed to $UV_{254\ nm}$ radiation followed by 1 h $UVA_{366\ nm}$ treatment; the extent of surviving spores was quantified as colony forming units after 48 h. Whereas there is no difference between A4 and *cryA*Δ, the UV resistance of *uvsB*Δ is increased by overexpression of *cryA* (~6-fold). Vertical bars indicate the standard deviations.

light and produces more cleistothecia under $UVA_{360-370\ nm}$-light. The loss of *cryA* causes a loss of light sensitivity that is specific for the near-UV and blue spectral region (Figure 4, A and B). The ~20-fold rise of the thresholds at 370 and 454 nm in the low-irradiance region might seem modest in view of the complete loss of photoreceptor function. We assume that the residual light sensitivity of *cryA* between 10^{-3} and 10^{-1} μmol m^{-2} s^{-1} (370 and 454 nm) is caused by phytochrome, which also absorbs light <500 nm (Vierstra and Quail, 1983a,b). The situation is similar in *Arabidopsis thaliana*, in which the loss of cryptochrome causes in blue light a modest, i.e., 20-fold, threshold rise that is thus comparable with our data (Figure 4, A and B; Galland, unpublished data; Guo *et al*., 2001). In addition, White Collar-type photoreceptors could mask the impact of the cryptochrome loss and might account for the fact that the *cryA* phenotype is restricted to an apparently small "irradiance window" in the respective photon fluence-rate response curves (Figure 4, 370 and 454 nm). Together, the phenotype of the *cryA* mutant indicates that this gene, in addition to its role in light-independent repression of sexual transcription, is also involved in photodifferentiation and that *A. nidulans cryA* could function as a near-UV/blue-light receptor as it is described for plant cryptochromes.

Photoreactivated DNA repair activity of CryA in UV-sensitive *E. coli* and *A. nidulans* suggest that CryA can repair DNA upon exposure to UVA light similar to other photolyase proteins. In agreement with our data, it has recently been shown for other proteins of the Cry-DASH protein family from bacterial, plant, and animal sources that they are actually photolyases with a high degree of specificity for cyclobutane pyrimidine dimers in single-stranded DNA. The CRY-DASH photolyase activity in general seems to be weaker in comparison with the photolyase activities of CPD photolyases (Selby and Sancar, 2006). The additional cryptochrome-like regulatory function of a photolyase is a novelty for this class of proteins. An analysis of the photospectroscopic properties of CryA should provide additional hints about the mechanism of CryA function.

It is difficult to speculate why a dual photolyase/cryptochrome protein has evolved in *A. nidulans*. One reason might be that light control of development is also mediated by other light receptors. Current data collected through photobiological, genetic, and biochemical experiments indicate the importance of the WHITE COLLAR gene products in blue-light sensing of lower eukaryotes, including fungi. In the *A. nidulans* genome, there are two WC orthologues that might be involved in blue-light–dependent development. Therefore, CryA action might not be required for the entire blue-light spectrum and is restricted to UVA light. It is tempting to speculate that the existence of other active blue-light receptors might have resulted in a slowdown evolution of the *A. nidulans* cryptochrome. Most other organisms usually encode two or more cryptochrome/photolyase-like genes, some of which have photolyase activity and some cryptochrome function. The closely related ascomycete fungus *N. crassa* genome encodes one photolyase and one CRY-DASH protein. In the available genome sequences from aspergilli, only one CRY/photolyase-like protein each can be retrieved from *A. nidulans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus oryzae*, and *Aspergillus terreus*. However, *A. clavatus* and *A. niger* encode two genes, one gene for a photolyase activity and one gene for a putative CRY-DASH. This might be the result of a gene duplication that did not occur in most aspergilli. Alternatively, it might also be possible that some time after the divergence of an *Aspergillus* progenitor from the *Neurospora* progenitor, *Aspergillus* has lost the CRY/photolyase-like gene, e.g., CRY-DASH, through chromosome rearrangements. The loss of the CRY-DASH protein might have caused the endogenous DNA photolyase to gain regulatory functions without losing its DNA photolyase activity, which might have slowed down its evolution to an exclusive and completely functional cryptochrome.

Finally, we propose that *A. nidulans* CryA combines the function of a negative regulator of sexual development upon integration of environmental factors, near-UV and blue light, with the function of a photolyase of the class I CPD photolyase subfamily (Lin and Todo, 2005). We assume that CryA has the ability to transmit a UVA-light signal to the nucleus to repress the *veA*-mediated initiation of a feedback loop between *nsdD* and *rosA* that is important in Hülle cell formation and sexual development. The same wavelength is also required for photoreactivation of DNA photolyase activities. Furthermore, we speculate that CryA might be the first example of a true dual-function protein that acts as a photolyase and a cryptochrome and accordingly represents a missing link between the established CRYs and DNA photolyases. Our findings suggest that characterized photolyase proteins might also possess regulatory functions.

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